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Use of Human Diploid Cell Cultures for Primary Isolation of Respiratory Syncytial Virus.* (30738)

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Studies of children and adults with acute respiratory infections have shown that several families of viruses, including the Parainfluenza viruses, Respiratory Syncytial (R.S.) virus and the Rhinoviruses play important roles in the etiology of such illnesses. In working with these viruses, it has been the experience that no single cell culture system can be considered optimum for isolation and propagation of all of these agents. Thus, the cell culture systems considered best for primary isolation of the Parainfluenza viruses are those derived from the kidneys of Old World monkeys, those for the Rhinoviruses, human embryo kidney, or human fetal diploid cells, and those for R. S. virus, continuous lines derived from human carcinoma tissues, particularly HEp-2.

Studies of children with acute respiratory illnesses have been conducted in this laboratory, in which specimens from patients were inoculated into cultures of Rhesus monkey kidney cells, human fetal diploid cells, and HEp-2 cells. In the course of this work it appeared that primary isolation of R. S. virus could be accomplished as well with the human fetal diploid cells, as with HEp-2 cells. The following report presents detailed comparisons of R. S. virus isolation rates and infectivity titers observed when specimens from patients were inoculated in both HEp-2 and human fetal diploid cell cultures. The results of these comparisons suggest that human fetal diploid cell strains may be considered appropriate for the primary isolation of R. S. virus.

Materials and methods. Cells. Strain WI-38: diploid cell derived from human embryonic lung, female(1). Strain Hel-1: human embryonic lung from a 14-week-old embryo —University of Chicago. This diploid cell was established according to the method of Hayflick(1). HEp-2: human epidermoid carcinoma, larynx(2).

Media and solutions. Growth: Eagle's Minimum Essential Medium (MEM), supplemented with 10% calf, plus 5% fetal calf

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serum for growth of diploid cells, and with 10% horse serum for growth of HEp-2 cells. These media contained 18 mg% $NaHCO_3$.

Maintenance: 50% Medium 199 and 50% MEM with 1% chicken serum for WI-38 cells and 1% fetal calf serum for Hel-1 cells. HEp-2 cells were maintained with MEM + 5% chicken serum. These media contained 36-108 mg% $NaHCO_3$. Sera in the maintenance media were heat-inactivated (56°C × 30 min).

Specimen collecting: Phosphate buffered saline (PBS) + 0.5% bovine serum albumin.

Penicillin, streptomycin and amphotericin-B were present at 250 units, 250 μg, and 1.0 μg/ml respectively in the growth, maintenance media, and collecting medium.

Diploid cells were propagated according to the method of Hayflick *et al*(1). 16 × 125 mm culture tubes were seeded with 5-10 × 10^4 cells, incubated at 36°C for 2 to 3 days and, after a confluent sheet had formed, washed one time with Hanks' balanced salt solution (BSS) and placed on maintenance medium. HEp-2 cells have been in continuous propagation in this laboratory since 1958. Tubes were seeded with 4-8 × 10^4 cells, incubated at 36°C for 1 to 2 days, washed 2 times with BSS, and placed on maintenance medium.

Specimens for virus isolation were obtained from infants and children seen in the clinics and wards of the University of Chicago Hospitals and Clinics. Specimens were obtained by vigorously swabbing the nose ond oropharynx with a dry cotton applicator and wringing this out in collecting medium. Specimens were kept at room temperature, or 4°C until inoculated later in the day that they were obtained; they were then stored at —90°C.

Isolation procedures. In the 1962-63 season, comparisons were made on the basis of 0.2 cc inoculated into one tube of HEp-2 and one tube of human diploid strain Hel-1. In the 1963-64 season the inoculum was increased to 0.3 ml and WI-38 substituted for the Hel-1 cell cultures. HEp-2 cultures were incubated stationary at 36°C and diploid cultures were incubated on roller drums at 33°C. Tubes were viewed for cytopathic effect (CPE) every 3-4 days for 10 days when they were passed to new tubes for an additional 10 days of observation.

Virus identification. Viruses were passed in HEp-2 until maximum CPE was evident within 2-3 days following inoculation. The fluid from such cultures was then spun at 3,000 RPM for 10 minutes and 0.25 ml of supernate (undiluted or diluted 1:10) was placed in a 16 × 125 mm tissue culture tube. To this was added an equal volume of R. S. virus antiserum (ferret, postinfection), or PBS + 10% horse serum. These mixtures were incubated for 1 hour at room temperature and then 6 × 10^4 HEp-2 cells in growth media were added to the tubes; the tubes were capped, incubated stationary at 36°C and final reading made when the virus controls showed 75-100% CPE. Neutralization was considered significant if the serum-containing tubes showed less than 25% CPE.

Infectivity titrations. Serial 10-fold dilutions were made in PBS with 10% horse serum or MEM with 5% chicken serum; and 3 tubes were inoculated for each dilution. Virus growth was judged by development of cytopathic changes and 50% infectivity endpoints were calculated by the method of Kärber(3).

Results. CPE of R. S. virus in HEp-2 and WI-38. The typical CPE produced by R. S. virus in HEp-2 cells is shown in Fig. 1. Rounding of the cells may be prominent but formation of giant cells and syncytia is the distinctive feature. These changes are most readily seen at the margin of the cell sheet.

The CPE seen in diploid cells is demonstrated in Fig. 2 where a typical early lesion is shown. This early lesion is focal and may best be identified in the body of the cell sheet where the interruption of the normal even pattern of the monolayer is easily detected by scanning at 40× magnification. There is little rounding of the surrounding cells and the margin of the lesion has an indistinct, feathery appearance. Close inspection reveals a barely evident syncytium consisting of a thin veil of cellular material spanning all or a portion of the break in continuity of the cell sheet. Although giant cell and syncytium formation is not pronounced, the

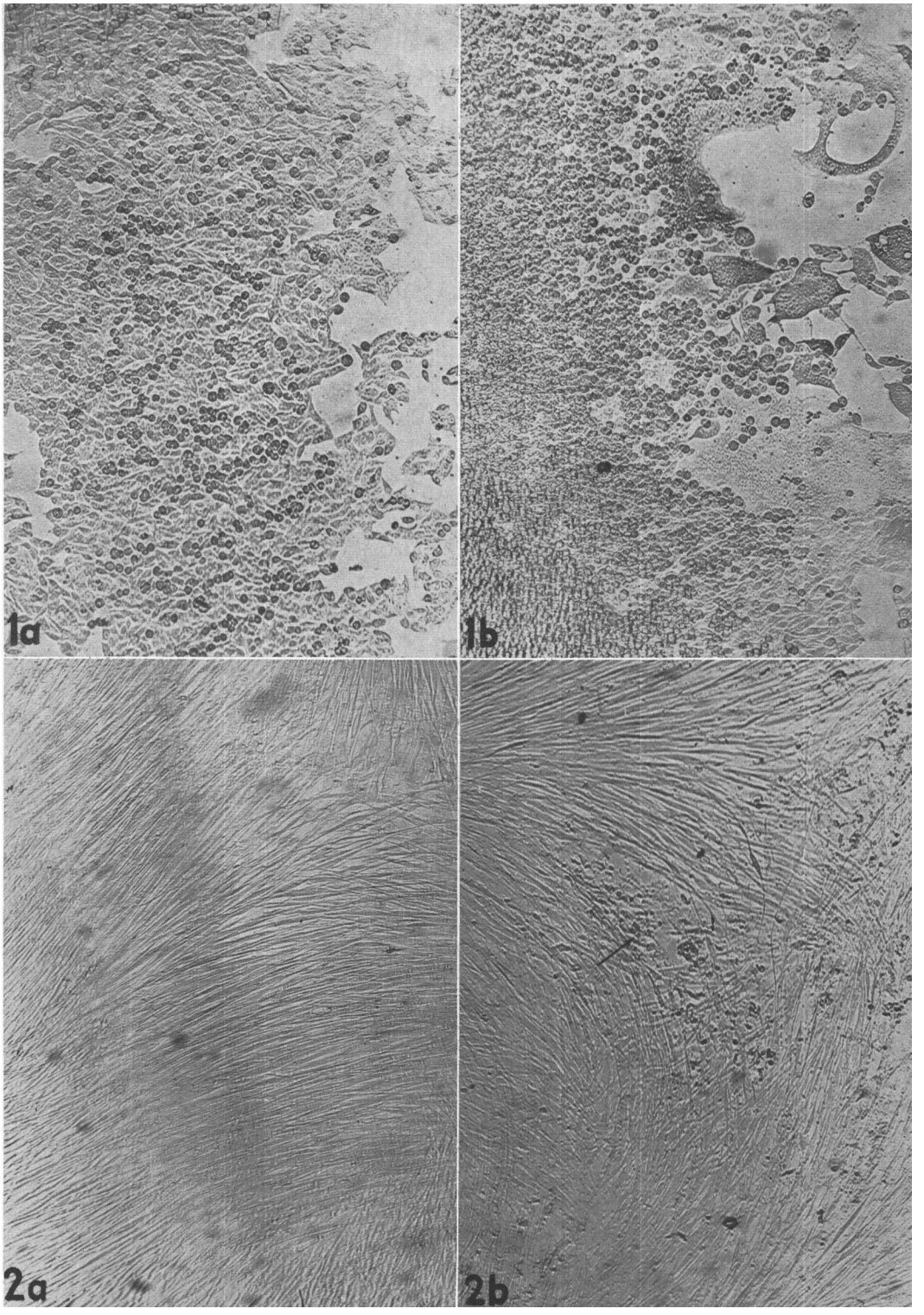

FIG. 1. a). Normal HEp-2: 60 ×, unstained. b). HEp-2 infected with R.S. virus. Note giant cell and syncytium formation and long, thin cytoplasmic strands bridging gaps between giant cells.

FIG. 2. a). Normal Diploid Cells, Strain Hel-1, 60 ×, unstained. b). Diploid Cells, Strain Hel infected with R.S. virus: Arrow indicates final lesion showing destruction of cells with faintly evident syncytium and amorphous cellular debris.

TABLE I. R.S. Isolations 1962-64 in Human Embryo Diploid and HEp-2 Cell Cultures.

Year	Total R.S. virus positive specimens (No.)	Cell cultures in which virus isolated: Diploid & HEp-2 (No.)	(%)	Diploid only (No.)	(%)	HEp-2 only (No.)	(%)
1962-63	73	59	81	7	9.5	7	9.5
1963-64	56	50	89	6	11	0	
Total	129	109	85	13	10	7	5

localized spread of the early cytopathic changes to contiguous cells suggests the importance of cell to cell spread in the propagation of this virus. These destructive changes progress to involve the entire cell sheet over a period of 2 to 4 days.

Comparative R. S. virus isolations in diploid and HEp-2 cell cultures. During the time of R. S. virus prevalence in 1962-1963 and 1963-64, 129 specimens were identified as R. S. virus positive on the basis of the isolation of this agent from either or both of the HEp-2 and diploid cell cultures (Table I). 73 of the positive specimens were in the 1962-63, and 56 in the 1963-64 season. Considering these isolations according to the type of cell in which virus was recovered it is seen that in 85% of specimens both cultures, diploid and HEp-2, were positive; while in 10% only diploid and in 5% only the HEp-2 cell cultures were positive. These data may also be arranged to indicate the effectiveness of each type of cell culture in detecting positive specimens (Table II); 90% were positive in HEp-2 and 95% positive in diploid cells. In all comparisons differences were small and well within the variation to be expected on the basis of inoculating multiple tubes with specimens that might contain virus at or near limit dilution.

Time for development of 1st CPE. Although HEp-2 and diploid cells were comparable in respect to overall isolation rates, they differed considerably in respect to the time required for viral CPE to become evident; this was usually shorter in the HEp-2 cultures. The time interval between inoculation and first CPE for each cell type is presented in Table III. Almost 50% of the HEp-2 cultures which were positive showed CPE by 5 days while this was true of only 8% of the diploid cell cultures. The majority of the diploid cell cultures became positive between days 6 and 10, so that this difference was less striking by day 10 when 74% of the diploid and 89% of the HEp-2 specimens had evidenced CPE. However, the slower development of CPE in diploid cells was evident in all time periods of the comparison.

TABLE II. Fraction of Specimens Positive in Each Type of Cell Culture.

Year	Total R.S. virus positive specimens (No.)	Positive in diploid (No.)	(%)	Positive in HEp-2 (No.)	(%)
1962-63	73	66	90	66	90
1963-64	56	56	100	50	89
Total	129	122	95	116	90

TABLE III. Appearance of R.S. Virus CPE: Comparison of Time Initial CPE Evident Hel-1 and HEp-2.

Days following inoculation	% of positives 1st evident in this time period: Diploid (Hel-1)	HEp-2
1- 5	8	48
6-10	66	41
11-15	17	8
16-27	9	3

Titration of virus in original specimens. Five specimens previously found to contain R. S. virus were taken from —90°C storage, rapidly thawed, centrifuged at 3,000 RPM for 10 minutes, and infectivity titrations of the supernate performed in HEp-2 and WI-38 cell cultures (Table IV). Infectivity titers ranged from undiluted to $10^{2.5}$ $TCID_{50}$/0.1 ml; small, but consistent differences in titer favoring the WI-38 cell cultures suggest a slight advantage in sensitivity for diploid cells.

Summary and conclusion. Human embryo diploid and HEp-2 cell cultures were compared in respect to efficiency in isolating

TABLE IV. Simultaneous Titrations of R.S. Virus Positive Original Specimens in WI-38 and HEp-2 Cell Cultures.

Specimen	Titer* WI-38	Titer* HEp-2
6206A	1.50	1.20
6249A	1.20	.80
6300A	2.20	1.20
5933-1.23	2.50	.80
6184B	undiluted	negative

* $TCID_{50}$/0.1 ml expressed as recip. $\log_{10}$.

R. S. virus. Although initial manifestations of viral growth were evident somewhat earlier in HEp-2 than in diploid cells, isolation rates in the two types of cell cultures were not significantly different. Comparative infectivity titrations of original patient specimens in HEp-2 and WI-38 cell cultures suggested the possibility of a slight advantage in sensitivity for the latter cell.

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Reversible Inhibition of DNA Virus Replication with Mithramycin.* (30739)

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Mithramycin, an antibiotic derived from cultures of Streptomyces, has been the subject of many studies as an investigational therapeutic agent in the treatment of cancer(1-5). Although the drug has a rather narrow range of therapeutic efficacy, principally limited to embryonal neoplasms, and a characteristic toxicological pattern(6), little is known of the mechanism of action resulting in cell death. This report deals with the effects of mithramycin on viral replication and shows that the antibiotic inhibits the synthesis of DNA but not RNA viruses.

Materials and methods. The effects of mithramycin† on several viruses in various tissue culture systems were studied (Table I). Mouse embryo fibroblasts obtained by trypsinizing 10-12-day Swiss mouse embryos were grown in one-liter Blake bottles in Eagle's medium (BME)(7) containing 100 units/ml penicillin, 100 μg/ml Streptomycin and 100 μg/ml Fungizone and supplemented with 5% calf serum. Rabbit kidney cells prepared in a similar fashion from the renal cortex of 2 kg albino rabbits were grown in BME containing 10% horse serum. "HeLa" and "L" cells were maintained in Blake bottles in BME with 5% calf serum and subcultured every 10-12 days. For inhibition experiments, cells were used within 24 hours of subculturing. Cell monolayers in 60 mm plastic petri dishes were pretreated with mithramycin in BME for 4 hours prior to virus inoculation. Following virus absorption the cells were washed in Hanks' balanced salt solution and then overlaid with medium containing the inhibitor. After one or two virus growth cycles, the cultures were frozen, thawed and virus yields determined by titration using the plaque method(8). The yields with mithramycin were compared with titers in paired control cultures. All cultures were incubated at 37°C in a humid atmosphere at 5% CO_2, 95% air.

Initial experiments to determine the susceptibility of the different cell types to the

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