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In Vitro and In Vivo Observations on a Murine C-Type Virus

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From 40 discrete mouse tissue culture cell lines examined by electron microscopy or complement fixation, or both, for the presence of detectable virus, one (NCTC 4705), initiated and maintained on chemically defined medium, was chosen for a more extensive study. Virus-like particles (100 to 110 mμ), morphologically similar to previously reported immature and mature C-type leukemia virus particles, were found budding from the plasma membrane and free in the intracellular spaces of cells in tissue culture and in fibrosarcomas resulting from intramuscular implants of these tissue cultures. Complement-fixation tests for group reactive murine leukemia antigens were positive, with titers consistently higher to a broadly reactive anti-serum than to anti-Friend, anti-Moloney, or anti-Rauscher sera. The 4705 virus was neutralized by Gross antiserum, but not by the F-M-R antisera. When injected into DD, BALB/c, or C3H/He newborn mice, the virus thus far has manifested no leukemogenicity, though virus from tumor extracts and tissue culture medium has been shown to be capable of infecting C3H and Swiss mouse embryo tissue cultures and successfully replicating in them. The role of the virus in accelerating or inducing neoplastic transformation in NCTC 4705 is still not known. When it was introduced into NCTC 4823, a non-neoplastic cell line in other respects similar to NCTC 4705, 4823 manifested no signs of neoplastic transformation after harboring the virus more than 300 days in vitro.

Long-term tissue cultures with their general tendency to undergo "spontaneous" neoplastic transformation (12, 16, 20, 21, 48, 49) provide an excellent and readily available source of material for the study of the transforming process. The potential of this method has been greatly enhanced by the development of chemically defined media (19, 38, 54), providing an even greater control of the in vitro environment, and thus of the extracellular factors. Several long-term cell lines have been adapted to defined medium (18, 55), and recently Andresen et al. (2) succeeded in initiating and continuously growing pooled mouse embryo cells in vitro "in a completely defined medium without the addition of, or exposure to, insulin or any enzymatic preparation, such as trypsin." This latter investigation provided for the first time a cell line which, from its very inception, existed exclusively on a chemically defined medium.

The growing list of long-term tissue cultures which have been found to harbor virus or "virus-like particles" (9, 11, 26, 30) suggested the need for an investigation to determine whether these particles could initiate or facilitate the so-called "spontaneous" neoplastic transformation. On the other hand, the absence of at least detectable virus in many neoplastic cell lines would indicate that neoplastic transformation as such can be independent of virus influence. From among 40 tissue culture sublines tested by us (26) for the presence of virus by complement fixation (CF) or electron microscopy (EM), or both, one, NCTC 4705, initiated on chemically defined medium and positive for CF and EM virus testing, was especially interesting. Preliminary study (25) suggested that the virus belonged to the murine leukemia group; thus experiments were undertaken to test its virulence, infectivity, neoplasia-inducing capabilities, and antigenicity. These comprise the subject matter of this report.

MATERIALS AND METHODS

Tissue culture. The development of the specific chemically defined medium and the method of initiation of tissue culture line NCTC 4705 from pooled minced cells of 22 embryos removed from three C3H/HeN females after 13 days in utero have been described in detail elsewhere (2, 18). Intraocular implants from cell cultures 910 days in vitro were made into five irradiated (425 r, whole-body exposure) weanling C3H/HeN mice by the technique of Grobstein (22). From one of these, a 1-mm³ portion of a

resulting tumor was then implanted into the left thigh muscles of each of three mice of the same age and strain. These tumors were then routinely transplanted every 3 or 4 weeks, and samples were surveyed with an electron microscope.

CF. At various stages of the experiment, the cell line was tested for the presence of mouse leukemia group-reactive CF virus antigens by Janet Hartley (National Institute of Allergy and Infectious Diseases, Bethesda, Md.), using rat antisera described previously (27, 45). Tests were also conducted under contract with Microbiological Associates, Inc., for the presence of Sendai virus, pneumonia virus of mice (PVM), reovirus, Theiler's GD VII virus, polyoma virus, K virus, mouse adenovirus, mouse hepatitis virus, and lymphocytic choriomeningitis (LCM) virus. (These tests were carried out by J. C. Parker at Microbiological Associates, Inc., Bethesda, Md., under NCI contract SA 43-PH4356.)

Virus preparation. Attempts to isolate virus from resulting tumors were made following essentially the procedure reported by Moloney (35). To recover virus from culture fluids, about 70 ml of medium (NCTC 135) which had been kept for 3 days on cell cultures grown in T-60 flasks were pooled and either first passed through a Selas 03 filter or centrifuged for 10 min at 1,000 rev/min in an IEC no. 269 rotor to remove cells and debris. The filtrate or supernatant fluid was then centrifuged at 29,000 rev/min for 60 min in a no. 30 rotor in a Spinco L2-65. The resulting pellets were either resuspended and combined in a small amount of phosphate buffer (0.1 M, *p*H 7.3) for liquid nitrogen storage until used for further study or fixed for electron microscopy.

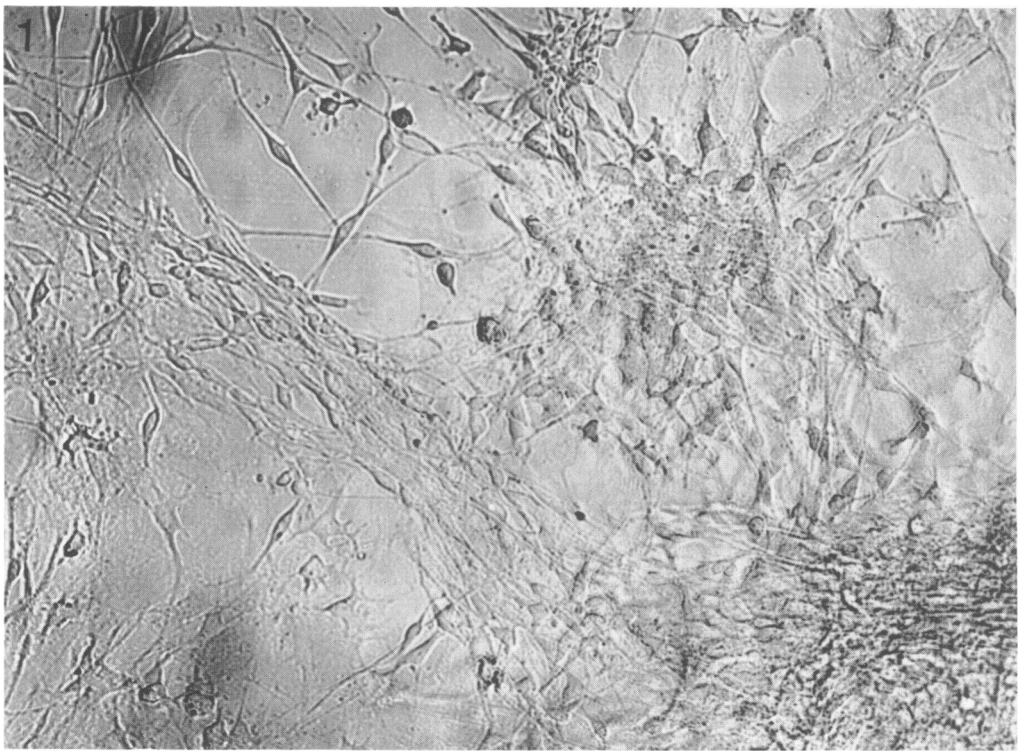
Electron microscopy. In the preparation of tissue for the electron microscope, three different fixation methods were used. All tissue cultures were initially fixed while still attached to the surface of the flask. After removing the culture medium, ordinarily 1% glutaraldehyde (GA) in phosphate buffer (46) was poured on the cell sheet and left there for 1 hr. The GA fixation was sometimes omitted to check the effect on the cells of the various fixation methods. GA at 1% was also used on viral pellets, but the concentration was increased to 3% for tumors. Wherever GA was utilized, the tissues were then rinsed three times in buffer for a total of 30 min and transferred to chrome-osmium (C-O) for 1 hr (10). During the last buffer rinse, the tissue cultures were scraped loose from the floor of the flasks with cellophane; the contents of two or three T-15 flasks were pooled and centrifuged at 1,800 rev/min in an IEC no. 269 rotor for 10 min. The resulting pellet was broken into small pieces and thus carried through the subsequent process, which included in all cases immersion in 0.5% aqueous uranyl acetate (UA) at *p*H 5.0 for 60 min prior to alcohol and propylene oxide dehydration. On a few occasions, the tissue was passed through 10% neutral Formalin for 1 hr after the C-O and before the UA treatment. All tissues were left overnight in propylene oxide and Epon-Araldite mixture (50:50) and finally embedded in Epon-Araldite (34). Sections were cut on a Reichert ultramicrotome with a diamond knife, picked up on bare 200, 300, or 400 mesh grids, stained with uranyl acetate and lead citrate (42), and viewed in a Siemens Elmiskop 1A electron microscope.

Virus infectivity. Attempts to infect stock cultures of NCTC 4823 were carried out on fresh monolayers in T-15 flasks by replacing the used culture medium with 3.0 ml of NCTC 135 plus 10% fetal calf serum to which had been added 0.1 ml of a 1-g equivalent concentrate of virus suspension prepared as described previously (35). The cells were incubated in the virus-containing medium at 37.5 C for 24 to 36 hr, when the culture medium was renewed. Infectivity was determined by CF testing of the infected cells for the presence of antigen and by EM for the presence of morphologically detectable particles.

RESULTS

EM and CF testing. The established cell line, NCTC 4705, exists as a multilayered sheet of predominantly fibroblast-like cells which have been grown exclusively on chemically defined medium (Fig. 1, 2). Investigation with a light microscope revealed a high degree of hypotetraploidy, characterized by various chromosomal abnormalities described in detail by Andresen et al. (2). In addition to this aneuploidy, multinucleate cells are also present and there exists a degree of cytoplasmic vacuolization in most of the cells, along with a rather high nucleocytoplasmic ratio and pronounced cytoplasmic basophilia. These features are more apparent under an electron microscope (Fig. 3, 4, 5). The endoplasmic reticulum is expanded in several places and may account for some of the apparent vacuolization; the very large number of free ribosomes probably contributes to the intense basophilia. The most striking ultrastructural feature of the NCTC 4705 tissue cultures, however, was, as previously reported (25), the presence in large quantities of viruslike particles approximately 100 to 110 mμ in diameter. Most often they were found intercellularly and appeared to be similar to mature C particles, having an electron dense nucleoid with a diameter of about 65 mμ (Fig. 3, 4, 5, 13). Frequently, particles were seen to bud off the plasma membrane (Fig. 6) or from intracytoplasmic membranes (Fig. 7, 8). Morphologically, the particles resembled the murine leukemia viruses in both their mature and immature forms (Fig. 13, 17, 18, 19, 20). In addition, intracytoplasmic intracisternal A particles were also encountered (Fig. 12), though more frequently in tumors than in tissue culture. These particles seem to be limited by a unit membrane of the endoplasmic reticulum which is itself immediately adjacent to an inner electron-dense ring-shaped core with an electron-lucent center.

Once the cell line had been established and routinely subcultured, rapidly growing fibrosarcomas were easily induced within 3 weeks by intraocular



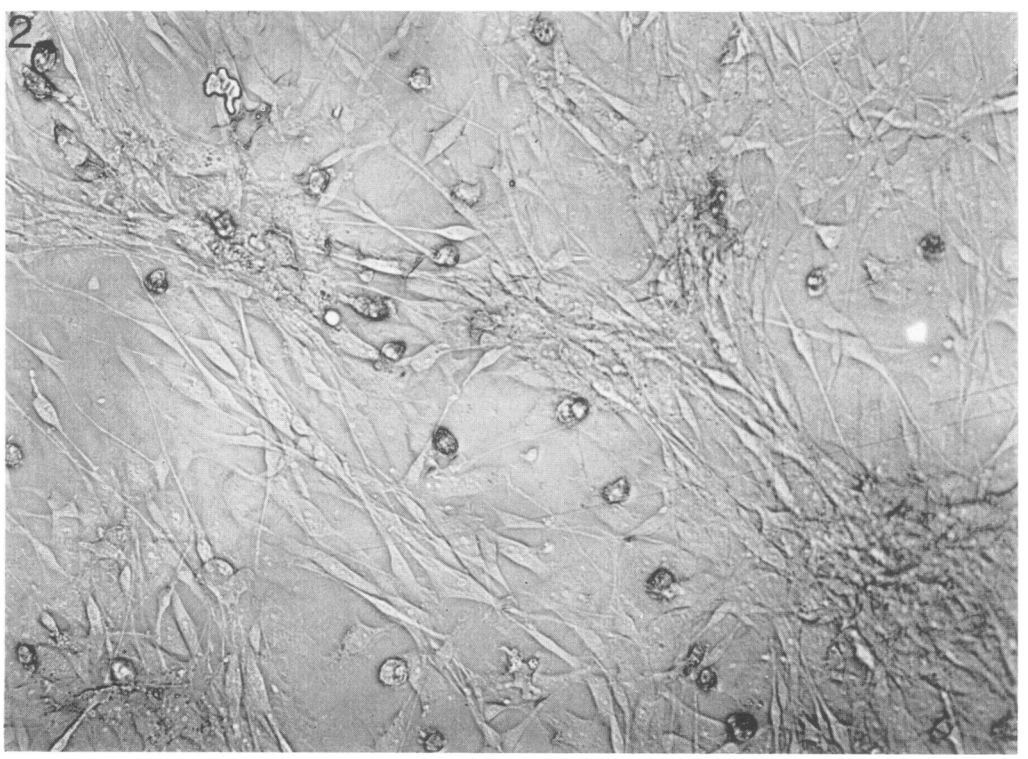


FIG. 1. *Light micrograph of NCTC 4705 after 253 days in vitro.* × *200.*
FIG. 2. *Light micrograph of NCTC 4705 after 547 days in vitro.* × *200.*

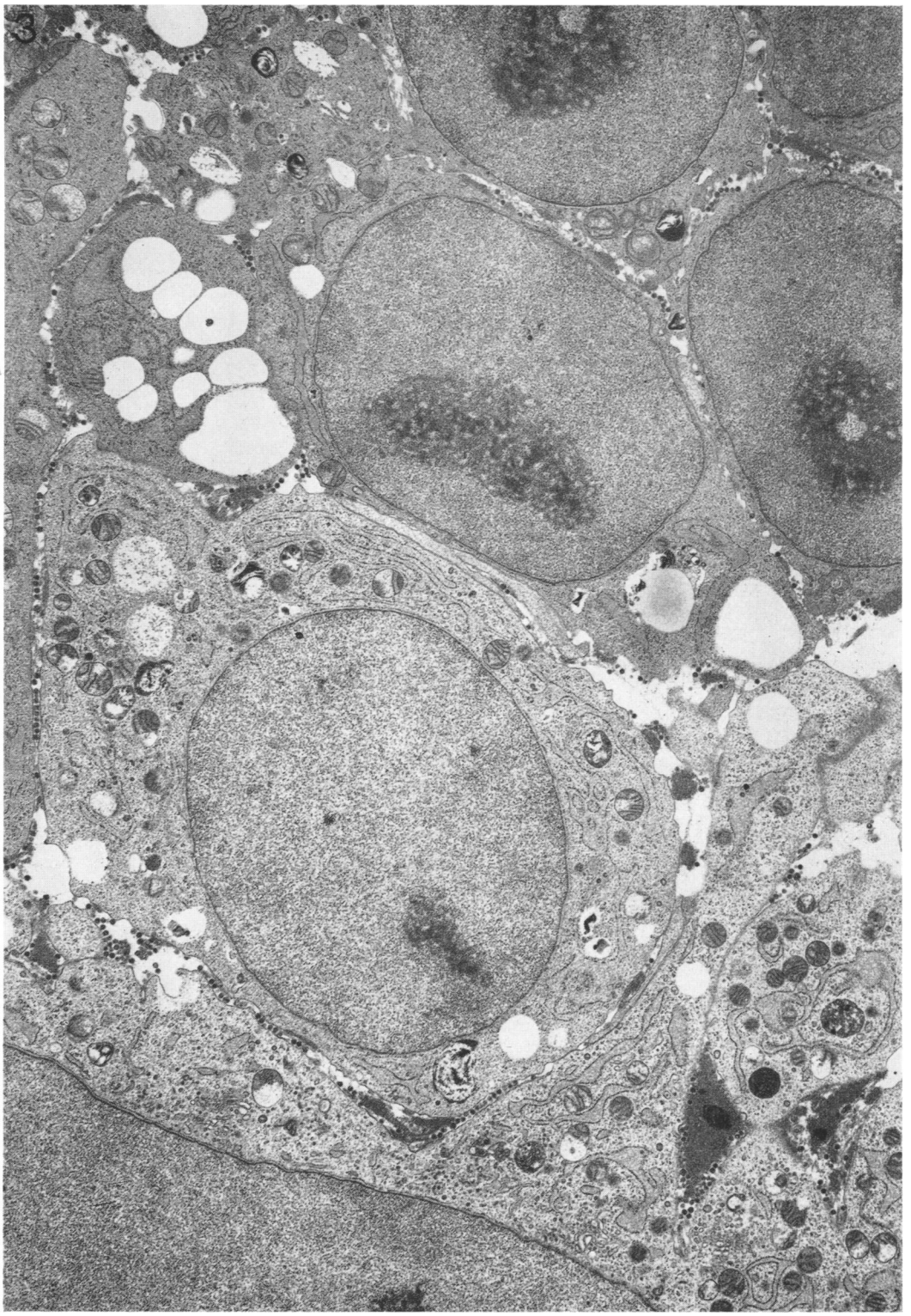
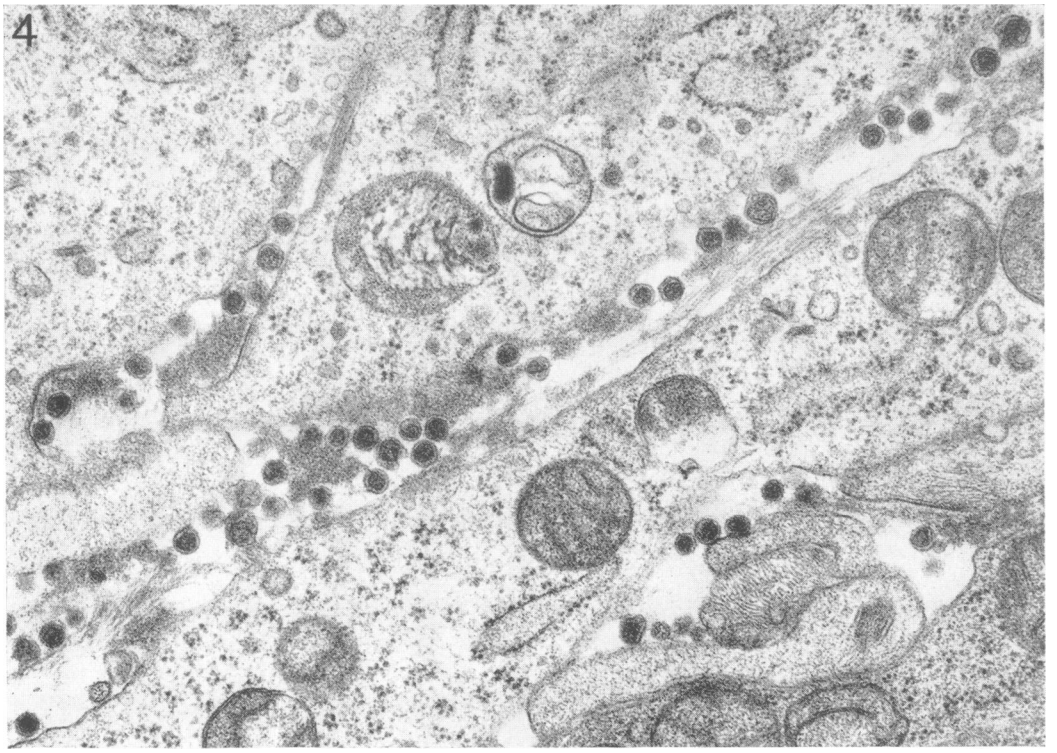


FIG. 3. *Low-power micrograph of NCTC 4705. Nuclei are large and nucleoli prominent; several vacuoles are visible and much of the endoplasmic reticulum is somewhat expanded. Around the cells, in the intercellular spaces, several virus particles can be seen.* × *7,000.*



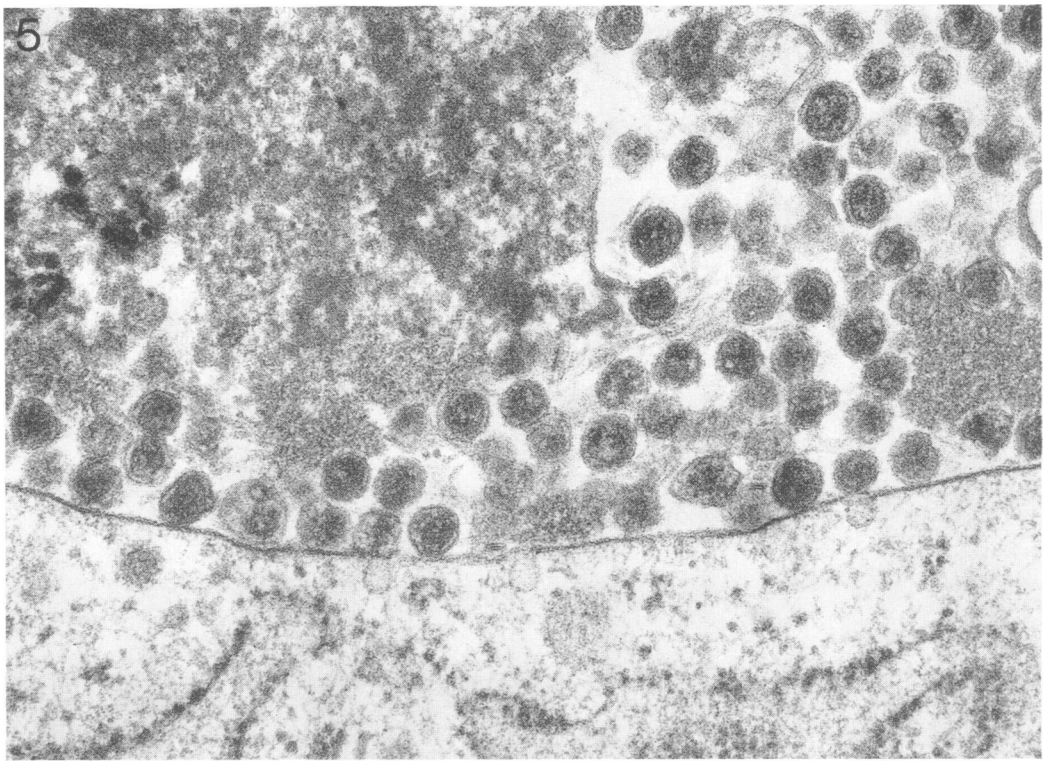


FIG. 4. *Typical mature C-type particles between cells of NCTC 4705. Many ribosomes unattached to membrane lie in the cytoplasm of the cells.* × *35,000.*

FIG. 5. *Higher magnification of several extracellular mature C particles in NCTC 4705.* × *70,000.*

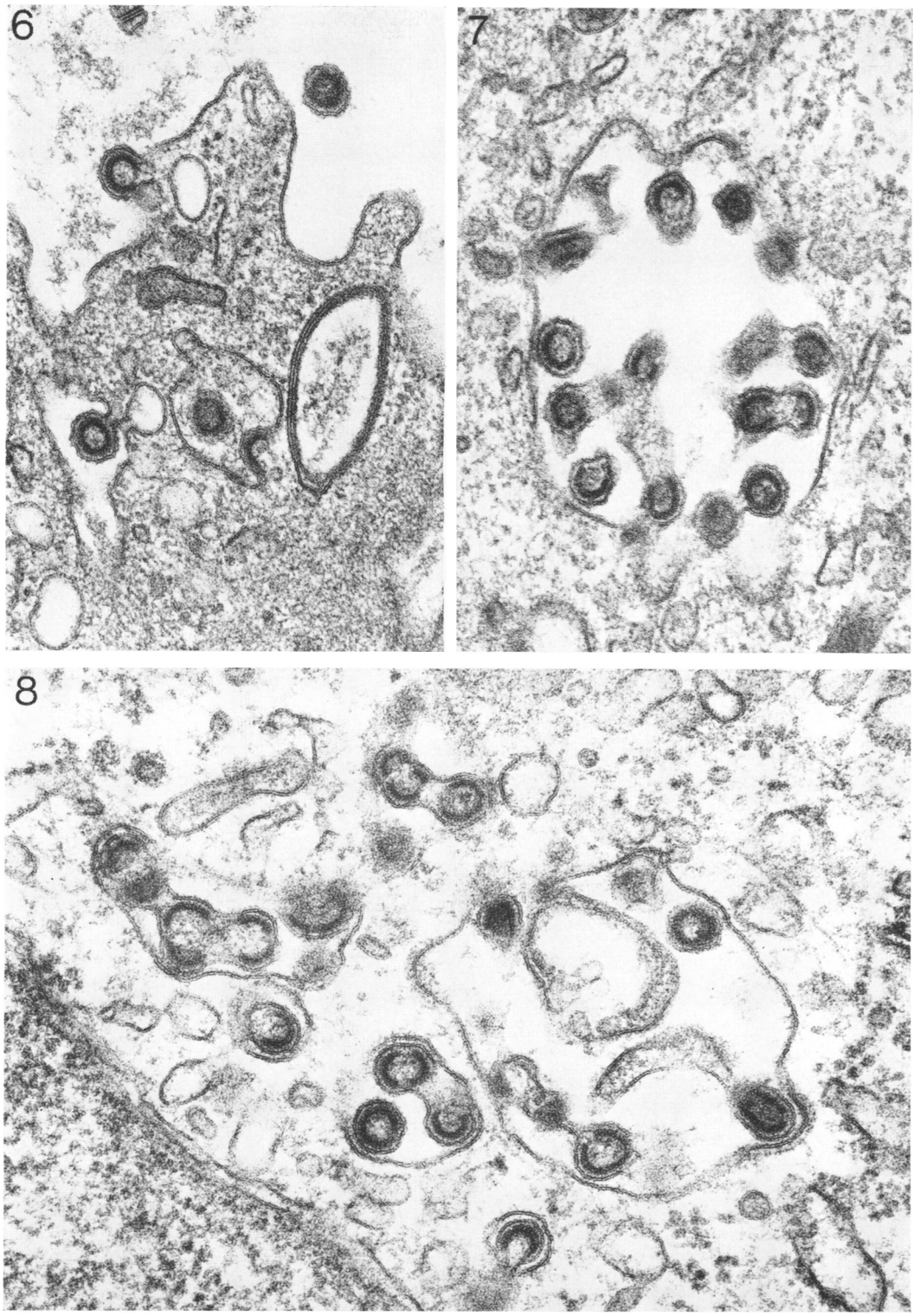


FIG. 6. *Immature C-type particles budding from plasma membrane of intramuscular transplant of fibrosarcoma resulting from implantation of NCTC 4705 tissue culture cells. Note also particle budding off membrane of vacuole.* × *80,000.*

FIG. 7. *Immature C-type particles in intracytoplasmic vacuole of tumor cells.* × *80,000.*

FIG. 8. *Same as Fig. 7.* × *80,000.*

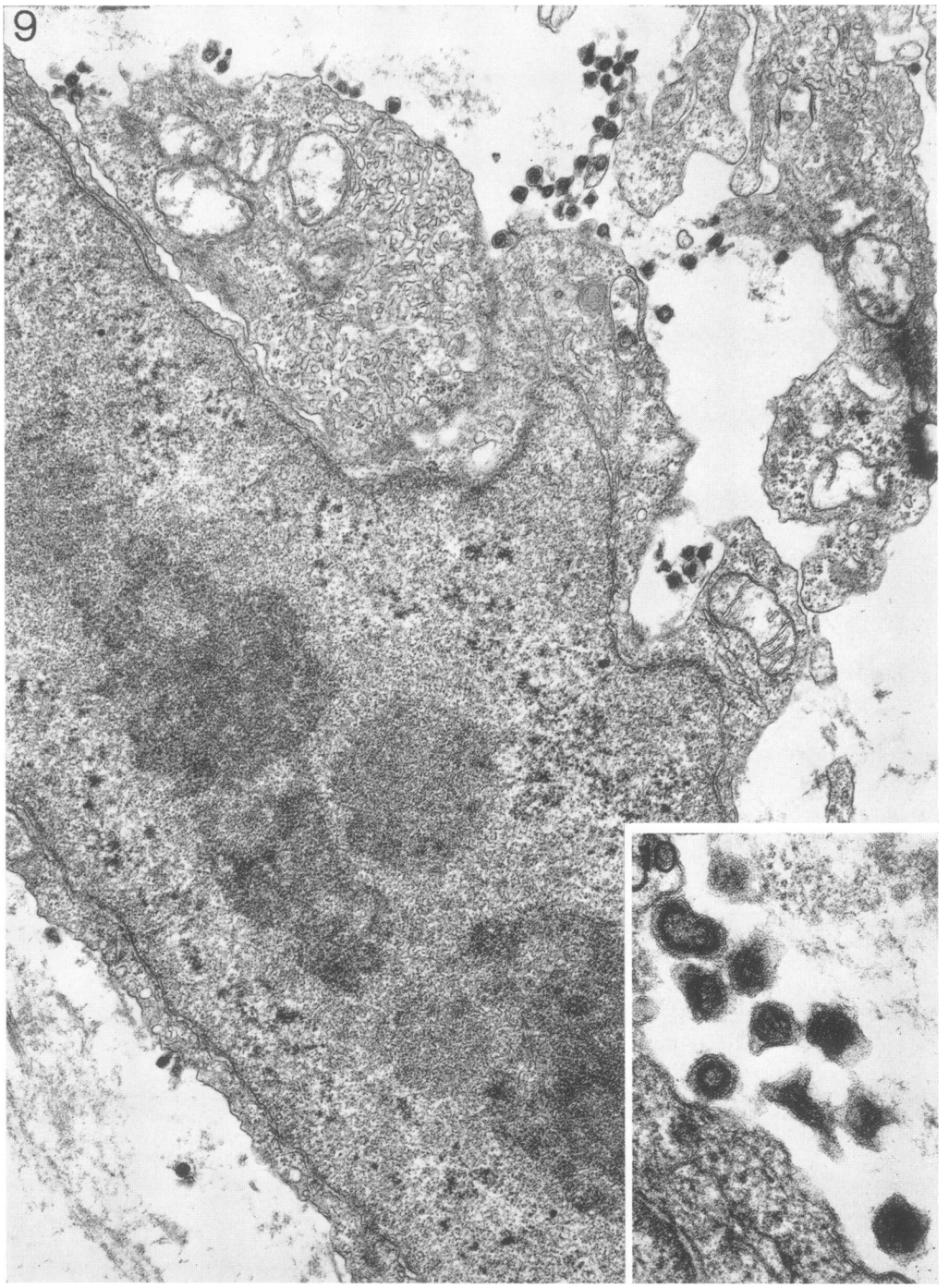


FIG. 9. *Mature and immature C-type particles in intraocular tumor which arose from implant of NCTC 4705 at 53rd subculture.* × *21,000.*

FIG. 10. *Higher magnification of particles from same intraocular tumor.* × *80,000.*

or intramuscular implants of cells or by tumor transplants. Cells from the 53rd in vitro subculture induced intraocular tumors in five irradiated C3H/HeN weanlings within 15 days, although the original attempt had required 10 months in non-irradiated animals from cells 417 days in vitro and not yet considered an established cell line. From one of these intraocular tumors, 1-mm^3 pieces of tumor were implanted intramuscularly into three C3H/HeN weanlings. These are now passaged every 4 weeks and represent our source of tumor-derived virus. At every passage, the tumor was examined with an electron microscope for the presence of virus (Fig. 9, 10) and periodically tested by CF for murine leukemia virus CF antigen. All of these observations have been unequivocally positive. Figure 11 schematically represents the general plan pursued in these investigations.

Hartley et al. (27) developed CF methods suitable for the detection of murine leukemia antigens. Several subcultures of NCTC 4705 have been tested in this system against Moloney virus rat antiserum and against more broadly reactive sera for mouse leukemia virus group-reactive CF antigens, obtained from rats carrying transplanted tumors induced by Gross virus or Moloney sarcoma virus. The procedure is carried out in two stages. In the first (or direct) test, extracts prepared from frozen and thawed or homogenized material (tissue culture or tumor) are examined for the presence of mouse leukemia virus antigens. Subsequently, the second (or recovery) test checks for the ability of the original material to induce CF antigen production in Swiss mouse embryo tissue cultures. The results of CF tests on 4705 or its derivatives are summarized in Table 1.

All of the tested subcultures were positive to a broadly reactive serum obtained from rats carrying transplanted tumors induced originally by Gross virus. The ability of frozen and thawed NCTC 4705 cells to induce CF antigen production in Swiss mouse embryo tissue culture is readily apparent from data in Table 1. Antigen was usually detected after one recovery passage and always by the second passage.

Samples of tumor originally initiated by intraocular implant of NCTC 4705 were tested as 10% tumor extracts for CF antigen at the second and sixth intramuscular transplant generations. As in the recovery test for NCTC 4705, antigen response in the direct test on the tumor was positive with both FMR and broadly reactive antisera, though at least fourfold higher with the broad.

NCTC 5597 and 5604, originally subcultures of NCTC 4823, were assigned their new culture numbers after having been successfully infected with virus extracted from the third tumor passage

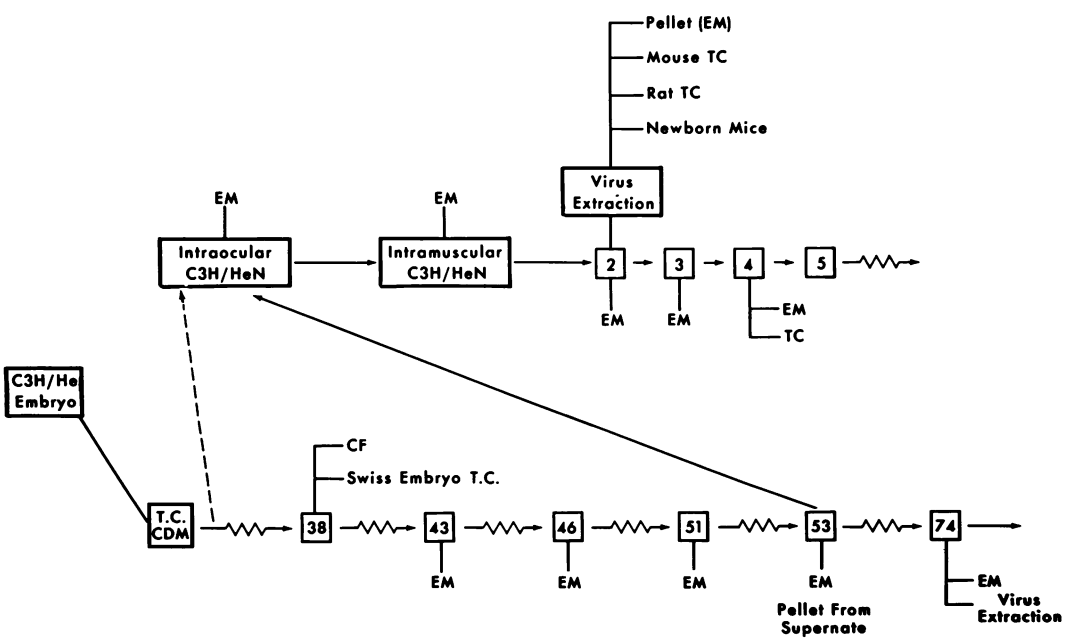


FIG. 11. *Schematic representation of the general plan pursued in the NCTC 4705 investigation. The lower boxes signify the tissue culture generations and the upper boxes tumor transplant generations. At the 53rd subculture, cells were implanted into the eye of C3H/HeN weanling mice and the resulting tumors were the source of subsequent tumor transplants. The cultures marked "EM" were examined with the electron microscope.*

TABLE 1. *Results of complement-fixation tests on NCTC 4705 and various tissue cultures infected with 4705 virus*

Culture no.	Generation[a]	Complement fixation		
		Direct test	1st passage	2nd passage
4705	10	+	+	+
	38	+	+	+
	47	+	+	+
	62	+	+	
	82	+	+	
5597[b]	4	+	+	
	10	+	+	
5604[c]	2	+	+	
	6	+	+	
5780[d]	3	+	+	
5781[e]	3	+	+	
5642[f]	6	+	+	
	11	+	+	
Tumor	T2	+		
	T6	+		

[a] For cultures other than NCTC 4705, "Generation" signifies the number of subculture generations *after* the virus had been experimentally introduced.

[b] NCTC 4823 + 4705 virus prepared from tumor in citrate buffer.

[c] NCTC 4823 + 4705 virus prepared from tumor in phosphate buffer.

[d] NCTC 4823 + 4705 virus prepared from tissue culture medium in the presence of antibiotics.

[e] NCTC 4823 + 4705 virus prepared from tissue culture medium without antibiotics.

[f] Cells from 4705 tumor put back into tissue culture on fetal calf serum.

of 4705 (see Table 1). NCTC 4823 itself was initiated from a pool of C3H/HeN minced mouse embryos and maintained on culture medium NCTC 135 plus 10% fetal calf serum. This line has been in culture over 1,700 days; it has been consistently negative to CF and EM tests for the presence of virus, and continues to remain non-neoplastic as far as can be determined by the absence of invasive, progressively growing tumors after intraocular or intramuscular implantation of cells into isologous mice. Thus, NCTC 4823 was selected as the control culture for determination of the infective and transforming capabilities of the 4705 virus.

Four different attempts to infect cell line 4823 were made with virus prepared in different ways: (i) virus prepared in citrate buffer from the second intramuscular passage (NCTC 5597); (ii) virus prepared in phosphate buffer from the fourth intramuscular passage (NCTC 5604); (iii) virus prepared from tissue culture medium from the 89th subculture with penicillin-streptomycin added to the infecting medium (NCTC 5780); (iv) same as iii, but without antibiotics (NCTC 5781). In all of these tests, ampoules of the same batch of virus previously stored in liquid nitrogen were given CF and EM tests for the presence of antigen or virus particles. As Table 2 shows, all of these attempts were successful and we thus developed four sublines of 4823 which have been infected with 4705 virus.

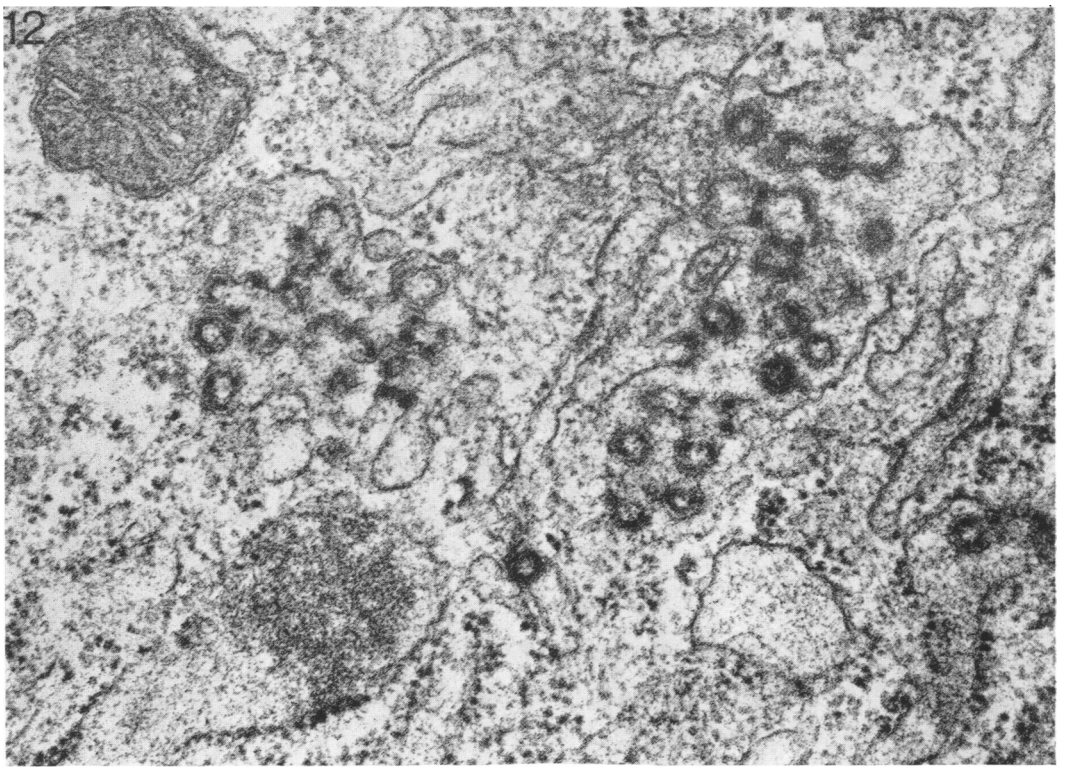
Since NCTC 4705 cells were grown exclusively in chemically defined medium, the possibility existed that the cells might propagate virus more effectively in a serum-supplemented medium. Therefore, a piece of tumor from the same source as the virus extraction in phosphate buffer (ii above) was put back into tissue culture on NCTC 135 plus 10% fetal calf serum. At culture generations 6 and 11, these cells were tested by CF and EM, but no significant differences could be observed between cultures of 4705 grown on chemically defined medium and those on medium with serum added. It would seem then that the higher titers observed in the CF recovery tests are more

TABLE 2. *Attempts to infect various tissue culture lines with NCTC 4705-derived virus*

Cell line no.	Virus subculture generation	Complement-fixation test[a]		C Particles (EM)	Remarks
		Direct	Recovery (1st passage)		
5597	4	2	16	+	4823 + V[b]
	8	8	>16	+	(citrate)
	12	8	>16	+	
5604	2	±	>16	+	4823 + V
	6	8	>16	+	(phosphate)
5780	3	>2		+	4823 + V(TC)
5781	3	>2		+	4823 + V(TC)
5689	1	−	−	−	Rat kidney
	2	−	−	−	fibroblasts
	3	−	−	−	
89124	3		>32	+	SMETC + V
21704	—		>32	+	SMETC + V

[a] Expressed as reciprocal of CF antigen titer.

[b] V = 4705 derived virus.



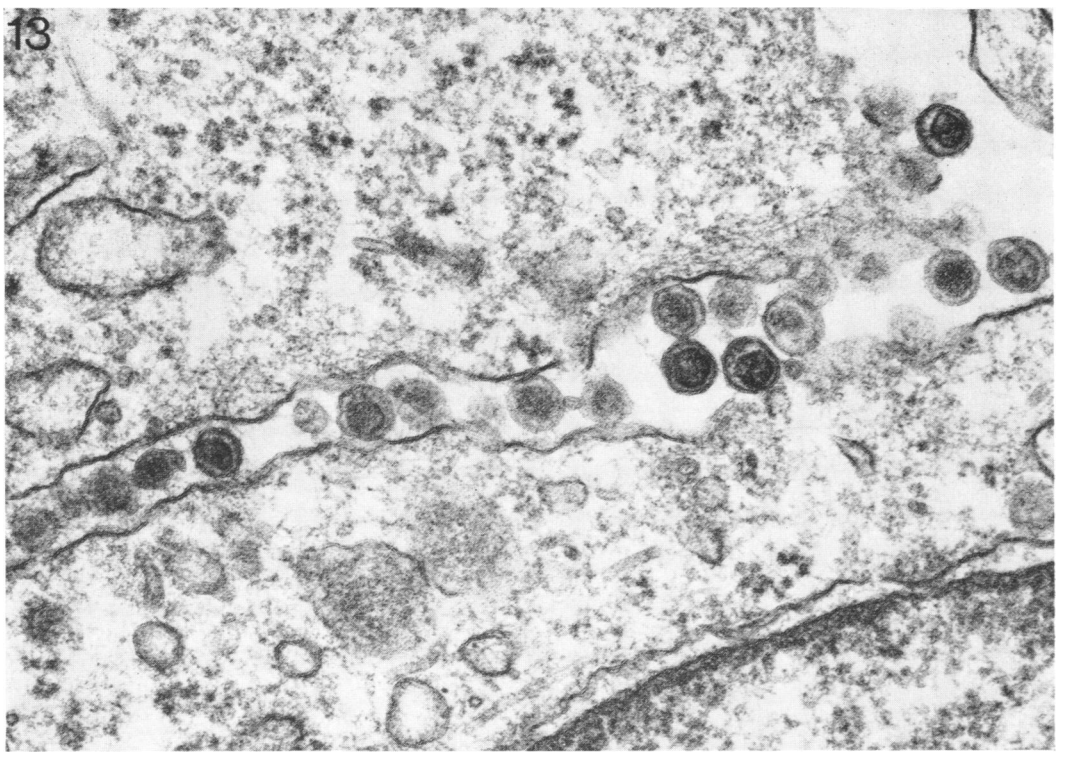
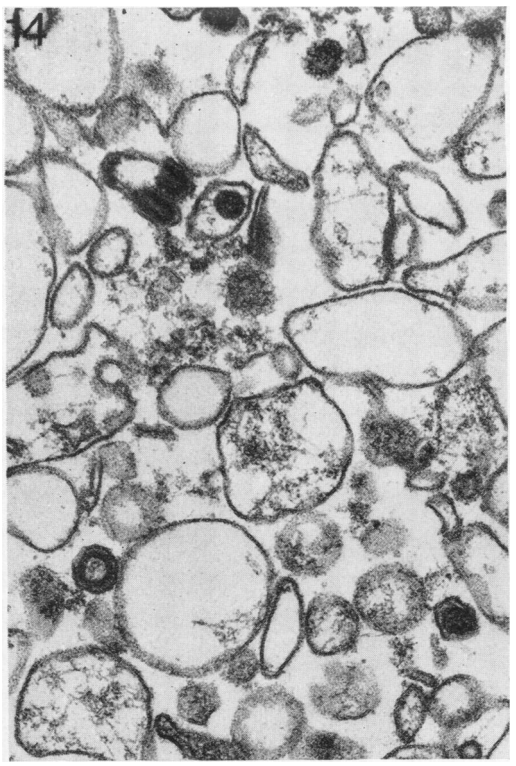
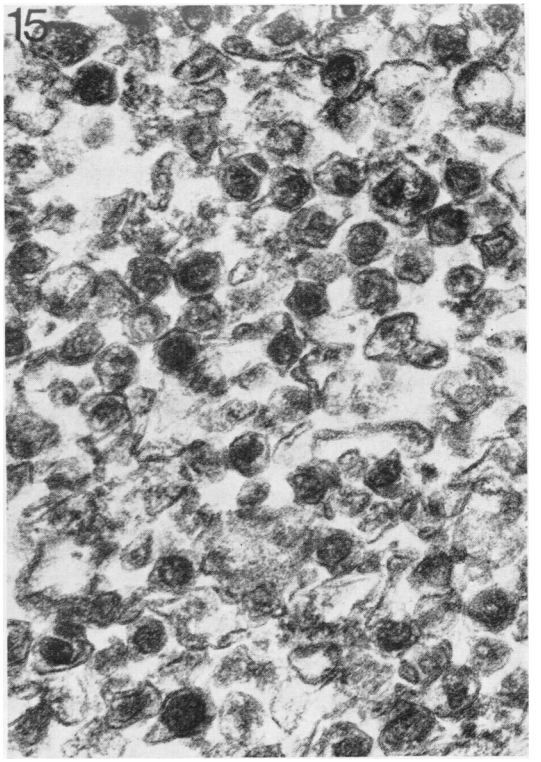


FIG. 12. *Intracisternal A-type particles in endoplasmic reticulum of 4705-induced fibrosarcoma.* × *60,000.*

FIG. 13. *Group of mature C-type particles between two cells of NCTC 4823 in third subculture after exposure to virus isolated from 4705-induced fibrosarcoma.* × *70,000.*





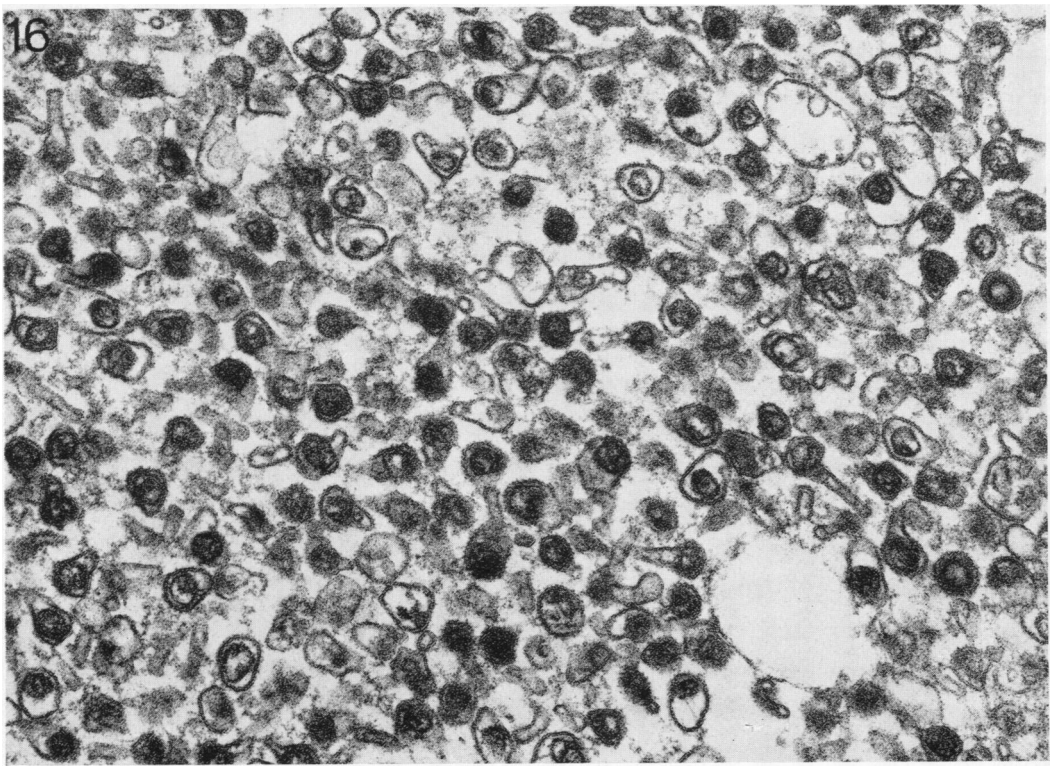


FIG. 14. *Thin section through pellet of a virus isolation from 4705-induced fibrosarcoma. Note small number of particles present.* × *60,000.*

FIG. 15. *Thin section through pellet obtained by centrifuging used medium from NCTC 4705 at 29,000 rev/min in a Spinco no. 30 rotor for 1 hr.* × *60,000.*

FIG. 16. *Thin section through pellet obtained by centrifuging used medium from Swiss mouse embryo tissue culture cells used as recovery test of CF done on NCTC 4705. Medium spun at 29,000 rev/min in a Spinco no. 30 rotor for 1 hr.* × *60,000.*

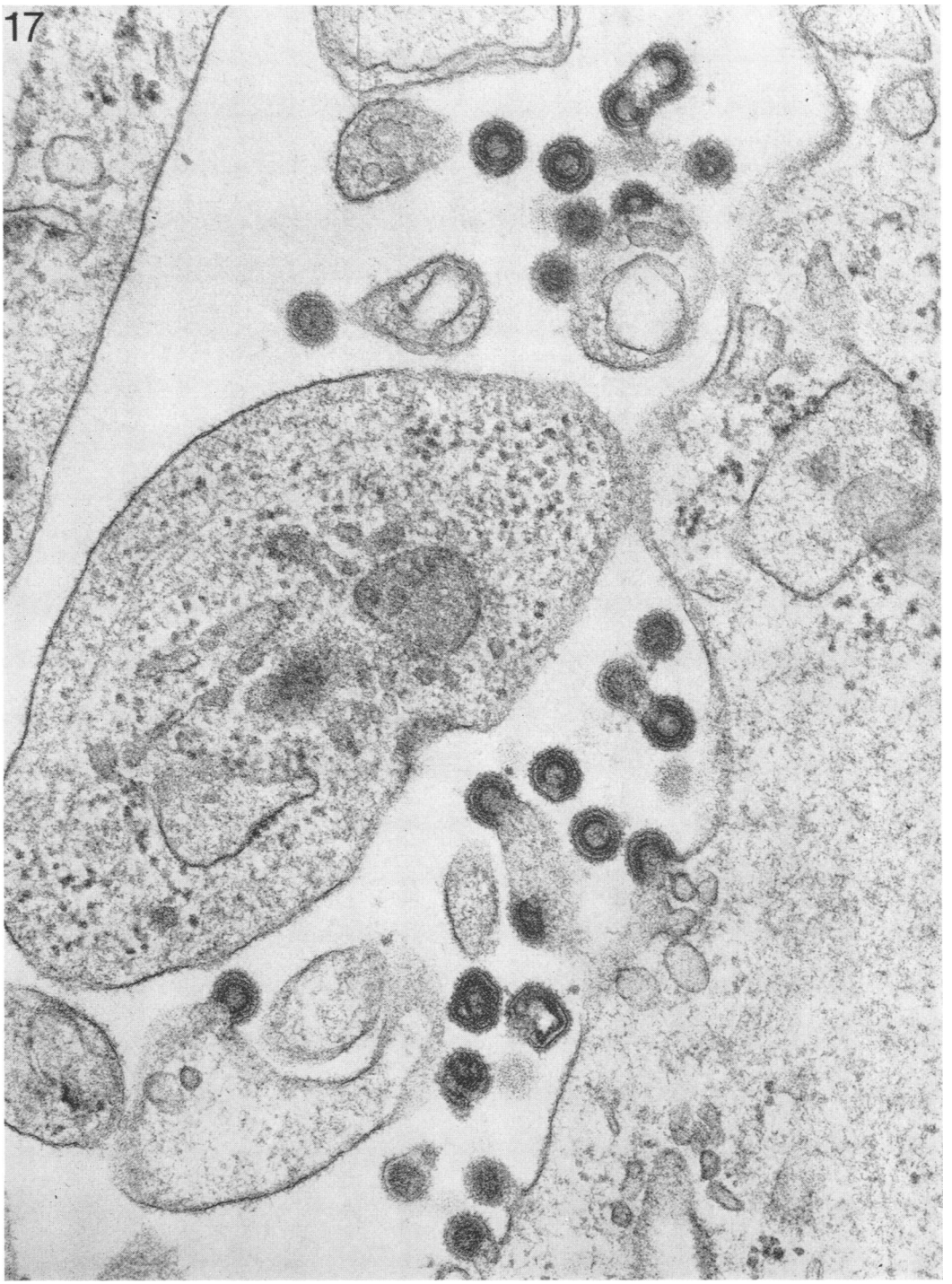


FIG. 17. *Swiss mouse embryo tissue culture cells from CF recovery test of NCTC 4705. Control cells were always negative.* × *80,000.*

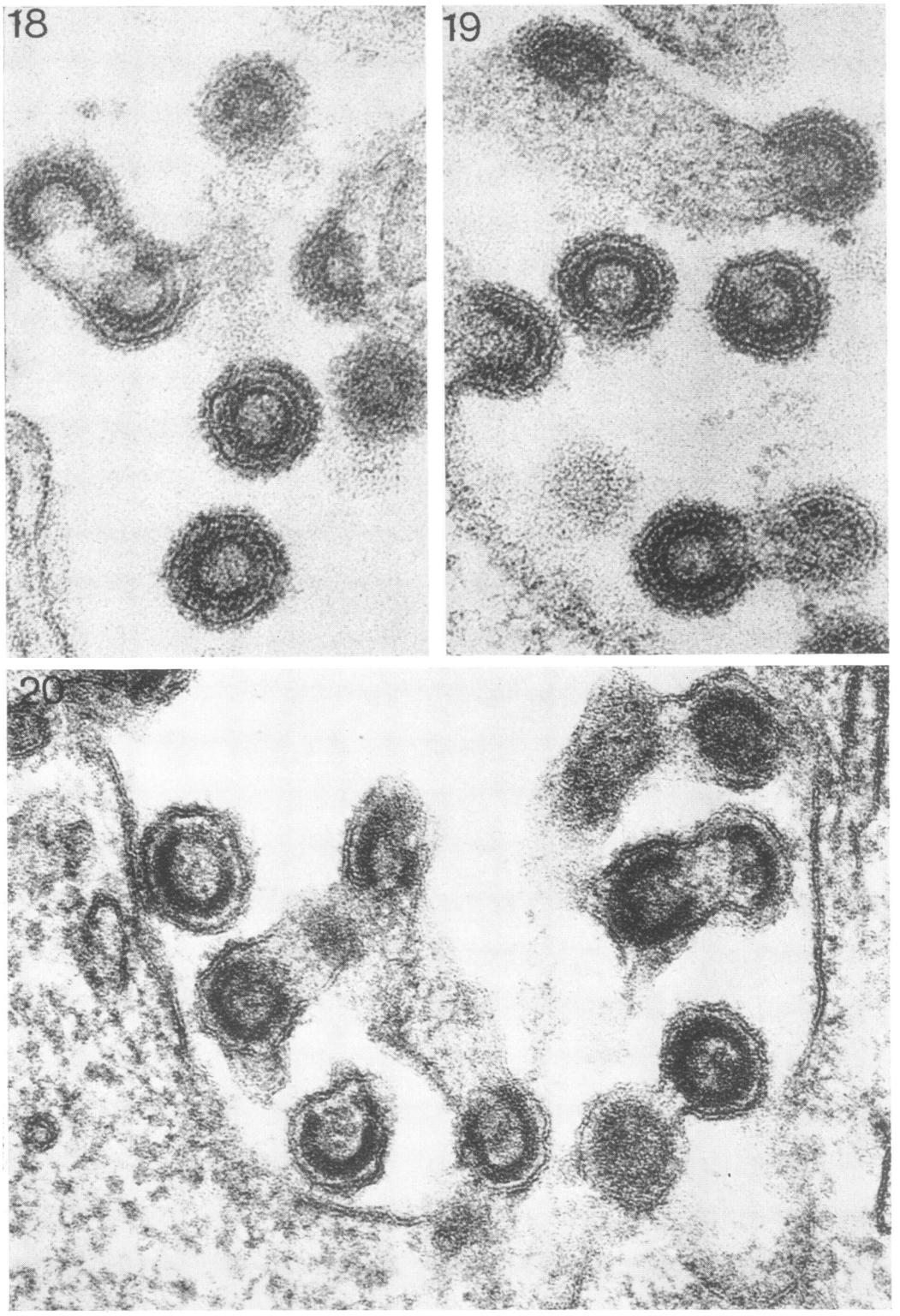


FIG. 18 and 19. *Higher magnification of immature C-type virus particles present in Fig. 17.* × *180,000.*
FIG. 20. *Immature C-type particles in intracytoplasmic vacuole of fibroblast from 4705-induced fibrosarcoma.* × *180,000.*

likely due to the Swiss mouse embryo cultures than to the fetal calf serum in the medium.

From a piece of tumor of the sixth intramuscular transplant, neutralization tests were carried out by Janet Hartley. In Swiss mouse embryo tissue cultures, the antigen-inducing capacity of a 1:2 dilution of the tumor extract was neutralized by a rat serum which also neutralizes Gross and AKR leukemia viruses, but not by rat sera which specifically neutralize Rauscher, Friend, or Moloney leukemia viruses, nor by control serum. The $TCID_{50}$ of a 10% extract of this tumor was found to be $10^{2.2}$ per 0.1 ml, based on CF antigen detectable by "broad" rat serum.

Source of virus. Once a transplantable tumor line had been established, two sources of virus were available: tumor tissue and cell cultures or medium, or both. The former source had the advantage of rapid growth and straightforward extraction procedures; the latter had a much lower probability of outside contamination from virus which might have been easily acquired during the serial passage of the tumor in mice. Likewise, virus pellets prepared from the medium were much more highly concentrated than those prepared from tumor by direct differential centrifugation procedures (Fig. 14, 15, and 16).

As mentioned previously, both sources of virus have yielded infective particles, as indicated by CF and EM. However, the minimal time before which the virus can be detected after infection has not been determined.

An additional source of virus was the Swiss mouse embryo cultures used for the virus recovery test in the CF procedure. This material has consistently shown higher titer than the direct test. In ultrathin sections of cell pellets, large numbers of extracellular particles were readily visible adjacent to the cells of the 4705 culture; yet, this was not the case with the Swiss mouse embryo tissue cultures, in spite of their normally higher CF titers. Thus, it was at first thought that the CF titer did not accurately reflect the quantity of virus particles present in the cultures. But when, in addition to the cells, the culture medium was also separately centrifuged, many more particles were found in the serum-containing medium (SMETC) than in the serum-free medium (NCTC 4705).

Infectivity of the virus. The ability of 4705-virus to infect virus-free, CF-negative tissue cultures has been previously mentioned. Our attempts have been limited to tissue cultures of C3H/HeN embryos (Fig. 13), Swiss mouse embryos (Fig. 17), and rat kidney fibroblasts (generously supplied by H. Duc-Nguyen of the National Cancer Institute). Virus infection was considered successful when the treated cultures gave positive CF tests and when both budding and mature C particles were detected by EM. On two separate occasions, rat kidney fibroblasts were exposed to 4705 virus, but CF and EM were negative for several subcultures of each subline. Table 2 contains the results of attempts to infect various culture lines with NCTC 4705-derived virus. All of the treated cultures of murine origin gave positive CF tests and possessed morphologically identifiable budding and mature C particles.

After it was found that 4705 virus was morphologically and antigenically similar to known leukemia viruses and that, when introduced into murine tissue cultures, it could infect them and in turn induce replication of infective virus, the next step was to test its effect in vivo. As a preliminary experiment, eight 3-day-old C3H/He, six 4-day-old DD, and six 4-day-old BALB/c mice were inoculated subcutaneously with 0.1 ml of a 1-g equivalent/ml of tumor-derived virus. Ten months later none of these animals showed any signs of leukemia. More recently, 70 C3H/He mice (1 to 2 days old) were inoculated intraperitoneally with 0.05 ml of a 1 g equivalent/ml of tumor-extracted virus. Three weeks later, one half of the animals were given a booster inoculation of the same concentration as the original. The virus used for inoculations had been previously stored in liquid nitrogen for 5 months. To ascertain its stability prior to injection into animals, one ampoule of virus was thawed and tested positively for direct and recovery complement fixation. The animals have carried the virus for 30 weeks with no visible effects.

As a result of these infectivity experiments, we have four sublines of NCTC 4823 containing the 4705 virus. Two of the sublines have been carried in vitro for more than 300 days and have shown no signs of morphological or neoplastic transformation, either by altered foci in the culture flasks or by production of invasive tumors after implantation into C3H/HeN mice. Cells were tested for malignancy after 68, 158, and 222 days in vitro by implantation into irradiated C3H/HeN weanling mice. After 4 months, the animals are still free from palpable tumors. NCTC 4823 has been the control in all of these experiments.

NCTC 4705 itself had, by the time of its first successful subculture, already undergone neoplastic transformation (2). It was not until the 43rd subculture that the cells were investigated with an electron microscope and the virus described, though its presence was indicated as early as the 10th subculture, when the CF test was first performed and found to be positive.

Recently, the 4705-virus prepared from an extract of tumor taken from a C3H/HeN mouse failed to function as a "helper" or "rescue" virus

for Moloney sarcoma virus. However, when the same virus was passaged three times through Swiss mouse embryo tissue cultures, it was able to act as helper virus (J. W. Hartley, *personal communication*). Further investigations are now underway on this aspect of the 4705 virus.

DISCUSSION

Several reports have appeared in the literature of viruses from both tumor and tissue culture morphologically similar to the C particle, which is characteristic of leukemia viruses. For the most part, the authors (9, 11, 30, 40) did not demonstrate biological activity and thus justifiably preferred the expression "viruslike particles." We have previously reported on 18 cell lines (of which 4705 is one) that have been positive for murine leukemia CF antigen and for the presence of C particles detectable by EM (26). The second part of the CF test is, as previously mentioned, a recovery test, and depends upon the ability of the CF-positive cells being tested (i.e., test cultures) to induce antigen production in a CF-negative and virus-free Swiss mouse embryo tissue culture. Investigation with an electron microscope of samples of Swiss cultures used in a positive recovery test has revealed budding and mature C particles like those in the test cultures. Such particles are consistently absent from the Swiss mouse embryo tissue culture controls. Thus, it can be concluded that the "viruslike particles" in these cultures are biologically active.

The inability of a leukemia-like particle to induce leukemia does not disprove the infective capacity of that particle. The 4705 virus provides an example of such a case. Although morphoogically similar to a leukemia virus, antigenically similar to a leukemia virus, and neutralized by antiserum to a leukemia virus, it does not cause leukemia when injected into newborn mice. It can, however, infect virus-free tissue cultures and successfully replicate in them.

Thus far, at least 14 murine leukemogenic viruses have been reported (37, 56), varying in source of isolation, host sensitivity, neoplastic cells affected, and, to some extent, in immunological properties. Whether these viruses represent, in fact, a single virus or a family of closely related viruses is still a point of contention. These relationships have been discussed by several investigators (23, 24, 36, 43, 44). No claim is made here that the 4705 virus represents still another leukemia virus. Immunological evidence would tend to indicate that it is strikingly similar to the Gross virus. However, no actual leukemogenic potential has been demonstrated, and, therefore, the present agent cannot at this time be considered a leukemogen.

Wright and Lasfargues (57, 58) and Bernard et al. (6) have shown the tendency of Rauscher virus towards attenuation in long-term tissue cultures. Since 4705 virus was originally tissue culture-derived and had been in vitro 866 days before it was injected into any mice, this may partially explain its lack of leukemogenicity. It may be possible to build up its virulence by continued blind passaging of thymus and spleen extracts after first inoculating newborns with the 4705 virus.

The capacity of several oncogenic viruses to induce malignant transformation in vitro has been reported. These include, among others, the Rous sarcoma virus (32, 51), polyoma virus (15, 33, 47, 53), avian myeloblastosis virus (4, 5), simian virus 40 (3, 41, 50), Moloney sarcoma virus (52), and Rauscher (14), Gross (29), and Friend (39) leukemia viruses. A critical review of much of the pertinent literature has been made by Sanford (49). The role of 4705-virus in the transformation of NCTC 4705 has not been determined. Since NCTC 4823 was derived from a similar source, it was hoped that introduction of 4705 virus into this culture would give some indication of its transforming capabilities. Thus far, 4823 infected with 4705 virus has remained nonneoplastic for more than 300 days in vitro. It is felt that the virus was present in NCTC 4705 from the inception of the culture, but its role, if any, in transforming this cell line must remain equivocal, especially since the virus has thus far failed to induce neoplastic transformation in the NCTC 4823 subline.

A further consideration must be that NCTC 4705 has been grown exclusively on chemically defined medium, whereas 4823 was and is on medium NCTC 135 (= chemically defined medium) *plus* 10% fetal calf serum. Evans et al. (17) and Black and Rowe (7) have reported that the serum constituents of the medium influence the rate of the neoplastic transformation. It may be that both the virus and the medium had some influence on the neoplastic transformation of NCTC 4705.

During the entire course of the investigation, the mouse leukemia antigen titer was generally higher in the Swiss mouse embryo tissue cultures used for the CF recovery test than in the C3H/HeN cultures of 4705. Since this difference did not seem to be a function of the culture medium, at least to the extent that 4705-virus titers were comparable in the exhausted medium from cultures of 4705 grown in chemically defined medium and in 10% (v/v) fetal calf serum, it might be that the 4705 virus can replicate more effectively in Swiss mouse embryo cells than in C3H. Such a phenomenon is not without precedent. Among others, Duc-Nguyen and Rosenblum (13), Compans et al. (8), and Holmes and Choppin (28)

have reported similar variations in virus titer with change of culture lines for mumps and SV_5 viruses.

Why mature extracellular virus particles remained in close association with 4705 cells and dispersed more freely in the medium in SMETC cultures is still not known. An explanation may come from the observation of both McQuilkin and Earle (31) and Abercrombie (1), who found that when serum was added to tissue cultures cell adhesiveness was reduced. Perhaps the presence of fetal calf serum in the SMETC permitted the virus particles to get free from the clumps apposed to cells pelleted from serum-free medium and thus escape detection when low-speed centrifuge pellets of tissue cultures were examined. Further work is necessary in this area.

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LITERATURE CITED

1. ABERCROMBIE, M. 1967. Contact inhibition: the phenomenon and its biological implication. The 1966 Decennial Review Conference on Cell, Tissue, and Organ Culture. Natl. Cancer Inst. Monograph **26**:249–273.
2. ANDRESEN, W. F., F. M. PRICE, J. L. JACKSON, T. B. DUNN, AND V. J. EVANS. 1967. Characterization and spontaneous neoplastic transformation of mouse embryo cells isolated and continuously cultured in vitro in chemically defined medium NCTC 135. J. Natl. Cancer Inst. **38**:169–183.
3. ASHKENAZI, A., AND J. L. MELNICK. 1963. Tumorigenicity of Simian papovavirus SV 40 and of virus-transformed cells. J. Natl. Cancer Inst. **30**:1227–1265.
4. BALUDA, M. A., AND I. E. GOETZ. 1961. Morphological conversion of cell cultures by avian myeloblastosis virus. Virology **15**:185–199.
5. BEAUDREAU, G. S., G. BECKER, R. A. BONAR, A. M. WALLBANK, D. BEARD, AND J. W. BEARD. 1960. Virus of avian myeloblastosis. XIV. Neoplastic response of normal chicken bone marrow treated with the virus in tissue culture. J. Natl. Cancer Inst. **24**:395–415.
6. BERNARD, C., D. SILVESTRE, J. TANZER, J. PERIES, AND M. BOIRON. 1965. Analyse d'un lignée cellulaire produisant de façon continue le virus leucémique de Rauscher *in vitro*. Exptl. Cell Res. **40**:513–526.
7. BLACK, P., AND W. P. ROWE. 1963. An analysis of SV-40 induced transformation of hamster kidney tissue *in vitro*. I. General characteristics. Proc. Natl. Acad. Sci. U.S. **50**: 606–613.
8. COMPANS, R. W., K. V. HOLMES, S. DALES, AND P. W. CHOPPIN. 1966. An electron microscopic study of moderate and virulent virus-cell inter actions of the parainfluenza virus SV5. Virology **30**: 411–426.
9. DALES, S., AND A. F. HOWATSON. 1961. Virus-like particles in association with L-strain cells. Cancer Res. **21**: 193–197.
10. DALTON, A. J. 1955. A chrome-osmium fixative for electron microscopy. Anat. Rec. **121**:281.
11. DAVID-FERREIRA, J. F., AND R. A. MANAKER. 1965. An electron microscope study of the development of a mouse hepatitis virus in tissue culture cells. J. Cell Biol. **24**:57–78.
12. DEFENDI, V., J. LEHMAN, AND P. KRAEMER. 1963. "Morphologically normal" hamster cells with malignant properties. Virology **19**:592–598.
13. DUC-NGUYEN, H., AND E. N. ROSENBLUM. 1967. Immuno-electron microscopy of the morphogenesis of mumps virus. J. Virol. **1**:415–429.
14. DUC-NGUYEN, H., E. N. ROSENBLUM, N. A. WIVEL, AND M. V. A. SMITH. 1967. Malignant transformation by Rauscher strain murine leukaemia virus. Nature **214**: 815–817.
15. DULBECCO, R. 1963. Transformation of cells *in vitro* by viruses. Science **142**:932–936.
16. EARLE, W. R., AND A. NETTLESHIP. 1943. Production of malignancy in vitro. V. Results of injections of cultures into mice. J. Natl. Cancer Inst. **4**:213–227.
17. EVANS, V. J., AND W. ANDRESEN. 1966. Effect of serum on spontaneous neoplastic transformation *in vitro*. J. Natl. Cancer Inst. **37**:247–249.
18. EVANS, V. J., J. C. BRYANT, H. A. KERR, AND E. L. SCHILLING. 1964. Chemically defined media for cultivation of long-term cell strains from four mammalian species. Exptl. Cell Res. **36**: 439–474.
19. EVANS, V. J., J. C. BRYANT, W. T. MCQUILKIN, M. C. FIORAMONTI, K. K. SANFORD, B. B. WESTFALL, AND W. R. EARLE. 1956. Studies of nutrient media for tissue cells in vitro. II. An improved protein-free chemically defined medium for long-term cultivation of strain L-929 cells. Cancer Res. **16**:87–94.
20. EVANS, V. J., G. A. PARKER, AND T. B. DUNN. 1964. Neoplastic transformation in C3H mouse embryonic tissue in vitro determined by intraocular growth. I. Cells from chemically defined medium with and without serum supplement. J. Natl. Cancer Inst. **32**:89–121.
21. GEY, G. O. 1941. Cytological and cultural observations on transplantable rat sarcomata produced by the inoculation of altered normal cells maintained in continuous culture. Cancer Res. **1**:737.
22. GROBSTEIN, C. 1950. Production of intra-ocular hemorrhage by mouse trophoblast. J. Exptl. Zool. **114**:359–374.
23. GROSS, L. 1964. How many different viruses causing leukemia in mice? Acta Haematol. **32**: 44–62.
24. GROSS, L., 1966. Are the common forms of spontaneous and induced leukemia and lymphomas in mice caused by a single virus? Conference on Murine Leukemia. Natl. Cancer Inst. Monogaph **22**:407–424.
25. HALL, W. T., 1966. Observations on a murine virus in *in vivo* and *in vitro* systems. J. Cell Biol. **31**:44A.

26. HALL, W. T., W. F. ANDRESEN, K. K. SANFORD, V. J. EVANS, AND J. W. HARTLEY. 1967. Virus particles and murine leukemia virus complement-fixing antigen in neoplastic and nonneoplastic cell lines. Science **156**:85–88.
27. HARTLEY, J. W., W. P. ROWE, W. I. CAPPS, R. J. HUEBNER. 1965. Complement fixation and tissue culture assays for mouse leukemia viruses. Proc. Natl. Acad. Sci. U.S. **53**:931–938.
28. HOLMES, K. V., AND P. W. CHOPPIN. 1966. On the role of the response of the cell membrane in determining virus virulence. Contrasting effects of the parainfluenza virus SV5 in two cell types. J. Exptl. Med. **124**:501–520.
29. JOACHIM, H. L. 1967. Neoplastic transformation of rat thymic cells induced in vitro by Gross leukemia virus. Science **155**:585–587.
30. KINDIG, D. A., AND W. H. KIRSTEN. 1967. Virus-like particles in established murine cell lines: electron-microscopic observations. Science **155**: 1543–1545.
31. MCQUILKIN, W. T., AND W. R. EARLE. 1962. Cinemicrographic analysis of cell populations in vitro. J. Natl. Cancer Inst. **28**:763–799.
32. MANAKER, R. A., AND V. GROUPÉ. 1956. Discrete foci of altered chicken embryo cells associated to Rous sarcoma virus in tissue culture. Virology **2**:838–840.
33. MEDINA, D., AND L. SACHS. 1961. Cell-virus interactions with the polyoma virus: the induction of cell transformation and malignancy *in vitro*. Brit. J. Cancer **15**:885–904.
34. MOLLENHAUER, H. H. 1964. Plastic embedding mixtures for use in electron microscopy. Stain Technol. **39**:111–114.
35. MOLONEY, J. B. 1960. Biological studies on a lymphoid leukemia virus extracted from Sarcoma 37. I. Origin and introductory investigations. J. Natl. Cancer Inst. **24**:933–951.
36. MOLONEY, J. B. 1962. The murine leukemias. Federation Proc. **21**:19–31.
37. MOLONEY, J. B. 1965. *In* Viral Etiology of Leukemia, Combined Clinical Staff Conference at the National Institutes of Health (W. Ray Bryan, Moderator), Ann. Internal Med. **62**: 376–399.
38. MORGAN, J. L., H. J. MORTON, AND R. C. PARKER. 1950. Nutrition of animal cells in tissue culture. I. Initial studies on a synthetic medium. Proc. Soc. Exptl. Biol. Med. **73**:1–8.
39. OSATO, T., E. A. MIRAND, J. T. GRACE, AND F. PRICE. 1966. *In vitro* malignant transformation of cells by Friend virus. Nature **209**:779–782.
40. PARSONS, D. F., M. A. BENDER, E. B. DARDEN, G. T. PRATT, AND D. L. LINDSLEY. 1961. Electron microscopy of plasma-cell tumors of the mouse. II. Tissue cultures of the X5563 tumor. J. Biochem. Biophys. Cytol. **9**:369–381.
41. RABSON, A. S., AND R. L. KIRSCHSTEIN. 1962. Induction of malignancy *in vitro* in newborn hamster kidney tissue infected with Simian vacuolating virus (SV40). Proc. Soc. Exptl. Biol. Med. **111**:323–328.
42. REYNOLDS, E. W. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. **17**:208–212.
43. RICH, M. A., J. GELDNER, AND P. MEYERS. 1965. Studies on murine leukemia. J. Natl. Cancer Inst. **35**:523–536.
44. RICH, M. A. 1966. Murine leukemia and its viruses. Conference on Murine Leukemia. Natl. Cancer Inst. Monograph **22**:425–437.
45. ROWE, W. P., J. W. HARTLEY, AND W. I. CAPPS. 1966. Tissue cultures and serologic studies on mouse leukemia viruses. Conference on Murine Leukemia. Natl. Cancer Inst. Monograph **22**: 15–19.
46. SABATINI, D. D., K. G. BENSCH, AND R. J. BARRNETT. 1963. Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. J. Cell Biol. **17**:19–58.
47. SACHS, L., AND D. MEDINA, 1961. *In vitro* transformation of normal cells by polyoma virus. Nature **189**:457–458.
48. SANFORD, K. K., W. R. EARLE, E. SHELTON, E. L SCHILLING, E. M. DUCHESNE, G. D. LIKELY, AND M. M. BECKER. 1950. Production of malignancy in vitro. XII. Further transformations of mouse fibroblasts to sarcomatous cells. J. Natl. Cancer Inst. **11**:351–375.
49. SANFORD, K. K. 1965. Malignant transformation of cells *in vitro*. Intern. Rev. Cytol. **18**:249–311.
50. SHEIN, H. M., AND J. F. ENDERS. 1962. Transformation induced by Simian virus 40 in human renal cell cultures, I. Morphology and growth characteristics. Proc. Natl. Acad. Sci. **48**:1164–1172.
51. TEMIN, H. M., AND H. RUBIN. 1958. Characteristics of an assay for Rous sarcoma virus and Rous sarcoma cells in tissue culture. Virology **6**:669–688.
52. TING, R. C. 1967. *In vitro* transformation of rat embryo cells by a murine sarcoma virus. Virology **28**:783–785.
53. VOGT, M., AND R. DULBECCO. 1960. Virus-cell interaction with a tumor-producing virus. Proc. Nat. Acad. Sci. U.S. **46**:365–370.
54. WAYMOUTH, C. 1959. Rapid proliferation of sublines of NCTC clone 929 (Strain L) mouse cells in a simple chemically defined medium (MB 752/2). J. Natl. Cancer Inst. **22**:1003–1017.
55. WAYMOUTH, C. 1965. The cultivation of cells in chemically defined medium and the malignant transformation of cells in vitro, p. 168–179. *In* C. V. Ramakrishnan [ed.], Tissue culture. Dr. W. Junk Publishers, The Hague.
56. WHO SCIENTIFIC GROUP ON VIRUSES AND CANCER. 1965. Viruses and cancer. WHO Tech. Rept. Ser. 295. World Health Organization, Geneva, Switzerland.
57. WRIGHT, B. S., AND J. C. LASFARGUES. 1965. Long-term propagation of the Rauscher murine leukemia virus in tissue culture. J. Natl. Cancer Inst. **35**:319–327.
58. WRIGHT, B. S., AND J. C. LASFARGUES. 1966. Attenuation of the Rauscher murine leukemia virus through serial passages in tissue culture. Conference on Murine Leukemia. Natl. Cancer Inst. Monograph **22**:685–700.