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Altered Amphibian Cell Morphology Following Treatment with Polyene Antibiotic and a Mammalian RNA Virus[1] (38258)

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The polyene antibiotic amphotericin B has been shown to induce permeability alterations in eukaryotic cells (1). Resulting membrane changes permit an increase in the uptake of a second drug by both fungal (2–4) and animal (4–9) cells. These findings prompted us to investigate the possibility of utilizing a polyene drug to facilitate the entrance of an unenveloped RNA virus into a normally nonpermissive host cell. The morphological alteration of haploid frog cells following treatment with the polyene antibiotic, mediocidin, and a mammalian RNA virus, Mengo virus, is herein reported.

Materials and Methods. Cell culture and media. A haploid frog cell line, ICR 2A, was grown at 23° in modified Leibovitz medium (Mod L-15) containing 10% fetal calf serum (FCS). The medium, before use, was diluted (40%) with sterile deionized water to correspond more closely to the tonicity of amphibian cells (10). The ICR 2A cell line was kindly supplied by Dr. J. Freed, Institute for Cancer Research, Fox Chase, PA. During viral infection and drug treatment, cells were maintained in Mod L-15 containing 2% FCS.

Virus. Stock Mengo virus was grown and assayed in L-M cells as described by Asculai and Kuchler (11). Viral titrations were made in Dulbecco's phosphate buffered saline (PBS) and 50 plaque-forming units per ml (PFU/ml) were inoculated into test cultures.

Polyene antibiotic. Mediocidin is a hexaene polyene antifungal antibiotic produced by *Streptomyces mediocidus* (12, 13). This antibiotic was made in our fermentation plant at the Institute of Microbiology, Rutgers University. Ten microliter quantities of mediocidin prepared in dimethyl sulfoxide (DMSO) were added to test cultures to yield final mediocidin concentrations of 1–10 μg/ml.

Drug and viral treatment of cells. Approximately one million ICR 2A cells suspended in 4 ml of growth medium were seeded into T-flasks of 25 cm^2 area and incubated at 23°. After 24 hr the growth medium was removed from semiconfluent cell sheets and replaced with maintenance medium. Sets of 12 T-flasks were divided into six groups designated as series A through F. The flasks in series A served as controls. Series B flasks received 10 μl of DMSO. Series C flasks received 10 μl of a mediocidin solution yielding final concentrations of 1, 2, 3, 5, or 10 μg/ml. Series D flasks were inoculated with 0.5 ml of PBS containing 50 PFU/ml of Mengo virus. The flasks in series E received 10 μl of mediocidin, yielding final concentrations of 1, 2, 3, 5, or 10 μg/ml, and 0.5 ml of 50 PFU/ml Mengo virus in PBS. Series F flasks received 10 μl of DMSO and 0.5 ml of 50 PFU/ml Mengo virus in PBS. Sets of four flasks in each series were incubated at 23°, 28°, or 30°. All of the flasks were observed daily over a 6 day test period for morphological changes.

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Cloning technique. Growth medium was removed from cells growing in 25 cm^2 T-flasks. The cell sheets were washed twice in Hank's balanced salt solution (HBSS). The upper side of the polyethylene T-flask was cut off, a penicillin cup was placed over specific colonies of cells, and 0.2 ml of a 0.2% trypsin solution was added. After 10 min, 0.8 ml of growth medium was added and the cells were resuspended and dispersed into 9 ml of cloning medium consisting of filtered conditioned medium and growth medium, 1:1. Approximately one thousand cells in 4 ml of cloning medium were then placed in 25 cm^2 T-flasks and left undisturbed for 14 days at 23°. Colonies of cells arising from single cells were then reisolated and maintained as cloned populations. Five cloned populations of morphologically altered ICR 2A cells (ICR 2A M/MV) were isolated from three separate T-flasks treated with 1 μg/ml of mediocidin and 50 PFU/ml of Mengo virus and 2 T-flasks treated with 2 μg/ml of mediocidin and 50 PFU/ml of Mengo virus and grown at 23°. Three cloned populations of ICR 2A cells surviving 1 μg/ml and 2 μg/ml of mediocidin were isolated from three separate T-flasks grown at 23° (ICR 2A M).

Viral rescue. Three techniques were utilized in an attempt to rescue Mengo virus from the five cloned populations of morphologically altered ICR 2A cells (ICR 2A M/MV). (1) Equal numbers (1×10^6) of ICR 2A M/MV and L-M cells were cocultivated in 60 × 15 mm culture dishes and grown at 28° or 36° in a CO_2 incubator. Culture dishes were observed daily over a 4 day test period for any signs of a cytopathogenic effect (CPE). (2) Five hundred thousand ICR 2A M/MV cells were seeded on L-M monolayers grown in 60 × 15 mm culture dishes. These cells were grown at 28° or 36° in a CO_2 incubator for 24 hr. The medium was then removed and the cells were overlaid with fluid consisting of 1.5% melted agar in maintenance medium. The plates were incubated for 2 or 4 days at 28° or 36° in a CO_2 incubator, after which the cells were stained with neutral red for plaque detection. (3) Two 75 cm^2 T-flasks of confluent ICR 2A M/MV cells were manually scraped and the cells were pelleted at 700 rpm for 10 min at 4°. Cells were then washed twice in HBSS and resuspended in 1 ml of PBS. Supernatant material was subsequently collected after sonication, freeze thawed twice in a methanol-dry ice bath and centrifuged at 2000 rpm for 30 min at 4°. This material was either diluted in PBS or applied directly onto confluent L-M cell sheets. After 30 min adsorption at 36° excess supernatant was removed and agar overlay medium added. Plates were stained with neutral red after 2 and 4 days to detect plaques.

Viral challenge. Five independently isolated clones of ICR 2A M/MV cells, obtained from ICR 2A cells treated with 1 μg/ml or 2 μg/ml of mediocidin and 50 PFU/ml of Mengo virus, three clones of ICR 2A M cells, obtained from ICR 2A cells treated with 1 or 2 μg/ml of mediocidin, and control ICR 2A cells were assayed for their sensitivity to vesicular stomatitis virus (VSV) infection. Growth medium was removed from semiconfluent cultures of ICR 2A or ICR 2A M cells and confluent cultures of ICR 2A M/MV cells grown in 25 cm^2 T-flasks. An inoculum of 0.5 ml of 60 PFU/ml of VSV (Indiana strain) diluted in Mod L-15 without fetal calf serum was applied to test cultures and allowed to adsorb for 1 hr at room temperature. Residual virus was then removed and 4 ml of maintenance medium was added to the T-flasks. Triplicate test cultures were then incubated at 23°, 28°, or 30° and observed daily for signs of a cytopathogenic effect. Control cultures were treated with 0.5 ml of Mod L-15 without fetal calf serum. VSV was kindly supplied by Dr. David H. L. Bishop, Rutgers University, Institute of Microbiology, New Brunswick, NJ.

Results. Selection and growth of morphologically altered ICR 2A cells. The morphology of the cells in control flasks (series A) was similar at 23° and 28° after a 6 day incubation period. Some dead cells were apparent in cultures incubated at 30°. The morphology of cells in the flasks treated with DMSO (series B), with Mengo virus

(series D) or with DMSO and Mengo virus (series F) was similar to the control cultures at the three incubation temperatures. In those cultures in which mediocidin even at 1 μg/ml, was added alone (series C) or in conjunction with Mengo virus (series E), 95% of the cells were detached after 24 hr. These floating cells were not viable. Total cell death resulted in 24 hr when cells were treated with 3 μg/ml or higher concentrations of mediocidin.

By the sixth day there were no surviving cells in any series C or E cultures grown at 28° or 30°. However, surviving cells were observed in both series C and E cultures grown at 23°. In these cultures, spent medium was replaced with cloning medium. After two weeks incubation at 23°, colonies of morphologically altered cells (Fig. 1c and 1d) were observed (epithelial as opposed to fibroblastic) in all series E cultures treated with 1 or 2 μg/ml of mediocidin and 50 PFU/ml Mengo virus. An average of 20 morphologically altered colonies were observed in each series E T-flask treated with 1 μg/ml of mediocidin and 50 PFU/ml Mengo virus, whereas an average of eight morphologically altered colonies were observed in series E T-flasks treated with 2 μg/ml mediocidin and 50 PFU/ml of Mengo virus. Only visibly normal colonies of cells (Fig. 1a and 1b) were seen in series C cultures (1 or 2 μg/ml mediocidin treatment, designated ICR 2A M). Similar numbers of colonies with a normal morphology were found after mediocidin treatment alone. At this point colonies of cells were removed and grown as separate cloned populations (Fig. 1a–d).

Three separately isolated clones of ICR 2A M cells surviving after the initial treatment with 1 μg/ml of mediocidin (ICR 2A Mla and ICR 2A Mlb) or 2 μg/ml mediocidin (ICR 2A M2) were assayed for their sensitivity to mediocidin. Two of the cloned populations ICR 2A Mlb and ICR 2A M2 were resistant to 3 μg/ml mediocidin when treated in maintenance medium. Progeny cells selected for increasing resistance to mediocidin are presently resistant to 5 μg/ml mediocidin, representing a fivefold increase in resistance. These cells have been passaged twenty times and still appear morphologically normal. In contrast, all five separately isolated ICR 2A M/MV clones consist of morphologically altered cells. These cells have maintained altered morphology for over twenty passages when grown at 23°.

Effect of temperature and viral challenge on ICR 2A M/MV cells. When ICR 2A M/MV cells from all five clones were transferred from 23° to 28°, or 30°, visible alterations were apparent. What appeared to be a CPE developed, as indicated by cell vacuolation and reduced survival as compared with control or ICR 2A M cells (Fig. 1e and 1f). ICR 2A M/MV cells at these elevated temperatures also appeared more fibroblastic than epithelial. Control or ICR 2A M cells grew well at 28° and were passaged five times without visible change or cell death. In contrast, ICR 2A M/MV cells were passaged only once and did not survive at 28°. Control, or ICR 2A M cells, survived as long as 2 weeks at 30°, with 4-day media changes, while ICR 2A M/MV cells survived only 4 days.

ICR 2A M/MV cells responded differently than ICR 2A or ICR 2A M cells when challenged with VSV at 28° or 30°. Total cell death resulted by day four in both control and VSV infected ICR 2A M/MV cells grown at 30°. The response of control and VSV infected cultures appeared similar: cells became vacuolated, detached and were nonviable. In contrast, ICR 2A and ICR 2A M cells exhibited a much reduced CPE at 30°. After 7 days postinfection signs of a CPE were evident in approximately 50% of the cells, while no signs of a CPE were apparent in control cultures. Cell death resulted after 5 days (4 clones) and 6 days (1 clone) when ICR 2A M/MV cells were infected with VSV and grown at 28°, whereas control cultures survived the seven day test. In contrast, both infected and control ICR 2A and ICR 2A M cells evidenced no sign of a CPE after 7 days at 28°. VSV infected ICR 2A, ICR 2A M or ICR 2A M/MV cells incubated for 7 days at 23° appeared normal.

Discussion. Morphological alteration of haploid frog cells following exposure to the

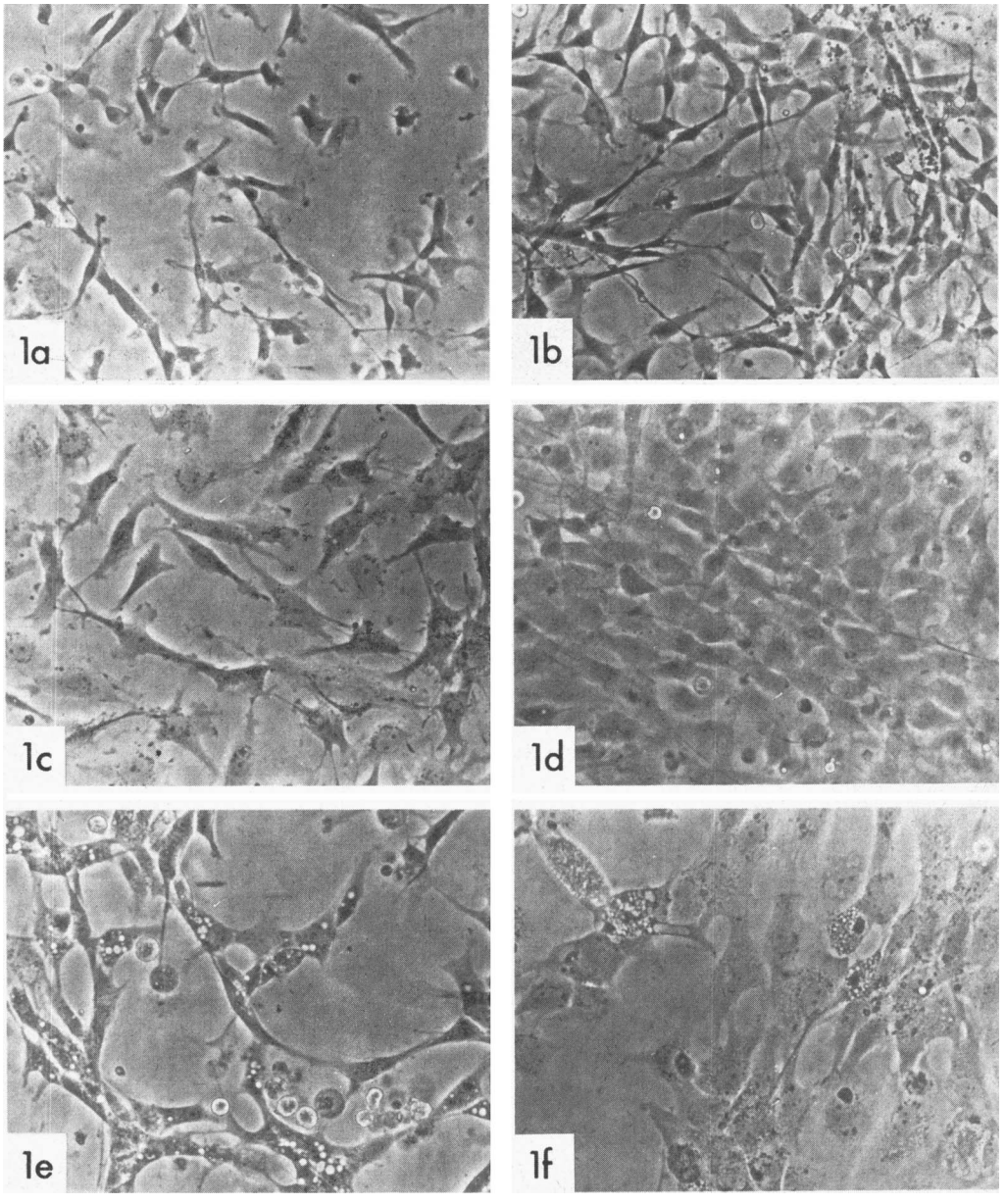

FIG. 1. Effect of mediocidin and mediocidin-Mengo virus on ICR 2A cells. (a) ICR 2A cells surviving treatment with 1 μg/ml mediocidin. (b) ICR 2A cells resistant to 5 μg/ml mediocidin. Both (a) and (b) are morphologically indistinguishable from control ICR 2A cultures. (c) Colony of morphologically altered ICR 2A cells grown at 23° 2 weeks after exposure to 1 μg/ml mediocidin and 50 PFU/ml Mengo virus. (d) Confluent monolayer of a cloned population of ICR 2A M/MV cells (mediocidin-Mengo virus). (e) and (f). Vacuolated ICR 2A M/MV cells from two different cloned populations after three days growth at 30°. 230×.

combination of a mammalian RNA virus, Mengo virus, and a polyene drug, mediocidin, has been demonstrated. The results indicate that both the virus and drug are necessary to induce the alteration. Mengo virus alone caused no cell death or change in morphology of cells at 23°, while, mediocidin alone exhibited marked toxicity without inducing any visible alteration in the morphology of surviving cells after subculture in the absence of the drug. The appearance of several distinct foci of morphologically altered ICR 2A cells in all cultures treated with 1 or 2 μg/ml of mediocidin plus Mengo virus and the absence of altered cells in drug or virus treated cultures argues against the possibility that these cells developed as a result of mutation. Karyotype analysis and differential response of ICR 2A M/MV cloned cell populations to VSV challenge also indicated that the altered morphology of these cells in comparison with control (ICR 2A) or mediocidin resistant (ICR 2A M) cells is not the result of selection for diploid variants. ICR 134, a diploid frog cell line developed from diploid (as opposed to haploid) Grass Frog embryo (*Rana pipiens*), cells are epithelial in morphology as opposed to fibroblastic (ICR 2A) and resistant to VSV infection at 28° or 30°. ICR 134 cells also fail to demonstrate the same temperature sensitivity responses of ICR 2A M/MV cells.

The reduced survival of ICR 2A M/MV cells at elevated temperatures in conjunction with visible alterations in morphology (return to a fibroblastic appearance) and vacuolation were suggestive of a CPE. However, attempts at rescuing Mengo virus from ICR 2A M/MV cells by cocultivation with an indicator strain (L-M), focal infectivity assay, or cell extract assay on L-M cells were unsuccessful. Our failure to rescue Mengo virus from ICR 2A M/MV cells suggests at least three alternative possibilities: (1) morphological alterations of ICR 2A cells as a result of infection by defective viral particles; (2) alteration in Mengo virus resulting from growth in a normally nonpermissive host at reduced temperature; and/or (3) noninfectious virus resulting from virus-drug interactions during or after infection.

Studies presently in progress also indicate differences in the membrane properties of ICR 2A M/MV cells as compared with ICR 2A or ICR 2A M cells. ICR 2A M/MV cells have a different pattern of cell surface glycoproteins and also differ in their ability to be agglutinated by conconavalin A. Further studies utilizing other viruses, polyene drugs and host cell systems are also in progress in order to extend the present experimental results.

Summary. Polyene antibiotics are agents known to damage cell membranes. The hexaene antibiotic, mediocidin, was used in an attempt to introduce a mammalian virus (Mengo) into normally nonpermissive ICR 2A haploid frog cells. Although no infectious virus could be recovered from mediocidin treated cells exposed to Mengo virus, a change in VSV susceptibility, temperature sensitivity and an apparently permanent change in morphology took place—from fibroblastic to epithelial—in the cells cultured at 23°. Neither the polyene antibiotic nor the virus used alone was capable of inducing these alterations in ICR 2A cells.

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