Effects of Cell Fusion on Production of the Mouse Mammary Tumor Virus—Immunofluorescence Study[1] (35363)

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The mouse mammary tumor virus (MTV) is an RNA virus whose replication in cell cultures derived from spontaneous mouse mammary tumors can be easily demonstrated by electron microscopy and immunofluorescence (1). However, despite numerous attempts to infect normal mouse cells with an MTV of known potency, evidence of replication has not been obtained (2, 3); furthermore, no other species, except the mouse, has ever been found to carry a virus morphologically identical to MTV.

The relative ease with which the cell fusion technique of Harris and Watkins (4) was applied to the successful infection of naturally resistant chicken and hamster cells with poliovirus (5) made it intriguing to investigate the fate of MTV in a fused cell system. Accordingly, we attempted to determine by immunofluorescence: (i) whether fusion of the mammary tumor cells with normal mouse cells would either repress or enhance MTV production; (ii) whether fusion of mouse cells with normal cells of another species would convey comparable results; and (iii) whether mouse/nonmouse cell mixtures and resulting hybrids would be more compatible with a nonmouse host to which they might transmit MTV.

Materials and Methods. Cell lines. The MTV-producing cell line used in this study was the certified cell line 51 (CCL-51, American Type Culture Collection, Rockville, Maryland) isolated in 1962 by J. Sykes from a spontaneous mammary tumor in a (C57BL×Af) F_1 hybrid mouse. This cell line, here referred to as MMT, is epithelioid and has shown evidence of continuous production of MTV by electron microscopy and bioassays (6).

Fusion of the MMT cells with two normal mammary cells lines, MG4 and RMG, was attempted: (i) MG4, derived 3 years ago from the mammary glands of a C57BL mouse in its early first pregnancy, showed a large population of fibroblasts intermixed with some epithelioid and amoeboid cells; (ii) RMG, derived in 1968 from the mammary glands of an early-pregnant Amsterdam/IMR rat, consisted of nearly equal populations of fibroblasts and epithelioid cells. These two lines, tested over the years by electron microscopy, gel-immunodiffusion and immunofluorescence, have consistently been free of MTV and leukemogenic viruses.

Procedure. Fusion was induced with Sendai virus (initial titer 32,000 HAU/ml) inactivated by β-popiolactone (7). This virus, obtained through the courtesy of Dr. Warren Nichols,[2] was diluted in Hanks' balanced salt solution and used in 1-ml aliquots titrating 8000 HAU/ml.

Following trypsinization, MMT and normal mammary cells were mixed together in equal numbers, spun at low speed for 5 min and resuspended in 1 ml of the Sendai-virus solution. Cell clumping occurred while the cell suspension was held at 4° for 20 min. After an additional 30 min in a 37° water bath, the cells were gently spun down and resuspended in enough culture medium for distribution of 5-ml aliquots containing 2×10^6 cells into a series of T30 Falcon plastic flasks. Control cultures prepared with an equal density of nonfused cells were made from each cell line. The culture medium used throughout these experiments was Eagle's

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MEM with 20% fetal calf serum and 20 μg of insulin[3]/ml.

Detection of MTV. The indirect method of cell-membrane immunofluorescence described for tissue culture by Klein *et al.* (8) was used to detect MTV. Antisera against MTV and Gross leukemia virus were prepared according to the procedure of Nowinski *et al.* (9). The high specificity of the MTV antiserum obtained by inoculation of density-gradient purified virus provided a method of great sensitivity; a percentage count of the fluorescing cells (based on a total of at least 200) was taken as a standard measurement of virus production. Leukemia virus antiserum was used as one of the controls to ascertain that the eventual production of a leukemia virus would not obscure the production of MTV in the same cell population.

Each cell suspension was also prepared for whole-cell mount electron microscopy, using the negative staining technique. This procedure, recently developed by Kramarsky *et al.* (1), permits a quantitative evaluation of MTV production which can be compared with fluorescence results.

Compatibility test. Ten days after treatment by Sendai virus, the mixed cell cultures and their controls were independently injected subcutaneously into newborn Amsterdam/IMR rats; as in earlier work (10) the rats received 2×10^6 cells suspended in 0.1 ml of culture medium. The degree of tolerance was determined by the relative time in which the injected cells regressed at the site of inoculation.

Results. Cells containing 2 to 5 nuclei were readily observed 1 day after fusion. Because the MMT cells are epithelial and have a large spherical nucelus, they were distinguishable from the associated MG4 or RMG cells which were elongated and have an oval nucleus of smaller size (Figs. 1 and 2). Microscope slide cultures fixed and stained 48 hr following exposure to inactivated Sendai virus showed that about 20% of the cells in the MMT/MG4 population were multinucleated. Only 7%, however, could be recognized as true heterokaryons. This percentage was somewhat higher in the MMT/RMG combination where, out of a total of 35% multinucleated cells, the ratio of heterokaryons was roughly estimated at 1:3.

After 10 days' cultivation several nuclear abnormalities, such as enlargement, multilobation, nuclear pulverization, fragmentation of chromatin, were commonly observed in both of the fused cell populations (Figs. 1–3). Such morphological aberrations were found with less frequency in the MMT cells, which are neoplastic, and rarely if at all in the normal MG4 and RMG cell lines.

Fluorescence. When MMT cells were stained with fluorescein-labeled MTV antibody, the antibody became bound to the virus produced at the cell membrane thus inducing a bright fluorescent ring around the positive cells placed in the UV beam of a fluorescent microscope (Fig. 4). In an MMT population, the ratio of positive cells routinely computed over a 1-year period has varied between 10 and 25% (Table I).

After fusion of MMT with MG4 mouse mammary cells 63, 75, and 84% of the cells were found positive in three independent experiments (Fig. 5). The intensity of fluorescence was stronger than with MMT cells alone, suggesting a stimulation in the production of MTV antigen.

In contrast, fusion of MMT with RMG rat-mammary cells gave a ratio of 15, 18, and 28% positive cells, respectively. The fluorescence was intense in some groups of cells but faint in many others (Fig. 6). This appears to indicate a stimulation of MTV antigen production in the MMT cells and their homokaryons but not in the rat cells.

Leukemia-virus antigens have not been detected in MMT or any of the fused cell lines.

Electron microscopy. The results obtained by immunofluorescence have been confirmed by whole-cell mount electron microscopy. Up to 85% of the MMT/MG4 fused cells have shown B particles budding from their external membrane, whereas only 36% of the MMT/RMG presented a comparable activity. The MMT cells remained in the 20% range.

[3] Crystallized insulin, bovine pancreas, 22.5–23.01 IU/mg; Mann Research Laboratories, New York, New York 10006.

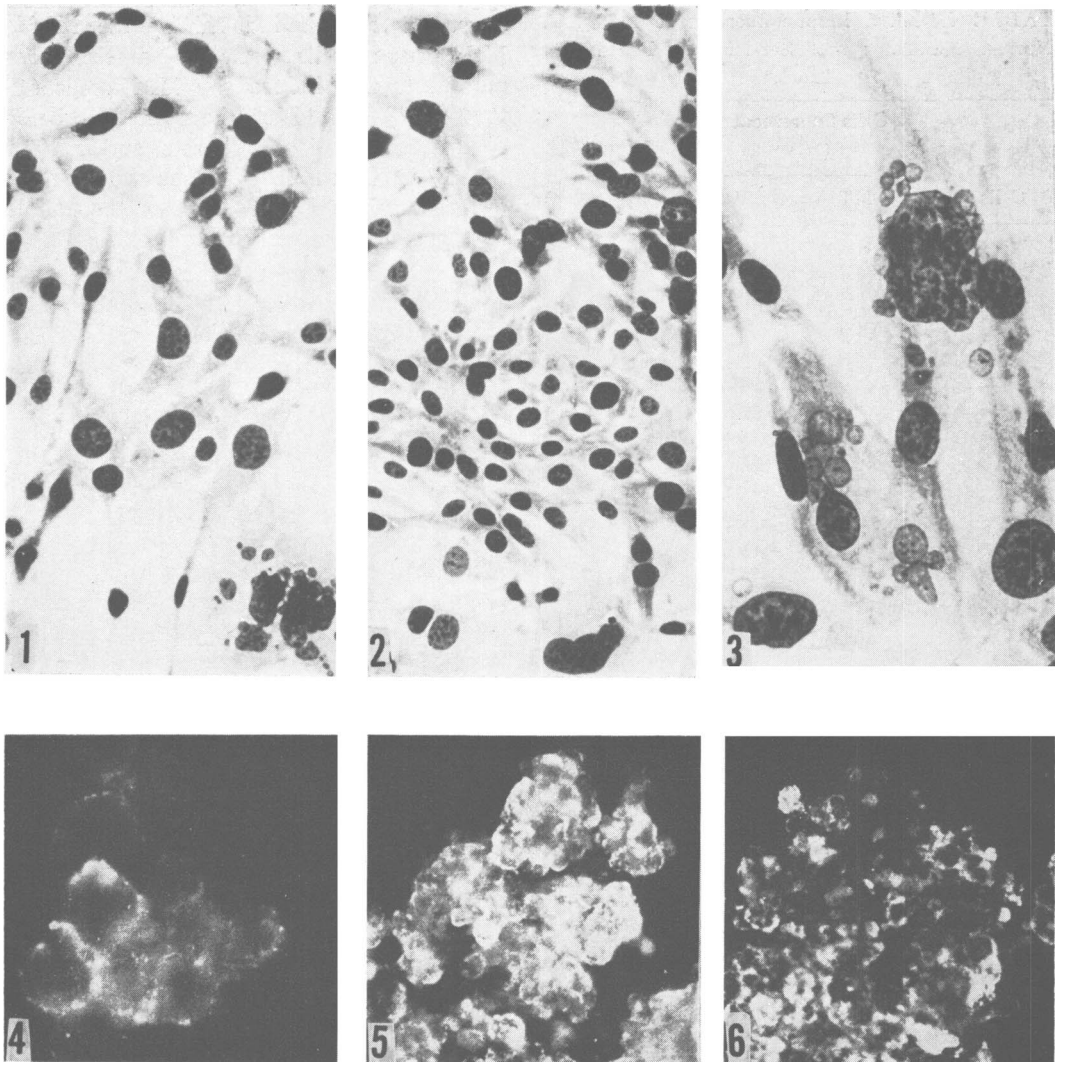

FIG. 1. Normal mouse mammary cells fused with mouse mammary tumor cells (MG4/MMT): The tumor cells have large nuclei, less dense than the nuclei of the normal cells which are smaller and deeply stained. Jenner-Giemsa stain of a culture 10 days after fusion; ×510.

FIG. 2. Normal rat mammary cells fused with mouse mammary tumor cells (RMG/MMT): As in Fig. 1, the nuclei of the tumor cells can be distinguished from the nuclei of the normal rat cells. Jenner-Giemsa stain; ×400.

FIG. 3. MG4/MMT fused cells showing nuclear fragmentation and incorporation of normal nuclei: Jenner-Giemsa stain; ×1600.

FIG. 4. Mammary tumor virus antigen demonstrated by immunofluorescence at the surface membrane of freely suspended MMT cells; ×1300.

FIG. 5. Fluorescence in MG4/MMT cultures 10 days after fusion. Brightness, as indicator of antigen, is intense in most of the cells; ×400.

FIG. 6. Fluorescence in RMG/MMT cultures 10 days after fusion. Some cells show an intense fluorescence while many others do not ×400.

TABLE I. MTV Immunofluorescence in MMT, Normal Mammary and Sendai-virus Fused Cell Lines.[a]

Cells	Cells fluorescent (%)		Electron microscopy (% cells with B particles)
	MTV	LV[b]	
MMT	10–25	0	20–30
MG4	0	0	0
RMG	0	0	0
MMT/MG4	63–75–84	0	68–82–85
MMT/RMG	15–18–28	0	30–27–36

[a] MMT, MG4, and RMG cell lines were tested about twice a month by fluorescence and whole-cell mount electron microscopy. Shown is the range of MTV positive cells found by these 2 techniques over a 1-year period. The average ratios for the two fused cell lines were determined in three independent experiments.

[b] LV, leukemia virus.

Tolerance test. Five days after their inoculation into Amsterdam/IMR newborn rats, the MMT cells formed small, well-circumscribed nodules at the site of injection. These nodules progressed in size until the 10th day, then gradually regressed and disappeared between the 15th and 20th day. Nodules were not induced by the inoculation of normal mouse MG4 or rat RMG cells.

After fusion of the MMT cells with either MG4 or RMG all the rats inoculated had progressing nodules 10 days later. Regression was delayed if compared to that of the MMT nodules; it began 20 to 30 days post-inoculation and was complete within 40 days. The nodules induced by the MMT/MG4 fused cells grew to a size comparable to that induced by MMT cells (1.5 to 2 cm^3) but ¼ of the rats died before complete regression. All rats survived the MMT/RMG combination even though nodules grew up to twice the size of the nodules induced by the former mixture (Table II).

Discussion. These experiments indicated a significant increase of MTV production when the MMT cells were fused with normal mammary cells from the same species. A comparison of the number of positive fluorescent cells with the ratio of heterokaryons found in the stained preparations clearly suggested that an MTV antigen was present on the membrane of many more cells than the actual MMT cells and their fused derivatives. That the antigen was MTV virions was demonstrated by whole-cell mount electron microscopy. It is therefore to be assumed that normal mammary cells which heretofore had been resistant to infection by MTV *in vitro* were infected by the procedure of cell fusion.

This is surprising, considering that B particles which once before had been observed to enter normal mouse embryo cells in less than 1 hr were unable to replicate (11). It is not impossible that a complementary factor (enzyme or perhaps a helper virus), produced by the MMT cells, is required for their multiplication.

In contrast, the smaller number of fluorescent cells obtained following fusion of MMT

TABLE II. Subcutaneous Inoculation of Newborn Amsterdam/IMR Rats with Fused Cell Lines.[a]

Cells	No. of newborn rats inoc.	No. of nodules at		Dead	Total regression (days)
		5 days	10 days		
MG4	24	None	None	0	
RMG	36	None	None	0	
MMT	91	91		12	10–20
MG4/MMT	33	10	33	9[b]	29–37
RMG/MMT	40	18	40	10	30–42

[a] Each newborn Amsterdam/IMR rat received 2.5×10^6 cells sc from the corresponding cell line. The cells were inoculated 1 week after fusion.

[b] Seven of the rats died as a result of fast-progressing, invading tumors 12 days after inoculation.

cells with normal rat cells revealed an incompatibility of the rat cells with MTV. In effect, despite a percentage of heterokaryons higher in MMT/RMG than in MMT/MG4, the ratio of fluorescent-positive cells remained substantially low and the electron microscope showed smooth external cell membranes without budding particles. The uneven and sometimes strong fluorescence which was observed in some cell clumps might be explained by the formation of several MMT homokaryons and some growth stimulation induced by this fusion process.

Whether allogenic or heterogenic, cell fusion produced cell mixtures which were better tolerated when transplanted into rats. The best tolerance was observed towards MMT/RMG transplants where, in at least two instances, large tumor nodules became permanently established. One of the recipients was a female whose milk was tested for MTV by gel-immunodiffusion through all subsequent lactations. Failure to detect MTV seems to confirm the incompatibility of rats to the mouse virus. In contrast, the early death of some of the MMT/MG4 recipients remains unexplained; their autopsy did not reveal any major lesions except the tumor itself.

Summary. Cell fusion between mouse mammary tumor cells and normal mouse cells not only enhanced the production of MTV, as demonstrated by immunofluorescence and electron microscopy, but also appeared to induce infection of normal nonfused cells. In cultures of mouse tumor cells fused with normal rat mammary cells, a definite species specificity of the mouse MTV has been shown, since this virus seemed to replicate exclusively in the mouse tumor cells. In both cases, cell fusion appeared to lower the compatibility barrier of rat recipients to mouse cell transplants but a subsequent infection of the host by MTV could not be demonstrated.

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1. Kramarsky, B., Lasfargues, E. Y., and Moore, D. H., Cancer Res. **30**, 1102 (1970).

2. Lasfargues, E. Y., Murray, M. R., and Moore, D. H., Nat. Cancer Inst. Monogr. **4**, 151 (1960).

3. Lasfargues, E. Y., Pillsbury, N., Lasfargues, J. C., and Moore, D. H., Cancer Res. **30**, 167 (1970).

4. Harris, H., and Watkins, J. F., Nature (London) **205**, 640 (1965).

5. Enders, J. F., Holloway, A., and Crogan, E. A., Proc. Nat. Acad. Sci. U.S.A. **57**, 637 (1967).

6. Sykes, J. A., Whitescarver, J., and Briggs, L., J. Nat. Cancer Inst. **41**, 1315 (1968).

7. Neff, J. M., and Enders, J. F., Proc. Soc. Exp. Biol. Med. **127**, 260 (1968).

8. Klein, G., Clifford, P., Klein, E., Smith, T., Minowada, J., Kourilsky, F., and Burchenal, J. H., J. Nat. Cancer Inst. **39**, 1027 (1967).

9. Nowinski, R. C., Old, L. J., Moore, D. H., Geering, G., and Boyse, E. A., Virology **31**, 1 (1967).

10. Lasfargues, E. Y., Kramarsky, B., Sarkar, N. H., Lasfargues, J. C., Pillsbury, N., and Moore, D. H., Cancer Res. **30**, 1109 (1970).

11. Sarkar, N. H., Lasfargues, E. Y., and Moore, D. H., J. Microsc. (Paris) **9**, 477 (1970).

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