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Serological and Structural Properties of Mason-Pfizer Monkey Virus Isolated from the Mammary Tumor of a Rhesus Monkey

(primate cancer/oncogenic RNA virus/simian foamy virus/visna virus/viral antigens)

ROBERT C. NOWINSKI, EUGENE EDYNAK*, AND NURUL H. SARKAR

Sloan-Kettering Institute for Cancer Research, New York, N.Y.; and the Institute for Medical Research, Camden, New Jersey 08103

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ABSTRACT **We describe here serological and structural properties of a virus, Mason-Pfizer Monkey Virus (M-PMV), isolated from a simian mammary tumor. This virus is morphologically similar to the known oncogenic RNA viruses (oncornaviruses). It has a 60–70S RNA, and its replication is inhibited by actinomycin. Antisera prepared against the virus isolated by density-gradient centrifugation identify at least two viral structural antigens. Immunodiffusion studies show that this virus is serologically unrelated to three types of simian foamy viruses, visna virus, and the known oncornaviruses. Immunofluorescence reveals that the structural proteins of the virus are synthesized cytoplasmically. Although M-PMV productively infects human cells *in vitro*, serological analysis does not show the presence of M-PMV antigens in human neoplasia.**

Chopra and Mason (1) have recently described a virus (M-PMV) found in a spontaneous mammary tumor of a Rhesus monkey that shows several morphological characteristics of the oncornaviruses (oncogenic RNA viruses; see ref. 2). The observation of Spiegelman *et al.* (3) that this virus contains an RNA-directed DNA polymerase increases the likelihood that this agent may be the first oncornavirus described in primates. The close evolutionary relationship between man and the monkey invites a detailed analysis of this virus to determine its relationship to known oncornaviruses and to search for antigens of M-PMV in human neoplasia.

MATERIALS AND METHODS

Source of M-PMV

A tissue culture line of mixed monkey embryo–monkey mammary tumor cells producing M-PMV was kindly provided by Chas. Pfizer and Co., Inc. Virus was concentrated from tissue-culture fluid by ammonium sulfate precipitation (50% saturation) and then layered upon a sucrose density gradient (15–60% sucrose in 0.1 M Tris·HCl–0.1 M NaCl buffer, pH 8.0). After centrifugation for 3 hr at 119,000 × *g* SW 27 rotor), a visible virus band was observed at density 1.16 g/ml. This band was diluted with Tris–NaCl buffer and pelleted by centrifugation for 1 hr at 119,000 × *g*.

Abbreviations: AvLV and AvSV, avian leukemia and sarcoma viruses; MuLV and MuMTV, murine leukemia and mammary tumor viruses; HaSV, hamster sarcoma virus; FeLV, feline leukemia virus.

* Present address: Department of Surgery, University of Pennsylvania Medical School, Philadelphia, 19104.

Antisera

The viral antigen used to prepare antisera came from 1500 ml of culture fluid. 1 mg of Tween 80 plus 0.5 ml of ethyl ether was added to the virus pellet suspended in 0.5 ml Tris–NaCl buffer, and the mixture was shaken for 30 min in the cold. The aqueous and ether phases were separated by centrifugation at 1000 × *g* for 20 min; the aqueous phase, dialyzed overnight against Tris–NaCl buffer to remove residual Tween 80 and ether, was used to immunize a (W/Fu × BN)F_1 rat. The initial inoculum was emulsified in an equal volume of complete Freund's adjuvant and given subcutaneously at several sites. Subsequent immunizations were given at three weekly intervals and consisted of antigen emulsified in an equal volume of incomplete Freund's adjuvant, also injected subcutaneously at several sites. Serum was obtained 1 week after the third and fourth immunizations.

To remove antibodies against normal tissue components, the antiserum was absorbed with lyophilized extracts of normal monkey liver (30 mg/ml of antiserum) and lyophilized fetal calf serum (30 mg/ml of antiserum). Antiserum was mixed with the lyophilized powders and incubated overnight at 4°C; the resulting precipitate was removed by centrifugation at 1000 × *g* for 30 min.

Reference precipitating antisera prepared against the *group-specific* (*gs*) antigens of avian sarcoma virus (AvSV) (4), murine leukemia virus (MuLV) (5), murine mammary tumor virus (MuMTV) (6), hamster sarcoma virus (HaSV) (7), and feline leukemia virus (FeLV) (8) have been described. Viral antigens for immunodiffusion tests were prepared from ether-treated pellets of density-gradient isolated avian myeloblastosis virus (AvLV), MuLV, MuMTV, HaSV, and FeLV.

Immunodiffusion tests

For immunodiffusion analysis, soluble cellular extracts were prepared from tissues homogenized in 10 volumes of 0.85% NaCl solution for 3 min at medium speed in a "Virtis 45" homogenizer. The soluble proteins were obtained after centrifugation for 1 hr at 119,000 × *g* and were concentrated by lyophilization or vacuum dialysis. Tissue powders of lyophilized samples were reconstituted in a minimum volume of distilled water (giving a protein concentration of about 200 mg/ml) and used as antigen in immunodiffusion. Double diffusion (Ouchterlony) tests were performed in 2% Noble agar slides ("Immunoplate" Pattern C, Hyland Laboratories). The slides were left at room temperature in a humidified chamber. Optimal precipitation occurred in 24 hr.

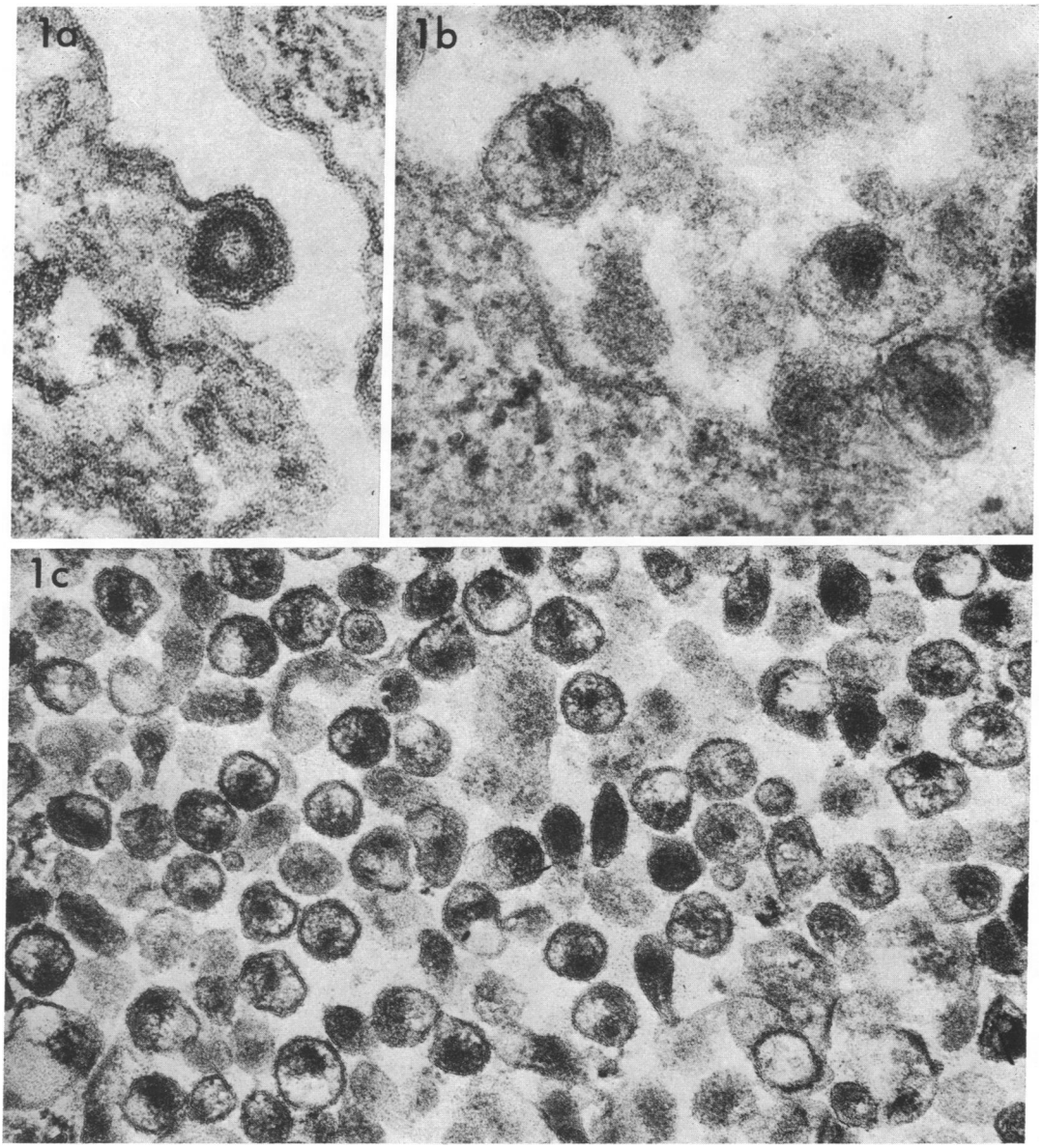

FIG. 1. *a*, Thin section of a budding virion. Note that in the budding virion the nucleocapsid is coiled in the form of a hollow sphere. Dalton's chrome–osmium fixation, stained with uranyl acetate and lead citrate. ×150,000. *b*, Thin section of extracellular virions. The supercoiling of the nucleocapsid has collapsed, resulting in a condensed nucleoid. ×150,000. *c*, Thin section of virions purified in a density gradient. The virions show different degrees of condensation of the nucleoid inside the viral membrane. Particles with eccentric and central nucleoids are seen. ×76,000.

Immunofluorescence tests

The indirect immunofluorescence test on fixed cells was used for localization of intracellular viral antigens (for details, see ref. 9). Monolayers of various tissue-culture lines were grown for 48 hr in individual wells on glass microscope slides and fixed by immersion of the slides in acetone at room temperature for 15 min. The fluorescenated *Goat anti-rat γ-globulin* (Hyland Laboratories) and absorbed viral antiserum (see above) were both used at 1/20 dilution.

RESULTS AND DISCUSSION

Electron microscopy of virus-producing cells

Fig. 1 shows M-PMV budding from the surface of infected monkey embryo cells. The nucleocapsid of the budding virion assumes a symmetry (a hollow sphere) characteristic of the oncornaviruses (Fig. 1*a*). Particles liberated into the extracellular fluid undergo a morphological transition (Fig. 1*b*), whereby the nucleocapsid condenses [for details, see (10)].

Electron microscopy of M-PMV

A visible band of M-PMV was recovered from 500 ml of tissue-culture fluid. The viral nature of this band was confirmed by electron microscopy. Fig. 1*c* shows a thin section of a pellet of M-PMV isolated by density-gradient centrifugation. These particles do not clearly fit into a category of B- or C-type morphology since the nucleoid tends to be more disperse than that seen in other oncornaviruses.

In negative-staining (Fig. 2), the membrane of most particles appears smooth, similar to the smooth surface seen with leukemia–sarcoma viruses; this is in contrast to the spike-covered surface of murine mammary tumor virus. Occasionally, particles with irregular projections on the viral sur-

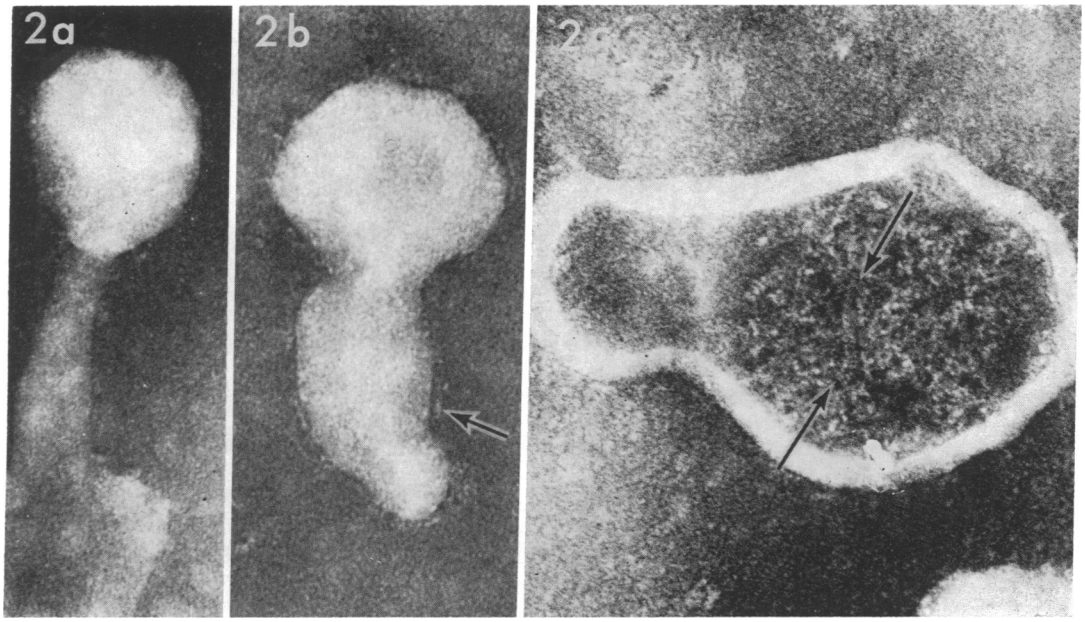

FIG. 2. *a*, Negatively stained M-PMV (phosphotungstate, pH 7.0). The head and tail configuration of this particle is an artefact caused by drying; nevertheless, it is a structural feature that is characteristic of the oncornaviruses. Note the absence of any surface projections of this particle. ×240,000. *b*, Negatively stained M-PMV. This particle has poorly defined small projections on its surface. Only a small number of particles (<5%) in the simian virus preparation show this surface structure. ×240,000. *c*, Internal structure of the M-PMV. This figure shows a damaged virion into which phosphotungstic acid has penetrated. The nucleocapsid is a helical structure (*arrows*), and is similar to the nucleocapsid of known oncornaviruses. ×300,000.

face also are observed. These projections are similar to those on the surface of avian myeloblastosis virus.

In most virions the nucleoprotein strands (30 Å in diameter) are observed randomly oriented in the nucleoid, but in some instances, the strands are actually coiled in a helix 80 Å in diameter (Fig. 2*c*). A nucleocapsid helix of the same dimensions has been described in AvLV, MuLV, MuMTV, and FeLV (ref. 2, and to be published).

Sucrose velocity sedimentation of viral RNA

Sucrose velocity sedimentation of [^{3}H]uridine-labeled RNA extracted from M-PMV reveals two species of RNA, a 60–70S component and a smaller 4S species (see Fig. 3).

Effect of actinomycin on viral replication

Fig. 4 shows that the addition of 0.5 μg/ml of actinomycin D to the culture medium inhibits viral production.

Assay for RNA-dependent DNA polymerase

The virus preparations described here were tested by Dr. Jeffrey Schlom (Columbia University) and found to contain the RNA-directed DNA polymerase that is characteristic of oncornaviruses (11).

In summary, M-PMV has several features that are similar to those of known oncornaviruses; it has a 60–70S RNA, it has the enzymes necessary for reverse transcription, the morphological features of its replication are essentially the same as those of the known oncornaviruses, and its replication is inhibited by actinomycin. Yet the biological activity of the virus is still unknown, and, for this reason, this virus cannot be classified with certainty as a mammary tumor agent. The possibility remains that this agent may be a leukemia virus.

Immunodiffusion tests with M-PMV and known oncornaviruses

Antisera prepared against M-PMV disrupted with Tween 80 and ether initially reacted in immunodiffusion tests with extracts of normal Rhesus-monkey tissues (separate extracts of liver, spleen, and kidney). Absorption of this antiserum with liver powder and fetal calf serum abolished all reactivity against normal monkey tissues and cells from an uninfected monkey embryo line (CV-1). This absorbed serum detected two viral antigens in ether-treated M-PMV and in extracts of monkey embryo cells infected with M-PMV†, but does not react with known oncornaviruses—MuLV, MuMTV, HaSV, and FeLV (Fig. 5) or AvLV antigens (not shown). In recip-

† A rabbit antiserum (kindly provided by Dr. S. Ahmed; Charles Pfizer and Co., Inc.) prepared against ether-treated M-PMV detects the same viral specificities as the antiserum described here. In immunodiffusion tests these antisera formed lines of identity with ether-treated M-PMV.

TABLE 1. *Human tissue extracts tested by immunodiffusion for M-PMV antigens*

Carcinomas:	Breast (52); rectum (3); squamous cell (1); kidney (1); embryonal (2); ovary (1); colon (2); stomach (1).
Sarcomas:	Liposarcoma (1); osteogenic (3); myxolipofibrosarcoma (2); leomyosarcoma (1).
Fetal tissues:	Whole fetus, 12 weeks (1); whole fetus, 14-week (1); whole fetus, 16-week (1); fetal parts from 16–24 weeks: lung (4); kidney (3); heart (1); stomach (1); thymus and spleen (1); testes (2); muscle (2); liver (2); ileum (1); placenta (1); brain (1); skin (2); colon (1).
Normal adult tissues:	Spleen (1); breast (6); breast cyst (1); ovary (1); ileum (3); colon (1); stomach (2); kidney (1); lung (1).

Numbers in parentheses refer to number of different preparations tested. All extracts were negative with M-PMV antiserum.

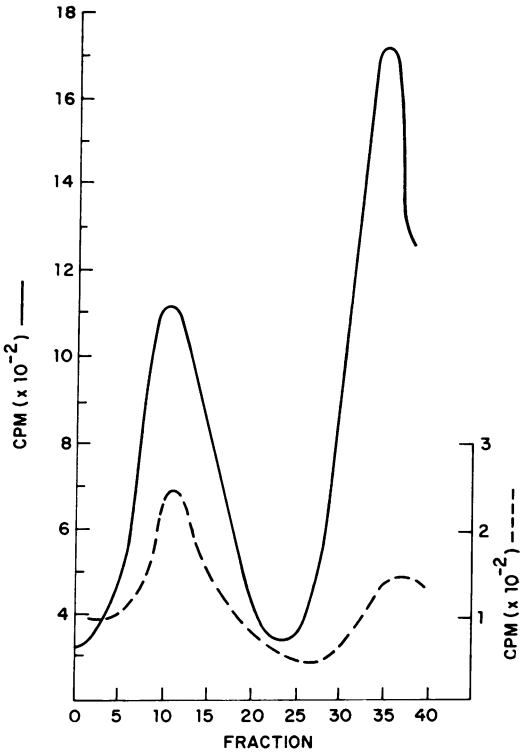

FIG. 3. Sucrose velocity sedimentation of RNA extracted from M-PMV. M-PMV contains the 60–70S and 4S RNAs that are characteristic of oncornaviruses. Cell cultures of monkey embryo infected with M-PMV and of rat embryo infected with MuLV (oncornavirus control) were incubated overnight with 20 μg/ml [^{3}H]uridine. 20 ml of fluid from each culture was first centrifuged at 1000 $\times$ *g* for 20 min and then layered directly upon a 20-ml sucrose density gradient (15–60%). The gradient (in the SW 27 rotor) was centrifuged for 3 hr at 25,000 rpm, the tube was punctured from the bottom, and 1-ml fractions were collected. The purified virus was pelleted and the RNA was extracted in 1 ml of Tris–NaCl buffer containing 0.5% sodium dodecyl sulfate, 1% mercaptoethanol, 0.01 M EDTA, and 0.05% yeast RNA carrier; this was then layered on a 5–20% sucrose gradient and centrifuged for 4 hr at 25,000 rpm (SW 27 rotor). The gradients were punctured from the bottom and 1-ml fractions were collected. After the addition of carrier (100 μg of bovine serum albumin per sample), the RNA was precipitated with trichloroacetic acid and collected on Millipore filters. MuLV ——; M-PMV- - - -.

rocal tests, ether-treated M-PMV did not react in immunodiffusion tests with potent antisera prepared against the *gs* antigens of AvSV, MuLV, MuMTV, HaSV, and FeLV. In addition, ether-treated M-PMV did not react with selected MuLV-antisera that detect the *MuLV-gs3* antigen (12), an antigen that is common to the mammalian leukemia–sarcoma viruses. The fact that M-PMV and MuMTV are serologically unrelated was further established when antisera prepared against isolated MuMTV proteins (and recognizing five serologically distinct antigens of MuMTV; see ref. 13) did not react with M-PMV antigens. *Rat anti-M-PMV* serum also was negative in immunodiffusion tests with four extracts of spontaneous feline mammary tumors.

Serological relationship of M-PMV to the simian foamy virus and visna virus

Recently it has been reported that simian foamy virus (14) and visna virus (15, 16) contain the RNA-dependent DNA

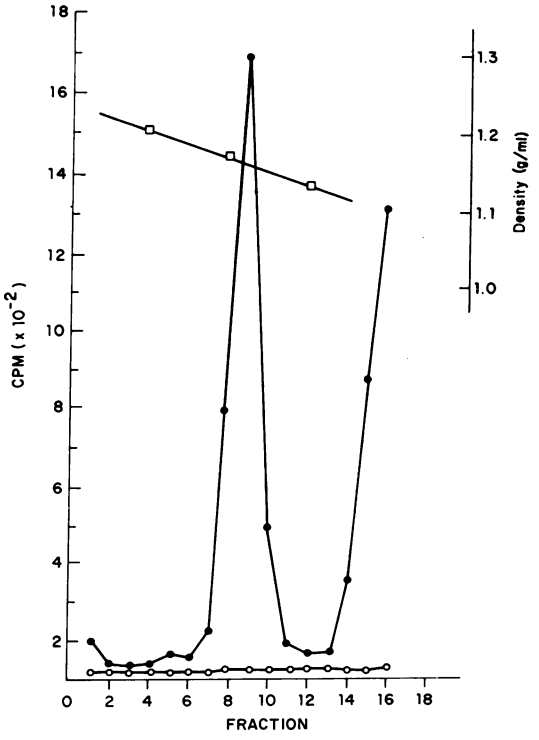

FIG. 4. Inhibition of M-PMV replication by Actinomycin D. Two replicate cultures of monkey embryo cells infected with M-PMV were labeled for 24 hr with 20 μCi/ml of [^{3}H]uridine. Actinomycin D (0.5 μg/ml) was added to one culture at the same time as label; the other culture served as an untreated control. Density-gradient centrifugation is described in Fig. 3. Actinomycin-treated O—O; untreated control ●—●.

polymerase that is characteristic of the oncornaviruses. In fact, it has been suggested that the simian virus described here might actually be a foamy agent (15).

Rat anti-M-PMV serum was tested in immunodiffusion and found negative with ether-treated preparations of simian foamy viruses (types 1, 2, and 3; kindly provided by Dr. S.

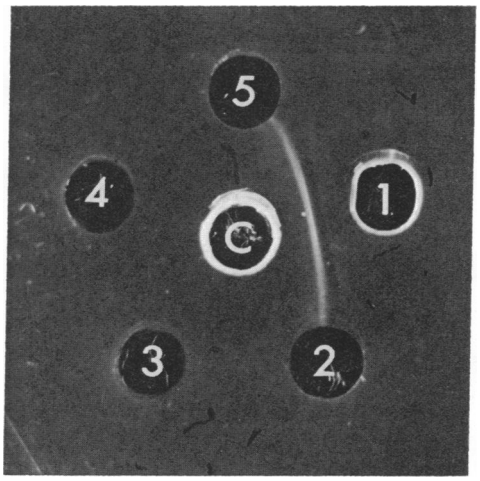

FIG. 5. Serological relationship of simian virus with known oncornaviruses. *Rat anti-M-PMV* (center well, *c*) detects two antigens in ether-treated M-PMV (peripheral well *1*) but does not react with antigens of murine leukemia virus (*2*), hamster sarcoma virus (*3*), feline leukemia virus (*4*), and mouse mammary tumor virus (*5*).

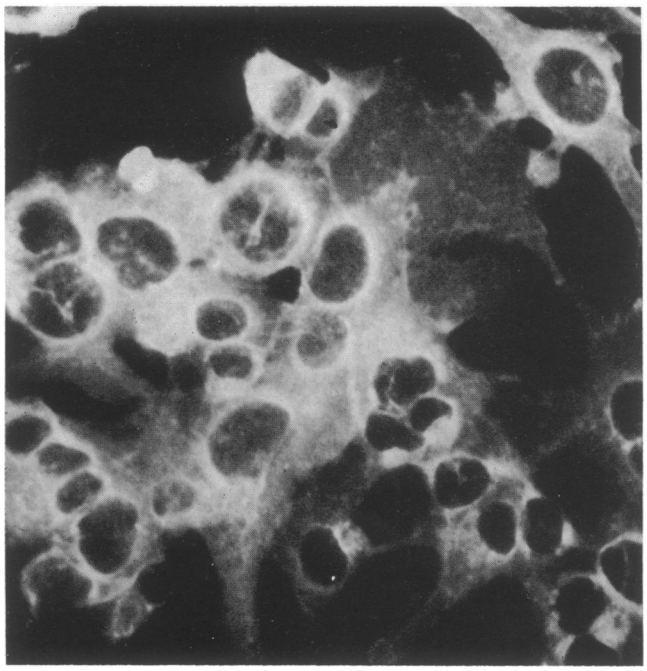

FIG. 6. Immunofluorescent localization of M-PMV antigens in infected monkey embryo cells. The viral antigens are restricted to the cytoplasm, with predominant occurrence in the perinuclear region; no nuclear fluorescence is observed.

Aaronson) and visna virus (kindly provided by Dr. D. Harter). Visna virus was tested also with antisera prepared against other oncornaviruses (see above list) and no cross-reactions were observed. Furthermore, visna virus did not contain the *MuLV-gs3* antigen.

Replication of M-PMV in human cells

The ability of M-PMV to replicate in human cells was established by infection of the normal fibroblast line WI38. The production of M-PMV in these cells was demonstrated by the incorporation of [^{3}H]uridine into particles at density 1.16 g/ml, and by the presence of M-PMV antigens in these particles.

Immunofluorescence tests with M-PMV antiserum

M-PMV antiserum reacted in IF tests with monkey embryo cells infected with M-PMV (Fig. 6), but not with a noninfected monkey embryo line. Similarly, this antiserum reacted with human WI38 cells infected with M-PMV but not with noninfected WI38. All reactions were cytoplasmic (perinuclear); no nuclear fluorescence was observed.

Immunodiffusion and immunofluorescence tests for M-PMV antigens in human tumors

Table 1 shows the result of immunodiffusion tests with extracts of human neoplastic tissue, fetal tissue, and normal adult tissue. No reaction was observed with 52 extracts of human mammary tumors or with 20 other neoplastic tissues, 25 fetal tissues, or 17 normal adult tissues.

Several human neoplastic lines maintained in tissue culture were screened by immunofluoresence tests for M-PMV antigens: five different breast cancer lines, an osteogenic sarcoma, and an ovarian tumor were negative.

The sera from 30 breast cancer patients were tested in immunodiffusion with ether-treated M-PMV. All but one of these sera were negative. The single positive reaction was directed against a component (gamma-fetal protein) of fetal-calf serum, a constituent of the culture medium in which the infected cells were grown. The immunological reactivity of cancer patients against gamma-fetal protein has been described previously (17).

Thus, immunodiffusion studies show that M-PMV is serologically distinct from the known oncornaviruses, the visna virus, and three types of the simian foamy agent. Preliminary immunological analysis has failed to reveal the presence of M-PMV structural antigens in human neoplasia. However, the possibility still exists that this virus could be present in human cells in a cryptic form—i.e., similar to murine sarcoma virus in transformed nonproducer cells (18).

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