Characteristics and Viral Susceptibility of an Embryo Cell Line from *Marmosa mitis* (36822)

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Marmosa mitis, a small South American pouchless marsupial (1), is a useful laboratory model for cytogenetic studies because its chromosomes are large and few in number ($2n = 14$). A colony of *M. mitis* was established at the Radiobiology Laboratory, University of California at Davis, in 1968, and a serially cultivated embryo cell line was developed during the past 2 yr. The *M. mitis* embryo (MME) cells have been serially passed for an average of 75 passages. This report describes the biological characteristics and viral susceptibility of this line of cells.

Materials and Methods. Medium. Eagle's minimal essential medium (Grand Island Biological Co., Grand Island, NY) supplemented with 2000 units/ml penicillin and 100 μg/ml streptomycin.

Trypsin solution (STV). The trypsin solution containing saline A, 0.05% trypsin and 0.025% Versene was prepared according to McClain and Hackett (2).

Preparation of M. mitis *embryo cells.* An embryo was obtained 1 day postpartum and washed several times in Eagle's medium. It was finely minced with scissors, and tissue pieces were suspended in 50 ml of STV, placed into a 250-ml Erlenmeyer flask with a magnetic stirrer and allowed to digest for 15 min. The flask was then removed from the mixer and allowed to stand while the larger tissue fragments settled to the bottom. The contents were filtered through a piece of sterile cheesecloth and 1.0 ml of fetal calf serum (FCS) was added to the supernatant before it was centrifuged at 400*g* for 15 min. The cell pellet was resuspended in Eagle's medium plus 10% FCS, dispensed into 3-oz Brockway prescription bottles, and incubated at 37°.

Cultivation of the MME cells. The *Marmosa mitis* embryo cell line that was developed originated from one of the 3-oz Brockway bottles. Predominantly, fibroblast-like cells could be seen growing out from small tissue clumps adhering to the glass at approximately 3 days. Media were replaced at 5 days, and at 8 days the cells growing out from the central clumps had virtually covered the entire glass surface. Cells were then washed once and removed with 8.0 ml of STV. After centrifugation at 400*g* for 10 min, the cell pellet was resuspended in 16 ml of Eagle's medium with 10% FCS (1:2 dilution) and 8.0 ml of the cell suspension were used to seed each of two 3-oz Brockway bottles. The approximate concentration was 1.0×10^4 cells/ml. Subsequent passages were made similarly. Passages were maintained at a 1:2 dilution ratio until about the tenth passage, at which time the growth rate had increased to a point where a 1:3 dilution could produce a confluent monolayer in about 3 days. Cultures were then transferred routinely at 4-day intervals. Cells were routinely tested for mycoplasma by culturing techniques and were uniformly found to be negative.

Preservation of MME cells. Cells were removed from 3-day-old cultures grown on 16-oz Brockway bottles with STV. After centrifugation at 400*g* for 10 min the pellet (1×10^6 cells) was then resuspended in 2.0 ml of a solution of 80% Eagle's medium, 10% FCS, and 10% dimethyl sulfoxide. The resulting suspension was dispensed into glass ampules, sealed, and frozen at −70°.

For reconstitution, ampules were immediately thawed in a 37° water bath. The cells from one ampule were diluted with 8.0 ml of

Eagle's medium plus 10% FCS and placed into a 3-oz Brockway bottle.

Growth curve. Eight milliliters of cells at a concentration of 3×10^4 cells/ml were dispensed into each 3-oz Brockway bottle. At timed intervals during the next 5 days, cultures were washed once with STV and the cells were removed from the glass with 7.5 ml of STV. After the addition of 0.5 ml of FCS, the concentration of cells/ml was determined by using a Coulter counter B (Coulter Electronics, San Carlos, CA). Duplicate cultures were counted for each sampling time.

Chromosome analysis. Fibroblast cultures were arrested at metaphase by the addition of 0.25 ml of colcemide (Difco)/5.0 ml of culture media. Following 3 hr of incubation at 27° the monolayer was treated with (STV) for 3–5 min. Clumped cells were gently broken up wih a pasteur pipette. Cells were washed in FCS, centrifuged, treated with 1% sodium citrate hypotonic solution, and fixed in 3:1 methanol:glacial acetic acid. Slides were prepared by flame drying and stained with Giemsa stain. Metaphase spreads from cultures in the 10th, 15th, and 20th passages were scored for number and morphology.

Viruses. The following viruses were used for susceptibility studies: vesicular stomatitis virus (VSV, New Jersey serotype), bovine viral diarrhea virus (BVDV, NADL strain), infectious canine hepatitis (ICH) virus, infectious bovine rhinotracheitis (IBR) virus, influenza A virus (WSN strain), polyoma virus, myxoma virus, polio virus (type 1, LSc), bluetongue virus.

Infection of MME cells. Marmosa mitis embryo cells between 50th and 60th passages were grown as monolayers. Cells were washed once with Eagle's medium prior to viral inoculation and were incubated for 1 hr at 37°, after which time 5.0 ml of Eagle's medium with 5% FCS were added to each culture. Cell cultures were examined daily for cytopathogenic effects (CPE). Cultures that showed CPE were frozen at −70° and assayed for viral titer. Contents of such cultures were used as inocula for second passage infections. Cultures in which no CPE were observed after 7 days were also frozen, assayed, and used as inocula for further passage in MME cells. All viruses were serially passed 3 times in MME cells.

Virus assay. Infectivity titers of vesicular stomatitis virus in pig kidney cells (PKH_{13}), bovine viral diarrhea virus in bovine bone marrow cells, infectious canine hepatitis virus in Madin-Darby canine kidney (MDCK) cells, IBR virus in MDBK cells, influenza A virus in MDBK cells, polyoma virus in 3T3 mouse cells, myxoma virus in rabbit kidney cells, polio virus in HeLa cells and bluetongue virus in sheep kidney cells were determined by the plaque technique under agar overlay (3).

Results. Cell morphology. Early passages of MME cells were composed mainly of fibroblast-like cells with a few epithelioid cells.

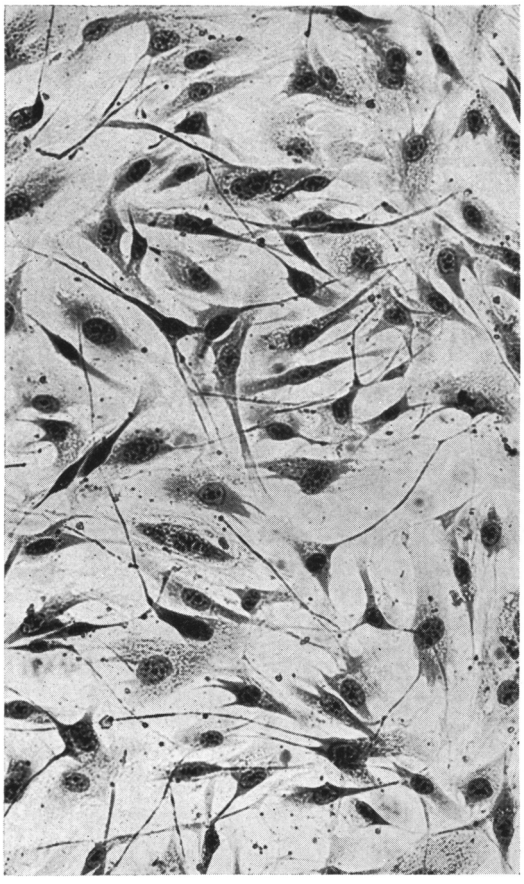

FIG 1. Morphology of MME cells. Cells were grown on coverslips, fixed with methanol, and stained with Giemsa.

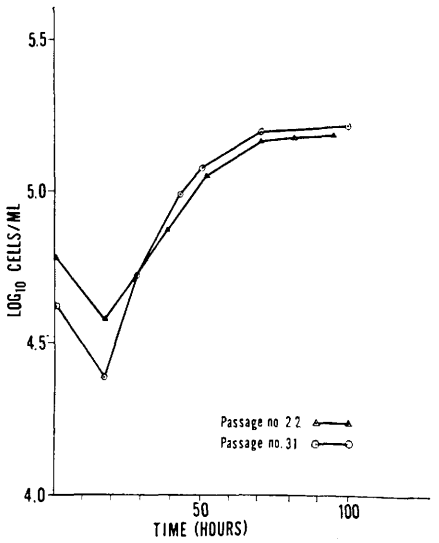

FIG. 2. Rate of growth of MME cells at two different passages. △, passage 22; ○, passage 31.

Continued passages of cells in culture did not alter cell morphology. The majority of cells were fusiform in shape with elongated protoplasmic processes of different lengths and numbers (Fig. 1). As cell density increased, a monolayer was formed, presumably due to contact inhibition. There was no "piling up" of cells as the culture aged; the monolayer remained on the glass for 10–14 days without requiring changes of medium.

Growth curve. Growth curves of MME cells were carried out at passages 22 and 31. There was a lag period of approximately 18 hr followed by rapid cell proliferation (Fig. 2). Cell replication virtually ceased by 72 hr. The doubling time of cells in these passages was about 20 hr.

Chromosome studies. Figure 3 shows a normal marmosa karyotype. Cytogenetic evaluation of cultures harvested at the 10th, 15th, and 20th passages showed an increasingly greater incidence of tetraploidy. The 10th passage had 8% tetraploid cells, the 15th had 28%, and the 20th contained 40% tetraploid cells. Other types of abnormalities were minimal and varied in nature. No particular type of aberration was consistently found.

Viral susceptibility of MME cells. Several representatives of RNA and DNA animal viruses were tested for their ability to replicate in MME cells. Each virus was passed three times in MME cells. Table I shows that vesicular stomatitis, influenza A, IBR, myxoma and bluetongue viruses were all capable of replication in MME cells. At the end of the third passage the titer of these viruses had increased at least 3 logs. Cytopathogenic effects were observed in all instances where viral replication took place.

Discussion. Continuous cell lines have been established from many mammalian species for cytogenetic and viral investigations. Most mammalian species have high chromosome numbers and complex karyotypes which are not ideal for cytogenetic studies. In this respect, cell lines from marsupials are extremely useful genetic tools. Most of them have diploid numbers of either 14 or 22 (4). The simplicity of the karyotype and the ease with which individual chromosomes can be identified make marsupial cell lines highly desirable for cytogenetic work. Permanent marsupial cell lines have been successfully established for the Tasmanian marsupial, *Potorous tridactylis* (5) and the marsupial mouse *Autechinus swainsonii* (6).

A line of embryo cells from *M. mitis*, a South American pouchless marsupial, has been established in our laboratory. The cell culture is easy to propagate and can be

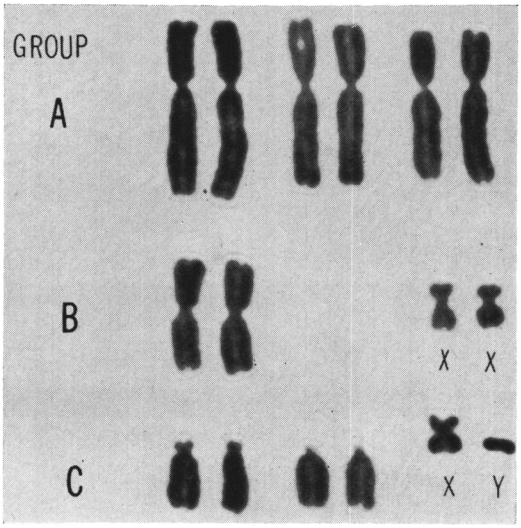

FIG. 3. Karyotype of MME cells showing normal diploid number and morphology.

TABLE I. Viral Susceptibility of *Marmosa mitis* Embryo (MME) Cells.

Virus	Titer of initial inoculum[a] (PFU/ml)	Passage no.	Titer (PFU/ml)	Theoretical titer[b] (PFU/ml)	Viral replication
Polio type 1, LSc	3.0×10^5	1	1.0×10^4	3.0×10^4	
		2	4.0×10^2	3.0×10^3	—
		3	$<10^1$	6.0×10^2	
Influenza A	3.7×10^5	1	7.0×10^3	1.5×10^4	
		2	5.0×10^4	6.0×10^2	+
		3	8.7×10^5	2.4×10^1	
Vesicular stomatitis	1.2×10^8	1	5.0×10^6	4.8×10^6	
		2	1.5×10^4	1.9×10^5	+
		3	7.0×10^6	7.6×10^3	
Bovine viral diarrhea	2.0×10^6	1	1.5×10^3	2.0×10^5	
		2	$<10^1$	2.0×10^4	—
		3	$<10^1$	2.0×10^3	
Bluetongue	5.0×10^3	1	2.5×10^3	5.0×10^2	
		2	7.5×10^2	5.0×10^1	+
		3	6.0×10^5	5.0×10^{-1}	
Polyoma	4.5×10^6	1	1.7×10^5	9.0×10^4	
		2	2.0×10^4	1.8×10^4	—
		3	3.5×10^3	3.6×10^3	
Infectious bovine rhinotracheitis	1.5×10^5	1	2.3×10^4	1.5×10^4	
		2	1.5×10^4	1.5×10^3	+
		3	1.4×10^6	6.0×10^1	
Infectious canine hepatitis	3.5×10^7	1	1.5×10^7	3.5×10^6	
		2	4.0×10^5	3.5×10^5	—
		3	1.5×10^4	7.0×10^4	
Myxoma	4.5×10^4	1	8.0×10^5	9.0×10^3	
		2	7.5×10^5	1.8×10^3	+
		3	5.0×10^5	3.6×10^2	

[a] The titer of each passage for all viruses was determined by plaque forming units (PFU).

[b] Theoretical titer is calculated from dilution of the initial inoculum, assuming no viral replication and inactivation.

stored in 10% dimethyl sulfoxide at —70° for prolonged periods of time without alteration in morphology and growth characteristics. The cells are found to be free of mycoplasma contamination and can be successfully passed for a minimum of 75 passages. The cells have a relatively short period of replication and have a doubling time of 20 hr. They exhibit contact inhibition and have been found to remain as a monolayer on glass for at least 10 days without any change of medium. This feature of marmosa embryo cells may make it feasible to assay viruses that take many days to replicate.

Marmosa embryo cells have a fairly wide viral sensitivity. Five of the nine viruses, each representing a member of a major group, replicated to high titers in MME cells. Cytopathogenic effects were also observed in these instances. The ability of MME cells to support the replication of certain viruses and not of others may be of diagnostic value. Since there are only 14 chromosomes in MME cells, this cell line will also be a useful tool in the study of chromosome alterations consequent to viral infections.

Summary. A cell line designated as MME, was established from the embryo of a pouchless South American marsupial, *Marmosa mitis*. The cells were mostly fibroblastic in shape and displayed contact inhibition. Cytogenic evaluation revealed a simple karyotype ($2n = 14$) and large chromosomes. Growth curves of MME cells at passages 22 and 31 showed a relatively short doubling time of 20 hr. The MME cells had a fairly wide viral spectrum. Vesicular stomatitis, influenza A, infectious bovine rhinotracheitis, myxoma and bluetongue viruses were all capable of replication in this cell line.

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Received June 19, 1972. P.S.E.B.M., 1972, Vol. 141.