JOURNAL OF VIROLOGY, June 1971, p. 753-758

Vol. 7, No. 6
Printed in U.S.A.

Analysis of the Fusion of XC Cells Induced by Homogenates of Murine Leukemia Virus-Infected Cells and by Purified Murine Leukemia Virus

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Received for publication 22 February 1971

The fusion of XC cells induced by murine leukemia virus (MuLV)-infected cells is also induced by homogenates prepared from the infected cells and by purified MuLV. The fusion-inducing factor appears to contain a heat-labile lipoprotein. No synthesis of specific macromolecules by the XC cells is necessary to obtain fusion. The results suggest that specific components of the viral particle are the activators for the fusion process and they may also be present in the membranes of infected cells.

Tissue culture cells of the XC cell line, a Rous sarcoma virus-induced rat tumor (14), undergo syncytium formation when placed in contact with mouse embryo cell cultures infected with murine leukemia virus (MuLV; reference 7). This mixed culture cytopathic effect has been utilized as an indicator system for detecting MuLV-infected cell lines (7) and as a rapid means of titrating these viruses in tissue culture (13). The syncytium formation is thought to be due to cellular fusion rather than to endomitosis (7), but the mechanism of the fusion and the biochemical stimulus for syncytium induction are unknown.

MuLV is released from the infected cells by a process of budding from the plasma membrane. It is thus possible that the activators for the fusion are either virus-specific components of the cellular membrane or the virus itself in the process of budding or adhering to the cellular membrane.

In this study, we report that intact, infected cells are not required but that cellular homogenates also induce the fusion. Furthermore, high concentrations of purified MuLV particles induce the fusion. The results suggest that components of the viral particle which may also be present in the membranes of the infected cells are the activators for the fusion process.

MATERIALS AND METHODS

Tissue culture. The XC cell line (7, 14) and MuLV (Moloney strain) (11) were obtained from J. Hartley and W. Rowe of the National Institute of Allergy and Infectious Diseases.

Cultures of 15- to 17-day-old National Institutes of Health (NIH) Swiss mouse embryos were obtained from Microbiological Associates, Inc. (Bethesda, Md.). Secondary cultures were planted and inoculated as previously described (5, 6). The mouse embryo cells and the XC cells were grown in Eagle's minimal essential medium with 10% fetal bovine serum (Grand Island Biological Co., Grand Island, N.Y.), glutamine (2 mM), penicillin (50 μg/ml), streptomycin (50 units/ml), polymyxin sulfate (100 units/ml), and mycostatin (50 units/ml). Both cell types were grown in 32-oz (0.946 liter) tightly stoppered, glass culture bottles and incubated at 37 C.

Preparation of fusion factor from infected mouse embryo cells. Cell cultures of mouse embryo cells were incubated 1 to 2 weeks after inoculation. The cells were removed from the monolayer by scraping and were suspended in hypotonic buffer (RSB), [0.01 M tris(hydroxymethyl)aminomethane(*p*H 7.4), 0.0015 M $MgCl_2$, 0.01 M NaCl]. The cells were pelleted at 700 × *g*, resuspended in RSB, and homogenized with 20 strokes of a tight fitting Dounce homogenizer. The homogenate was centrifuged for 10 min at 700 × *g*. The resulting 700 × *g* supernatant was centrifuged at 35,000 × *g* for 15 min and the pellet was suspended in RSB. Samples were treated as described. The resultant suspensions were recentrifuged at 35,000 × *g* for 15 min, and the pellets and supernatants were analyzed for fusion-inducing activity.

Development of cellular fusion. The XC cells were removed from the substratum by trypsinization and were planted at 1.2 × 10^6 to 1.4 × 10^6 cells per 60-mm plastic, tissue culture dish (Falcon Plastic, Div.

[1] Recipient of Public Health Service postdoctoral fellowship no. 1-F02-GA 35507-01.

of B-D Laboratories, Los Angeles, Calif.). The cell layer developed to a concentration of approximately 2.5×10^6 cells by 18 to 24 hr at 37 C in a humidified atmosphere containing 5% CO_2. The appropriate sample of the fusion factor was then added, and after 48 hr the cells were stained with hematoxylin.

Cell and membrane fractionation. A cell homogenate was centrifuged for 10 min at 700 × *g*, and the resulting supernatants were centrifuged successively at 11,000 × *g* for 10 min, 35,000 × *g* for 10 min, and 90,000 × *g* for 1 hr. To fractionate cell membranes, the 700 × *g* supernatant was made 30% (by weight) sucrose in RSB by addition of 65% sucrose. A discontinuous gradient (3) was formed by layering sucrose solutions in the following order: 3 ml of 65%, 7 ml of 45%, 7 ml of 40%, 10 ml of fusion factor suspension in 30% sucrose, 7 ml of 25%, and 4 ml of RSB on top, The gradient was centrifuged at 86,000 × *g* for 16 hr in a Spinco model SW 27 rotor. Visible bands of material were collected by means of a Pasteur pipette. The fractions were diluted 5- to 10-fold in RSB and pelleted at 35,000 × *g* for 1 hr. The pellets were suspended in 1 ml of RSB, and densities were determined by weight ratios relative to water. In all experiments, protein concentrations were determined by the method described by Lowry et al. (9) by using bovine serum albumin as the standard.

Purification of MuLV. Purified MuLV was obtained from Electro-Nucleonics Laboratories (Bethesda, Md.). It had been purified by use of one or two isopycnic sucrose gradients with zonal ultracentrifugation (16). In the first gradient, virus was collected at densities of 1.14 to 1.16 g/ml, whereas, in the second gradient, virus was collected at 1.15 to 1.16 g/ml. Viral particles from both collections were concentrated to 10^{11} to 10^{12} virus particles/ml. The final concentrations were determined by electron microscopy. The second banding removed some nonviral material. The concentrated, once-banded virus contained 0.8 to 1.0 mg of protein/ml (i.e., per 10^{11} to 10^{12} virus particles), whereas the concentrated, twice-banded virus contained 0.5 to 0.7 mg of protein/ml.

Electron microscopy. The 700 to 35,000 × *g* pellets were fixed in glutaraldehyde and osmium tetroxide (4) and embedded by the method described by Luft (10). The electron micrographs were taken by William Hall of Electro-Nucleonics Laboratories at ×19,000 magnification by using an Hitachi HU-11E electron microscope.

RESULTS

Description of fusion process. When XC cells are added to a monolayer of mouse embryo cells infected with MuLV, syncytium formation occurs. Our preliminary experiments showed that similar syncytium formation also occurs when infected mouse cells are added to XC monolayers. Within a few hours after addition of infected mouse embryo cells or their homogenates, but not uninfected cells or uninfected cell homogenates, to a monolayer of XC cells, cellular fusion is apparent (Fig. 1). The multinucleated cells become larger and more numerous with further incubation, and the fusion process is complete within 48 hr. The resulting cells can be divided into three general groups: (i) small multinucleated cells composed of 2 to 10 nuclei; (ii) larger cells composed of about 10 to 30 nuclei, often accompanied by vacuoles and occasionally nuclear fusion; and (iii) extremely large cells composed of an indeterminable number of nuclei with nuclear fusion, often extensive, and marked vacuolization.

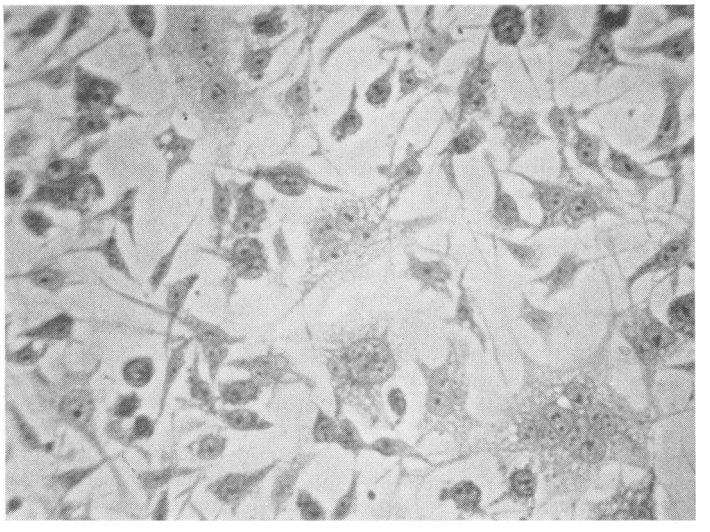

FIG. 1. *XC cells in the process of fusion. Cells were stained with hematoxylin at 5 hr after addition of the fusion-inducing factor. An identical sample caused +5 fusion in 48 hr in another culture.* × *250.*

The size and number of the multinucleated cells is dependent upon the amount of fusion factor added. Hence, a fusion index system based on the number of multinucleated cells and the number of nuclei per cell can be utilized as a method of quantitation. In this index system, fusion consisting of a small number of multinucleated cells and a few nuclei per cell is given a +1 rating, whereas fusion involving roughly one-half of the cells is rated +3. In the fusion rated +5, essentially all the XC cells are involved in the fusion, and numerous, extremely large, bizarre cells are apparent. Typical results are shown in Fig. 2 and 3. The presence of excessive nuclear fusion prevents the utilization of a more precise quantitative method such as that based on the exact number of nuclei per cell.

Distribution of the fusion activity. The distribution of the fusion-stimulating activity in the infected cell homogenates is shown in Table 1. The activity is detected in all of the particulate fractions but not in the 90,000 × *g* supernatant. The 11,000 to 35,000 × *g* fraction is most active. This fraction represents at least a fivefold purification of the fusion factor over the whole homogenate.

Various treatments of the homogenate were utilized in attempts to solubilize the fusion factors. The treatments used were sonic treatment for 2 min at 0 C (model W14OD, Sonifier Cell Disruptor, 50 w power), rapid freezing and thawing in RSB or in RSB plus 1.7 M KCl, and storage of the homogenate in RSB plus 0.001 M ethylenediaminetetraacetic acid overnight at 4 C. None of these treatments significantly alters the distribution, although they decrease the total amount of fusion caused by a given sample of the homogenate.

The density of the cytoplasmic membrane fractions containing the fusion-inducing activity was determined by discontinuous sucrose gradient centrifugation (Table 2). In the gradient system applied, the most active components band at a density of 1.15 g/cc with an additional band of activity of 1.18 g/cc. There are no visible bands other than those indicated.

Stability studies. The stability of the fusion-stimulating factors to treatment by various enzymes was tested. Samples of the 700 × *g* supernatant or 700 to 35,000 × *g* fraction (200 to 300 μg of protein) were incubated for 15 to 30 min at 37 C in the presence of one of the following enzymes: ribonuclease (0.5 μg/ml), deoxyribonuclease (25 μg/ml), trypsin (5 to 200 μg/ml), or neuraminidase (50 μg/ml). The trypsin treatment at concentrations as low as 5 μg/ml completely destroys the fusion-stimulating activity, whereas the other enzymes have no effect. Incubation overnight at 0 C with the lipase, steapsin (0.07%; Nutritional Biochemicals Corp., Cleveland, Ohio), also destroys the activity.

Treatment of the homogenates with ether followed by removal of the ether by bubbling with nitrogen completely destroys the activity.

The activity is stable during 10 to 15 min of

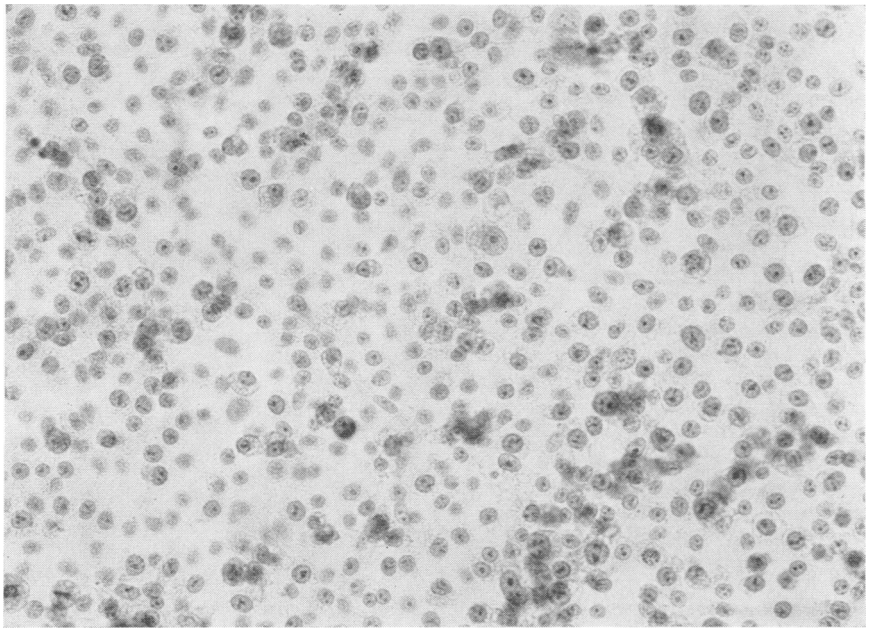

FIG. 2. *Control XC cell culture.* × *250.*

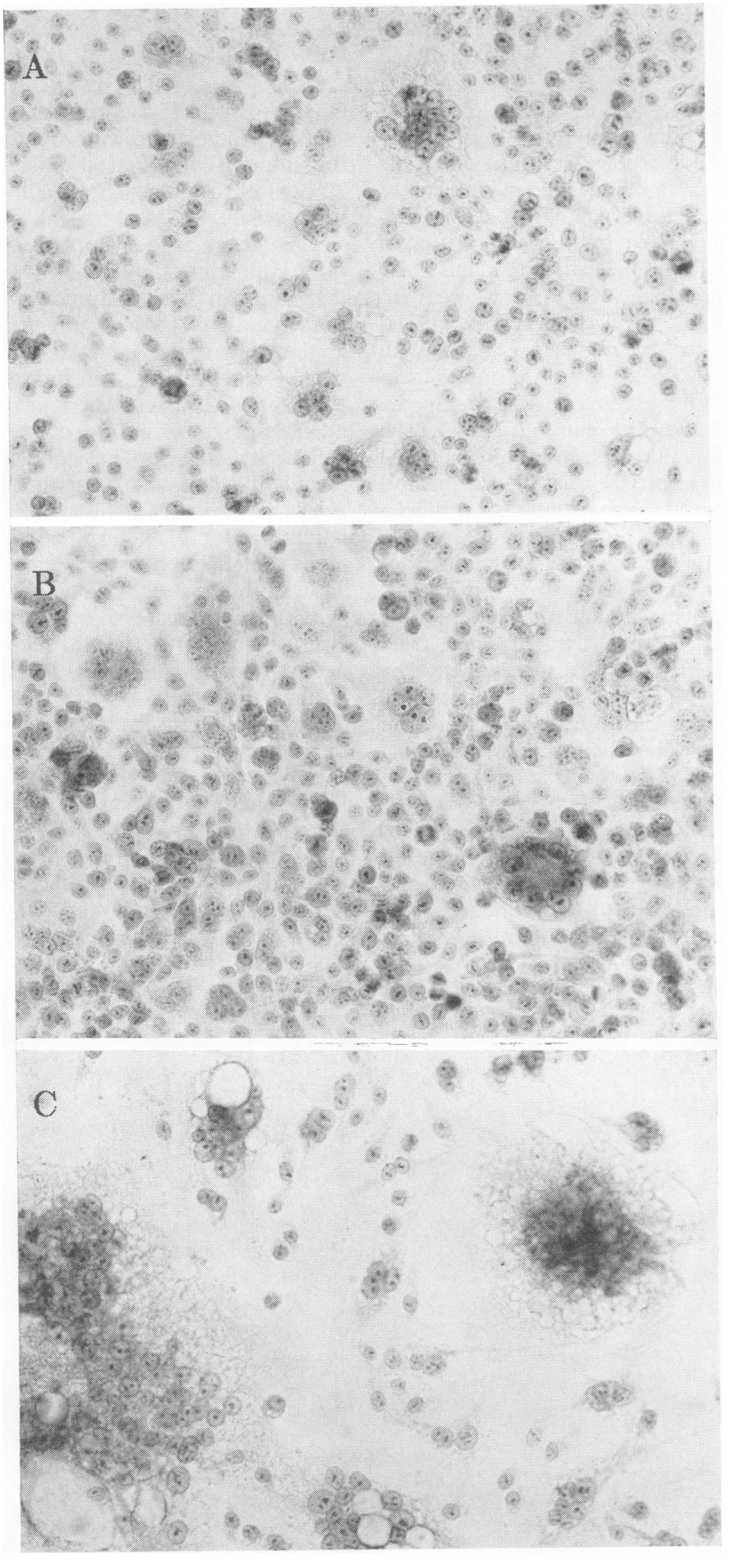

FIG. 3. *Syncytium induced by homogenates of MuLV-infected mouse embryo cells. Pictures show typical examples of the fusion index system employed in this study: (A) +2 fusion rating; (B) +3 fusion rating; (C) +5 fusion rating.* × *250.*

TABLE 1. *Distribution of fusion-inducing activity in mouse embryo cell homogenates*

Fraction	Amt of protein tested (μg)	Fusion index
Whole cell homogenate...	510	+3
700 × *g* Pellet.....	350	+2
700 × *g* Pellet.....	1,050	+4
700 to 11,000 × *g*.....	350	+5
11,000 to 35,000 × *g*.....	120	+4
35,000 to 90,000 × *g*.....	240	+2
90,000 × *g* Supernatant...	500	0

TABLE 2. *Density of cell homogenate fractions containing fusion-inducing activity*

Density (g/cc)	Amt of protein tested (μg)	Fusion index
1.099	97	0
1.100	97	+1
1.146	125	+3
1.181	230	+2

incubation at 37 or 44 C but is completely inactivated at 50 C. About 50% of the activity is destroyed during 6 hr of incubation at 37 C.

Fusion inducing ability of purified MuLV. Since MuLV is released from the cell by budding from the cellular membrane, it seems possible that virus particles in the process of budding or loosely adhering to the cell membrane may be present in the cellular homogenate and thus be the stimulus for syncytium induction. To check for such a possibility we tested purified virus for fusion-inducing ability and checked for the presence of viral particles in the homogenate. Extensive fusion is observed with the addition of large amounts (more than 10^9 viral particles per dish) of both once-and twice-banded virus (Table 3); however, negligible fusion is observed with smaller samples of the virus. The resulting multinucleated cells develop within a few hours after addition of the virus and are identical in appearance to those shown in Fig. 3. The twice banded virus is approximately five times more effective per milligram of protein than the most efficient particulate fraction from infected mouse embryo cells (Table 1); 25 μg of virus protein is required for a +4 fusion, whereas 120 μg of protein from the particulate fraction is required for similar levels of fusion. It appears that the fusion is caused by the virus and not by a cellular contaminant in the virus fraction since approximately equal amounts of fusion are obtained by using an equal number of particles of once-purified or twice-purified virus (*see* Materials and Methods). Also, the presence of a cellular contaminant banding at a density of 1.15 to 1.16 g/ml in sufficient amounts to induce the fusion is unlikely.

Electron microscopy was employed to estimate the number of viral particles in the cellular homogenate. Numerous viral particles are found in the 700 to 35,000 × *g* fraction of the infected mouse embryo cell homogenate. As many as 10^9 to 10^{10} particles are present in samples which cause extensive fusion. Since this is in range of the results shown in Table 3 for purified virus, it is indeed possible that the fusion caused by the cellular homogenates is due solely to the viral particles and not to specific components in the cellular membranes, but additional investigations are necessary for an unequivocal answer to this question.

Is there a requirement for cellular metabolic activity for fusion? XC cell cultures were incubated for 6 hr at 37 C with 10^{10} viral particles (once banded) and one of the following compounds: actinomycin D (0.5 μg/ml), puromycin (20 μg/ml), cycloheximide (10 μg/ml), rifamycin (120 μg/ml), or rat serum interferon (60 units in 0.1 ml rat serum). None of the added compounds inhibited or activated fusion, suggesting that specific synthesis of macromolecules or viral particles by the XC cells is not required for syncytium formation.

DISCUSSION

XC cells undergo syncytium formation when placed in contact with cells infected with MuLV. The syncytium formation is thought to be due to fusion rather than by endomitosis (7). The results of this study show that intact, infected cells are not required, but that homogenates of the infected cells and also purified viral particles will induce XC cell fusion.

MuLV is released from the cells by a budding process. Thus, viral specific products may remain as part of the cell membrane and induce the fusion, or viral particles in the process of budding or loosely adhering to the cell surface after budding may be responsible for the fusion. Virus-specific antigens are found in infected cell membranes (12). However, the large number of viral particles observed in the infected cell homogenates suggest

TABLE 3. *Fusion induced by murine leukemia virus*

No. of virus particles	Fusion index	
	Once banded	Twice banded
1×10^9	+1	+2
2×10^9	+2	+3
4×10^9	+4	+4
10×10^9	+5	+5

that the viral particles may be solely responsible for the fusion. Further investigation of the homogenates is necessary for an unequivocal answer.

Cellular fusion induced by various strains of Newcastle disease virus (NDV) is of one of two types, fusion from within or fusion from without (2). Since the fusion induced by MuLV (Moloney strain) is insensitive to inhibitors of macromolecular synthesis, the process would be considered fusion from without. However, it is possible that the fusion of XC cells induced by cells infected with other strains of MuLV (7) may be caused by a fusion from within process.

The factors responsible for the syncytium formation are heat labile, sensitive to trypsin, lipase, and ether but insensitive to ribonuclease, deoxyribonuclease, or neuraminidase. This suggests that proteins are required for the fusion process, but viral ribonucleic acid (RNA) or other nucleic acid species such as products of the RNA-dependent deoxyribonucleic acid polymerase (1, 15) are not. The sensitivity to lipase and ether indicates that lipids or lipoproteins may be important. However, the possibilities that the ether treatment may inactivate an important protein and the lipase may be contaminated with proteases cannot be overlooked. The integrity of the phospholipids of the viral membrane has been shown to be required for fusion of cells induced by NDV (8).

ACKNOWLEDGMENTS

We are grateful to J. Hartley and W. Rowe for supplying the XC cells and the MuLV and for many helpful suggestions, T. Pincus and M. Harrison for preparing the homogenates for the electron micrographs, and W. Hall of Electro-Nucleonics Laboratories for taking the micrographs.

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