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Virus Particles and Murine Leukemia Virus Complement-Fixing Antigen in Neoplastic and Nonneoplastic Cell Lines

Abstract. *Twenty-seven lines of murine tissue cultures derived from 12 different cell pools and grown on various media were examined with the electron microscope for morphologically detectable virus particles. They were also tested for complement-fixing mouse leukemia virus antigens and for recoverable virus. A 100-percent correlation between results obtained by these two methods is reported. An additional 19 lines from 8 different cell pools were examined for either virus particles or complement-fixing antigens. All lines were assayed for neoplastic transformation. Seven cell pools gave rise to lines showing evidence of contamination with leukemia virus. Since most of these lines had also undergone "spontaneous" neoplastic transformation in vitro, this virus cannot be excluded as a possible cause of the neoplastic change, or of influencing it. The remaining cell pools gave rise to lines with no evidence of contamination with leukemia virus; but most of these lines also underwent similar transformation. These results suggest that "spontaneous" neoplastic transformation can occur in the absence of detectable mouse leukemia virus.*

It is a rather general consensus of investigators that cells maintained in long-term tissue cultures tend to undergo "spontaneous" neoplastic transformation. "Spontaneous" is understood to mean any transformation to which a known causative agent has not yet been definitely ascribed (*1*). As part of a long-term study of such transformation, we have made attempts to find a virus-free cell line that could be studied through several preneoplastic and neoplastic passages to gain some insight into the transforming process. Virus-like particles, morphologically similar to those identified as the murine leukemia virus, have been described in certain long-established mouse tissue culture cell lines, notably in Earle's strain L cells (*2*), but no biological activity has been associated with them. In the course of screening several cell lines, a strong correlation appeared between observation of C-particles with the electron microscope (EM) and detection of group specific complement-fixing (CF) antigens of mouse leukemia virus. Furthermore, an attempt has been made to correlate these findings with the neoplastic state of the cells in vivo.

Cell pools and cell lines examined by either or both testing methods and their respective sources of origin are listed in Table 1. Because of the recently reported (*3*) inhibition or delay of neoplastic transformation by fetal calf serum, a constituent of the culture medium, the medium we used is also included. The span of days in vitro and the subculture generations during which the cell testings were made are given for each of the cell lines. Some of the lines in medium containing horse serum were transferred to medium with fetal calf serum for two fluid renewals (about 4 days) before the tests for CF antigens, since certain lots of horse serum appeared to be anticomplementary. Table 1 also shows the neoplastic or nonneoplastic state of cell cultures at the time of the CF test and EM examination. All tests for neoplasia were carried out by intraocular or intramuscular implantation of cells into isologous mice, and the only ones considered positive were those that subsequently developed invasive, progressively growing sarcomas. For the CF test, as applied to the murine leukemia viruses, we used the method described previously (*4*), except that the serums were from Fischer rats bearing transplants of a thymic lymphoma induced by Gross virus or of a sarcoma induced by Moloney sarcoma virus. These serums were selected for their high reactivity with a wide variety of mouse leukemia antigens and their complete negative reactivity with control tissue and tissue culture antigens (*5*). Two test procedures were used, a direct CF test on the specific cell line under investigation and a virus-recovery test that utilized the ability of mouse leukemia viruses to induce a group-reactive complement-fixing antigen in Swiss mouse embryo tissue cultures (SMETC) (*4*). Cells scraped from one fully sheeted T-60 flask or from two T-15 flasks were resuspended in 2 to 3 ml of culture medium and the suspension was divided into two parts. Sufficient fluid was removed from one portion to leave an approximate 5-percent suspension, which was then frozen and thawed twice and tested at a 1 : 2 dilution for CF antigen. Antigen titers, ranging from 1 : 2 to 1 : 8, were determined in a few cases. The second sample was frozen and thawed once, and 0.2 ml of the undiluted suspension inoculated into SMETC for virus isolation; all samples giving a negative result were passed at least once to fresh SMETC. Antigen titers in the positive cultures were generally higher than in the original cell line, averaging 1 : 8 or more. All cell lines were tested under code numbers and results were tabulated independently of EM observations.

Some of the cell lines (those marked with asterisks in Table 1) had previously been tested and found negative (by testing for the production of antibodies in mice) for the following viruses: pneumonia virus of mice, reo 3, Sendai, polyoma, K, mouse adenovirus, lymphocytic choriomeningitis, SV_5, rat virus, and Theiler's virus (*6*).

Cells that were examined with the electron microscope were first fixed as a monolayer in the culture flask with 1 percent glutaraldehyde in 0.1*M* phosphate buffer (*p*H 7.3) for 1 hour at room temperature. Thereafter, cultures were rinsed three times with phosphate buffer and then allowed to remain in fresh buffer for 30 minutes. Finally, the buffer was changed again and the cells from two T-15 flasks were scraped loose with a piece of cellophane, combined in one conical centrifuge tube, and spun in an International centrifuge (rotor 269) for 10 minutes at 1800 rev/min. The resulting pellet was cut into 1-mm^3 pieces, fixed again with

Dalton's chrome-osmium (*7*) for 1 hour, and left for 90 minutes in 0.5 percent aqueous uranyl acetate that had been brought to *p*H 5.0 with 1.0*N* NaOH. The tissue was rapidly dehydrated with ethanol and propylene oxide and then embedded in Epon-Araldite (*8*). Sections were cut on a Reichert ultramicrotome with a diamond knife, stained with uranyl acetate and lead citrate, and inspected with a Siemens Elmiskop IA electron microscope.

In general, two types of particles were observed in the cultures, corresponding to the so-called A_1-type (70-mμ diameter) and C-type (100-mμ diameter) particles of Dalton and Bernhard, respectively (*9*). In accordance with the most recent nomenclature (*10*), these are now called intracytoplasmic intracisternal A-particles and extracytoplasmic immature and mature C-particles. This A-particle, not to be confused with the intracytoplasmic A-type particle of the mammary tumor virus, has been found in many types of neoplastic and nonneoplastic cells and tissues, though no biologic activity has been demonstrated. Likewise, no actual developmental association or interrelation has been conclusively established between A-type and C-type particles. From previously published studies and from our own studies (to be published elsewhere) the infective agent of murine leukemia viruses is known to be the C-type particle in either its mature or immature form (*11*). Therefore, in the interpretation of data obtained with

Table 1. Data on cell lines examined for morphologically detectable virus particles by electron microscopy (EM) or for complement-fixing mouse leukemia virus antigens, or both, and for neoplastic or nonneoplastic state of cells. In two cases (cell pools 1 and 3) the cell pool was cloned and thus represents origin from one cell. Days in vitro refer to the total period in culture; number of subcultures, to the number of transfers in medium given in this table. NCTC, National Cancer Institute Tissue Culture; NP, neoplastic; NNP, nonneoplastic; ?, determination incomplete; for CF antigen, + is positive (3^+ or 4^+ reaction) at a dilution of 1:2 or higher, ± is partial fixation of complement, and − is negative.

Cell pool	Cell line (NCTC)†	Source	Medium‡	Days in vitro/No. of subcultures	State of cells	CF antigen		C-particles (EM)
						Direct test	Recovery test	
1	1742	C3H/He connective tissue	4	1955/102	NP			+
	2445		3	4343–4571/263–297	NP	+	+	+
	2472		3	4357–4606/248–269	NP	+	+	+
	2555		3	4407–4548/243–263	NP	+	+	+
	4555		1	5096–5330/72–104	NP	+	+	+
	4700		1	5330–5645/91–137	NP	±	−	+
	5025		1	5394–5461/48–58	NP	−	+	+
	3069	Tumor from 1742	3	3905–3987/206–218	NP	−	+	+
	4953		1	4806–4839/46–53	NP	−	+	+
2	4094	C3Hf/He parotid	3	657–782/14–28	NP	+	+	+
3	929	C3H/An connective tissue	4	9087–9338/745–875	NP	+	+	+
	2071		1	5853–9052/62–504	NP	−	−	−
4	5303*	C57BL/KaLw embryos	2	120/7	?	−	−	
	5303		2	280/16	NP			−
	5431		3	412/18	?	−	−	
	5476		3	412/13	NP	−	−	
5	4093	C57BL/KaLw embryos	3	598/26	NP	−	−	
6	5267*	ALB-M2T embryos	3	73/8	NNP	−	−	
	5267		3	317/38	NP			−
	5268*		2	38/4	NNP	−	−	
	5268		2	317/40	NP			−
7	5143*	C3Hf/HeN embryo	2	124–210/14–33	NNP	−	−	−
	5144*		3	124–245/14–37	NP	−	−	−
8	4075	C3H/HeN parotid	1	2202/137	NP	−	−	
9	5415	C3Hf/HeN embryos	3	41/5	NNP	−	−	
10	5433	C3Hf/HeN embryos	3	52/3	NNP	−	−	
11	5434	C3Hf/HeN embryos	2	52/4	NNP	−	−	
	5435		3	52/3	NNP	−	−	
12	5449	C3Hf/HeN embryos	2	42/4	NNP	−	−	
13	5404	C3H/HeN embryos	3	112–145/10–15	?	−	−	−
	5405		2	112–251/16–36	?	−	−	−
	5417		2	210–251/40–48	?	−	−	
	5418		5	210–251/28–34	NP	−	−	
14	4932*	C3H/HeN embryo	3	523–733/68–98	NP	−	−	−
	5075		3	283–348/19–27	NP	−	+	+
	4933*	Tumor from 4933	2	551–590/93–102	NP	+	+	+
	5458		2	33–51/5–8	NP	+	+	+
15	4705*	C3H/HeN embryos	1	658–1146/10–82	NP	+	+	+
16	5047	C3H/HeN embryos	1	690–893/1–6	?	+		+
17	4823	C3H/HeN embryos	2	750–896/101–121	NNP	−	−	−
	5597	4823 + virus from 4705	2	921–982/2–4	?	+	+	+
	5604		2	982/2	?	+	+	+
18	5190	C3H/HeN embryos	3	312–595/44–82	NP	−	−	
19	5093	C3H/HeN embryos	3	530–740/70–100	NP	−	−	
	5094		2	467–740/62–101	NP	−	−	
20	5255	C3H/HeN embryos	3	366–513/49–70	NP	+	+	
	5259		2	239–513/22–61	?	−	−	
	5289		2	303–513/32–62	NP	−	+	+
	5328		2	366–513/37–58	NP	+		

*Cell lines tested for other murine viruses. See text. †Cell lines 1742 and 2071 were examined by electron microscopy for virus particles by Dr. A. J. Dalton. ‡Medium 1 is chemically defined medium NCTC 135; 2, NCTC 135 plus 10 percent fetal calf serum; 3, NCTC 135 plus 10 percent horse serum; 4, 40 percent horse serum, 20 percent chick embryo extract, and 40 percent saline; and 5, NCTC 135 plus 5 percent fetal calf serum and 5 percent horse serum. For specific constituents see V. J. Evans *et al.* (*13*).

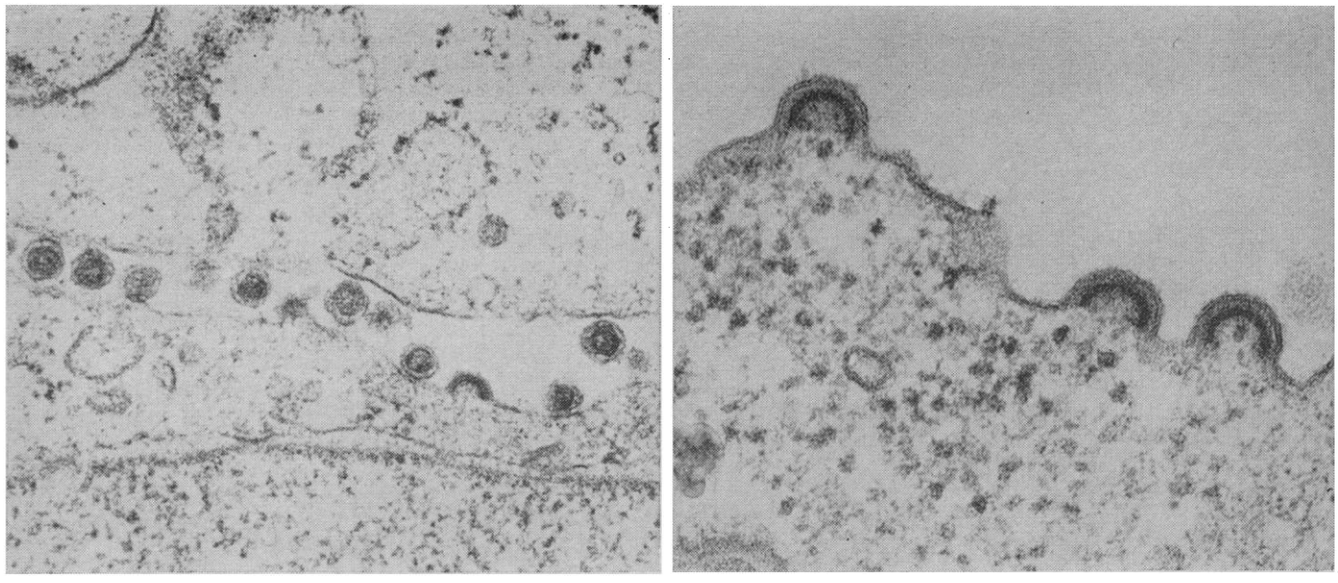

Fig. 1 (left). Section through pellet of cell line 4705 (43rd subculture). Several mature extracellular C-type particles are seen, along with a particle budding from plasma membrane (× 60,000). Fig. 2 (right). Section through pellet of cell line 5075 (4th subculture), showing three particles budding from plasma membrane (× 120,000).

EM only those cultures in which both budding and mature particles could be morphologically identified were considered to contain viruses. In the cell lines designated as positive (+) for C-particles (Table 1), it was fairly easy to find the virus in ultrathin sections, as Figs. 1 and 2 show; where the virus could not be found, however, one is always confronted with the possibility that the culture possessed particles that escaped detection because of either very low titer or poor sampling. When the virus was found, with but one exception, it was in the very first grid examined, as well as in all subsequent grids. Cultures in which budding or mature particles could not be found after a thorough search of two grids from each of five blocks were considered virus-free (at least of morphologically detectable virus). When data were tabulated, it was found that in all cases the presence of budding and mature C-particles was associated with the presence of CF antigen or virus recoverable by virtue of its ability to induce formation of CF antigen in Swiss embryo tissue culture cells, or both.

Although cell lines 4953, 3069, and 5025 gave negative direct CF tests, the virus-recovery tests were convincingly positive. Examination by EM revealed that these cell lines contained a preponderance of intracisternal A-particles but very few C-particles, either mature or immature. It is presumed that the virus titer was too low to provide detectable CF antigen but was sufficient to infect Swiss mouse embryo tissue culture cells. Cell line 4700 offers the only questionable correlation between EM and CF observations. Like the above lines, it was rich in A-particles but had even fewer morphologically detectable C-particles. This line contained low titer (1 : 1) antigen by the direct test, but no virus could be recovered in SMETC.

Because many of the cell lines used in this study have been carried for a number of generations before being tested for particles and CF antigen, the possibility must be considered that virus could have been introduced into the cultures from an extraneous source. It is important to note, however, that positive tests were obtained with lines maintained in media of different chemical composition and with different serum components, including two lines (4705 and 5047) initiated and carried exclusively on serum-free, chemically defined medium. Also, several cell lines were consistently negative, although they were carried in the same lots of medium and in the same environment as other cell lines that were positive. Viruses inducing murine leukemia virus CF antigen have been isolated regularly from primary or secondary tissue cultures of strains AKR, C58, and C3H/Fg mouse embryos and, independently of our study, have been recovered from a small percentage of strain C3H/HeN mouse embryos and from normal specimens of plasma or spleen of adult mice [see Parker *et al.* (*6*); (*12*)]. Therefore it seems likely that the virus we detected was present in the original pools of tissues from strain C3H mice. Also, all lines of cell pool 1 originated in culture from one cell. Three of these were recloned, and a fourth (3069) was cloned after growth of the cells in vivo. Yet all of these, after years of culture as independent lines or clones in vitro, showed evidence of contamination with leukemia virus. Our interpretation of these results is that virus present in the original isolated cell was vertically transmitted to all derivative lines and clones.

Data are not available to determine any temporal correlation between the presence of virus and neoplastic transformation in the naturally infected cell lines. Of possible value will be the long-term observation of cell line 4823 (non-neoplastic for more than 1000 days in vitro) and its subline, designated 5597 in Table 1, which was recently begun by infecting 4823 with cell-free virus extracted from tumors induced by implantation of line 4705 cells into strain C3H/HeN mice.

In correlating the neoplastic or non-neoplastic state of the cells with the EM or CF observations, it may be noted that 7 of the 20 cell pools gave rise to derivative lines that showed evidence of contamination with leukemia virus. In three cases (pools 3, 14, and 20), both positive and negative cell lines arose from the same pool of cells. Since one of these, strain 2071, was

derived from clone 929 of strain L and had been cultured for many years in chemically defined culture medium, it seemed possible that this type of medium failed to support replication of the virus. However, as previously mentioned, other cell lines initiated and continuously maintained on serum-free medium showed evidence of virus. It seems more reasonable to assume: (i) some heterogeneity in the original cell populations with respect to presence or absence of virus; (ii) failure of some cells to support continued virus replication; or (iii) sporadic contamination of cell lines from an extraneous source of virus—although the last seems rather improbable. With one exception, all of the cell lines showing evidence of leukemia virus had also undergone "spontaneous" neoplastic transformation in vitro. From these results, mouse leukemia virus cannot be excluded as a possible cause or influence in the neoplastic conversion of these particular cell lines.

The remaining 13 cell pools gave rise to lines that showed no evidence of contamination with leukemia virus, although many of these had undergone spontaneous neoplastic transformation in vitro. Others, such as lines 5415, 5433, 5434, 5435, and 5449, underwent conversion after the tests for CF antigens and viral recovery. These results suggest that spontaneous neoplastic transformation can occur in the absence of leukemia virus that is detectable by the two testing methods that we used. Eight of these cell lines also gave negative tests for the presence of 10 other murine viruses.

Since the demonstration of complement-fixing antigen in tissue culture cells infected with murine leukemia viruses, coupled with electron microscope observations, provides a means of detecting avirulent or poorly leukemogenic virus, it is now possible to select and monitor "virus-free" cell lines that are used in studies of preneoplastic and postneoplastic changes.

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Growth and Sporulation of a Pyrimidine Spore Color Mutant of Sordaria fimicola

Abstract. *A nonautonomous spore color mutant of* Sordaria fimicola *is a pyrimidine auxotroph that produces hyaline nonviable ascospores. Uracil, uridine, and cytidine are more effective growth factors than cytosine and thymine and, in high concentrations, render the mutant self-fertile by inducing the ascospores to resume development and maturation. Crosses with the unlinked arginine nonautonomus spore color mutant* st-59 *yielded the double mutant* st-59 pyr *that requires both arginine and a pyrimidine for growth, which indicates a lack of suppression of the pyrimidine requirement by the arginine locus.*

Ascospore color mutants of the homothallic ascomycete *Sordaria fimicola* (*1*) are divided into two groups—the autonomous mutants that maintain their charcteristic spore color in the hybrid ascus and the nonautonomous whose ascospores in the hybrid ascus are of wild phenotype. In the second group the ascospores can resume their development and maturation as a result of gene complementation in the hybrid ascus. One of the nonautonomous mutants, *st-59* (*2*), is an arginine-deficient auxotroph; its nutritional requirement and spore color are the function of a single locus. However, neither supplementation with arginine at various concentrations nor complementation in a heterokaryotic mycelium could induce the ascospores to mature in selfed asci of the mutant. It is not yet clear whether the amount of arginine that reaches the ascus in either case is a limiting factor or whether arginine is merely a growth factor that plays no direct role in the development of ascospores. In the latter case, it would be assumed that pleiotropic effects of the locus are the result of a block in biosynthesis of a precursor required for two divergent pathways, one for growth that yields arginine and the other for development of spores.

Four other auxotrophs (*3*) have been found to be partially or completely blocked in self-fertility as a result of the same gene that governs nutritional requirements. Two of them, *a-3* and *st-412*, are partially arginine-deficient and are closely linked to *st-59*. Thus, nutritional requirements of such auxotrophic mutants, along with their centromere-locus intervals, not only provide the best criteria for selecting self-sterile mutants at complex loci but also indicate an approach to the study of the role of amino acids, pyrimidines, and purines in self-fertility of homothallic

Table 1. Effect of pyrimidines on growth of *pyr* mutant of *Sordaria fimicola*. Values are averages of triplicate assays representing dry weights of mycelium (in milligrams) grown in 50 ml of minimal medium for 6 days at 26° ± 1°C.*

Conc. of supplement (μg/ml)	Dry weight of mycelium (in mg) after addition of			
	Uracil	Uridine	Cytidine	Cytosine
100	212.3	158.0	189.0	14.1
50	196.6	98.8	114.8	9.4
20	110.6	60.8	62.9	11.6
10	77.2	38.3	44.3	9.3

* The mutant failed to grow on minimal medium; on medium supplemented with uracil (100 μg/ml) growth of both wild type and mutant was about the same.