USE OF HEP-2 CELLS FOR GROUPING ENTEROVIRUSES

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WITHIN the enterovirus group of Picornaviruses, 63 serotypes have been recognized (*1–3*). Since numerous untypable strains are still being isolated in laboratories throughout the world, many new types may be awaiting description and official recognition. For diagnostic laboratories which routinely perform virus isolation tests, the difficult and time-consuming task of typing large numbers of enterovirus strains may discourage detailed study of this important group of disease agents.

Attempts to simplify the task of enterovirus identification have led to a number of new techniques and modifications or adaptations of older methods which may help to reduce time and expense. Various antiserum pools have been proposed (*4, 5*) as a means of decreasing the number of neutralization tests that must be performed, and pooling is practiced routinely in our laboratory at the Communicable Disease Center.

A number of enteroviruses cause hemagglutination of human erythrocytes, and these viruses can be typed by hemagglutination-inhibition. This technique has resulted in considerable saving in our laboratory (*6*). The complement fixation test also has been adapted to the identification of enterovirus isolates. At the Communicable Disease Center this test has often proved useful for typing ECHO type 4 strains, which are difficult to detect by the standard tube neutralization test, and for typing polioviruses in studies of oral vaccine (*7*). The preparation and standardization of typing antiserums for the Coxsackie group B types is now underway at CDC.

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The recent work of Dr. Hsiung (*8*) suggested a simple technique for preliminary grouping of enterovirus isolates which may reduce significantly the number of neutralization tests otherwise necessary. Dr. Hsiung showed that the enteroviruses ordinarily isolated in rhesus monkey kidney cell (RMKC) tissue cultures (all three types of poliovirus, all six types of Coxsackie group B and type 9 of group A, and ECHO types 1 to 27) can be grouped according to the comparative susceptibility of rhesus and patas monkey kidney cells, primary human amnion (HAm) cells, and HEp-2 cells, a line derived from a human carcinoma.

Patas kidney cells are not readily available, and the virus types which cause a cytopathic effect (CPE) in tissue cultures of this species include some infrequently encountered ECHO viruses as well as the ubiquitous polioviruses and Coxsackie group B types. HAm cells have a range of sensitivity almost as broad as that of RMKC cells, and in HAm cells some virus types produce CPE inconsistently. HEp-2 cells, however, proved "highly sensitive to the three types of polioviruses and to all six types of Coxsackie B viruses. In recent tests, none of the ECHO virus types produced CPE in HEp-2 cells" (*8*). HEp-2 cells seemed to have characteristics which might prove useful in the routine identification of these viruses.

Dr. Hsiung has employed the HEp-2 cell line in studies of field isolates (primary isolates of naturally occurring strains), but her published data include only results of tests with prototype strains. To determine the general HEp-2 cytopathogenicity of enterovirus types, taking into consideration strain variations within each type, all RMKC isolates made in our laboratory during the past year have been tested in HEp-2 cells. We hoped thereby to discover whether passage into tissue cultures of the HEp-2 cell

line was a useful step in enterovirus identification. Actually, we promptly recognized its value and reorganized our identification routine long before the data presented here were fully accumulated.

Materials and Methods

Tissue culture. The strain of HEp-2 cells maintained in our laboratory was derived from a sample sent to us by Dr. Albert Sabin in 1961, and it has been propagated since then essentially according to his recommendations. Dr. Sabin obtained the strain in 1959 from Microbiological Associates, Bethesda, Md., where it had been grown and maintained in medium 199 containing 10 percent calf serum and 0.125 percent $NaHCO_3$.

The cell stock is grown in 32-ounce prescription bottles, using a CDC modification of Eagle's Minimum Essential Medium (MEM) (*9*) with a final concentration of 10 percent calf serum, 0.11 percent $NaHCO_3$, and the antibiotics standard in our laboratory (penicillin, streptomycin, neomycin sulfate, and amphotericin B in final concentrations of 200,000 units, 100 mg., 125,000 units, and 1 mg. per liter, respectively). Growth medium is prepared as follows, and sterilized by Seitz filtration:

Material	*Number of milliliters*
Demineralized water	625
Earle's BSS (10 X), without $NaHCO_3$, with 20 mg. percent phenol red	100
Eagle's MEM vitamin solution (100 X)	10
Eagle's MEM amino acid solution (100 X)	10
L-glutamine (3 gm. percent)	10
Glucose (25 gm. percent)	10
Biotin (10 mg. percent)	10
Yeast extract + peptone solution (5 gm.+50 gm. per liter)	100
Calf serum (heated at 56° C. for 30 minutes)	100
$NaHCO_3$ (8.8 gm. percent)	13
Penicillin+streptomycin (200,000 U+100 mg. per ml.)	1
Neomycin sulfate (12,500 U per ml.)	10
Amphotericin B (1 mg. per ml.)	1

Cells are removed from growth bottles by decanting the growth fluid, replacing with 20 ml. of prewarmed (37° C.) trypsin solution per bottle, incubating for 5 to 15 minutes, and pouring the cell film into a flask. A suspension of cells is made by repeated gentle agitation, using a syringe and large-gauge needle or a Cornwall pipette. The trypsin solution is removed by centrifuging for 5 minutes at 1,000 rpm and decanting. The cells are then resuspended in 12.5 ml. growth fluid per 32-ounce bottle. The trypsin solution consists of 0.125 percent Difco 1:250 trypsin in CDC modified Eagle's solution with 2 percent calf serum, and 0.11 percent $NaHCO_3$.

The concentration of cells in the suspension obtained as above is determined by counting in the usual manner. A 32-ounce prescription bottle usually produces about 12.5 million viable cells. For the preparation of new stock cultures, 4 million cells in 40 ml. growth fluid are inoculated per bottle. For tube cultures, the cell suspension is diluted with growth fluid to 40,000 cells per ml. and each tube is inoculated with 1 ml.

The growth fluid in stock cultures is changed when the medium reaches pH 6.8, as indicated by phenol red. Two changes are usually necessary during a growth period of 4 to 6 days at 37° C., after which heavy, confluent cell films should be present.

Tissue culture tubes are incubated at 37° C. for 4 days without change of growth fluid. After that time, growth fluid is replaced by maintenance fluid. The tubes are incubated again for 24 hours, and the maintenance fluid is replaced with fresh fluid before use. Maintenance fluid is prepared in the same manner as growth fluid, except that 5 percent chicken serum is used instead of 10 percent calf serum and the concentration of $NaHCO_3$ is increased to 0.22 percent.

Virus strains. The standard prototype virus strains of each of the enterovirus types are the same as those used by Hsiung (*8*). They had all been produced in RMKC and had titers between 10^4 and 10^7 $TCID_{50}$ per 0.1 ml.

The field isolates had all been made in RMKC and were untitered. They were derived from several investigational studies of healthy children conducted by our Enterovirus Unit and from reference requests sent to the unit from a number of diagnostic laboratories. The original specimens had been collected during 1960, 1961, and 1962 from persons residing in many different States. However, the majority came from Atlanta, Ga., Miami, Fla., Buffalo, N.Y.,

Minneapolis, Minn., Seattle, Wash., and San Francisco, Calif.

Inoculation and examination of tissue culture tubes. Each RMKC isolate was inoculated into two HEp-2 and two RMKC tubes, 0.1 ml. per tube. Tubes were incubated at 37° C. for 7 days. They were examined microscopically, usually on days 1, 3, 5, and 7. If no CPE was noted in the RMKC control tubes (a rare circumstance), all tubes were discarded and the experiment was repeated. After CPE developed in HEp-2 tubes, it inevitably progressed to involve all cells in the film. If CPE was noted in HEp-2 tubes, the result was recorded as positive, and the HEp-2 isolate was processed for identification.

If CPE was not noted in HEp-2 tubes (but was found in RMKC control tubes), the result was recorded as negative and the tubes were frozen and thawed and the fluids pooled, and two new HEp-2 tubes were inoculated with this harvest, 0.1 ml. per tube. These second-passage tubes were examined and recorded in the manner described above. On occasion, a third passage was made if the second showed no CPE.

Virus typing. HEp-2 isolates were typed by a simplified neutralization procedure suitable for unknown strains of enterovirus types which characteristically produce a high titer of virus in tissue culture. This procedure was uniformly satisfactory in this study since only polioviruses and Coxsackie B viruses were found to be cytopathic for HEp-2.

An aliquant of each isolate was diluted 1:1,000 without prior titration, and 0.4 ml. of the diluted virus was added to tubes containing 0.4 ml. of each of two potent antiserum pools, one consisting of poliovirus 1–3 and the other of Coxsackie B3–5. The mixtures were incubated at 37° C. for 1 hour, and inoculated into three RMKC tubes each, 0.2 ml. per tube. In addition, three RMKC tubes were inoculated with 0.1 ml. each of the HEp-2 isolate without antiserum. Inoculated tubes were incubated at 37° C., and examined on days 2, 4, 6, and 8. If neutralization was not demonstrable, the test was run with an antiserum pool consisting of the less frequent types, Coxsackie B1, 2, and 6. The composition and priority of such pools may be, and have been, changed from time to time as the predominance of the virus types varies from place to place and from year to year.

Cytopathogenicity in HEp-2 cell tissue cultures of enterovirus strains isolated in rhesus monkey kidney cells

Virus type	Number of strains tested	Number of strains with CPE in two HEp-2 cell passages		Number of strains producing no CPE in two HEp-2 passages	Virus type	Number of strains tested	Number of strains with CPE in two HEp-2 cell passages		Number of strains producing no CPE in two HEp-2 passages
		1st	2d				1st	2d	
Poliovirus					6	13	0	0	13
1	66	65	0	1	7	48	0	0	48
2	96	94	2	0	9	24	0	0	24
3	203	193	10	0	11	35	0	0	35
Coxsackie					12	27	0	0	27
A 9	46	0	0	46	13	1	0	0	1
A 16	4	0	0	4	14	20	0	0	20
B 1	4	4	0	0	15	23	0	0	23
B 2	17	6	3	8	16	4	0	0	4
B 3	74	58	14	2	17	8	0	0	8
B 4	77	40	30	7	18	6	0	0	6
B 5	15	13	1	1	19	30	0	0	30
B 6	8	6	0	2	20	21	0	0	21
ECHO					21	4	0	0	4
1, 8	37	0	0	37	22	10	0	0	10
2	8	0	0	8	23	1	0	0	1
3	1	0	0	1	24	2	0	0	2
4	25	0	0	25	27	18	0	0	18
5	7	0	0	7	29	1	0	0	1

NOTE: CPE=Cytopathic effect.

When neutralization had been demonstrated with an antiserum pool, retesting was done in the same manner with the monovalent antiserums contained in the pool. All HEp-2 isolates were identified in this manner, after adenoviruses had been eliminated by their peculiar cytopathic effect and their identity confirmed by complement fixation test.

RMKC isolates which had failed to produce CPE in HEp-2 cells were identified by standard procedures, which included the use of antiserums against poliovirus 1–3 and Coxsackie B1–6.

Results

Prototype strains. Our results paralleled those of Hsiung (*8*). Poliovirus 1–3 and Coxsackie B1–6 produced complete cell destruction in first HEp-2 passage. Coxsackie A9 and ECHO types 1–27 and 29 produced no detectable CPE after two HEp-2 passages.

Field isolates. The results of tests on 984 identified enterovirus strains isolated in RMKC from stool samples or rectal swabs are shown in the table.

No strains of Coxsackie A9 and 16 or of any ECHO type produced CPE after two HEp-2 passages. No untypable strain (one which cannot be identified as one of the 63 recognized enterovirus serotypes) has been found which produces CPE in HEp-2 cells.

Of 365 poliovirus strains, 352 were cytopathic on first HEp-2 passage. Of the remainder, 12 strains of poliovirus types 2 and 3 produced CPE on second passage. One strain of poliovirus 1 did not produce CPE after second passage, although control RMKC films were destroyed, but it was cytopathic on third passage.

Coxsackie B strains were more variable; although a majority produced CPE on first HEp-2 passage, many did not. However, of 68 strains which failed on first passage, 48 produced CPE on the second. Of 20 strains remaining, only 1 strain of B3 was cytopathic on third passage. Coxsackie B4 was particularly variable. Almost half of 77 strains failed to produce CPE on first passage, and 7 failed on second and third passages.

An interesting and consistent difference between polioviruses and Coxsackie B viruses was noted when cytopathogenicity for RMKC and HEp-2 tissue cultures was compared. With polioviruses, CPE was usually first noted after 1 day of incubation in HEp-2 and after 2 days in RMKC, and was almost always complete on the day following its first appearance in either type of tissue culture. By contrast, Coxsackie B strains usually produced first evidence of CPE in RMKC on about the third day of incubation, and thereafter in HEp-2 cells on about the fourth day. Cytopathogenicity was not complete until about 2 days later.

Discussion

For the enterovirus laboratory using RMKC tissue culture as the basic medium for virus isolation, passage of isolates into HEp-2 cells is a simple technique for segregating unknowns into two major groups: (*a*) polioviruses and Coxsackie B viruses, and (*b*) ECHO types and Coxsackie A9. Since group *a*, consisting of only nine types and including most of those which have been most often associated with significant illness, produces CPE in HEp-2 cells, typing by neutralization test is reduced to easily manageable proportions. Furthermore, the HEp-2 cell line is easily handled and can be maintained in any laboratory that prepares tissue cultures.

Based on the experience reported above, our laboratory has adopted the use of two HEp-2 passages as a routine procedure. A third passage produces too little additional benefit to be justified. The usual routing for identification of RMKC isolates in the Enterovirus Unit is as follows:

1. All strains are first passed through HEp-2 cells. If enterovirus-like CPE is demonstrable, they are typed as described above. If adenovirus-like CPE is evident, their identity is confirmed by complement fixation test and they are typed only when indicated.
2. Strains negative for HEp-2 are tested by hemagglutination test; if positive in this test, they are identified by hemagglutination-inhibition (*6*). In this step, reoviruses are also identified.
3. Strains still remaining are titrated and tested by neutralization, using antiserum pools consisting of enterovirus types which ordinarily would not be positive in HEp-2 or hemagglutination tests. If neutralization by these

pools is not demonstrated, the strains are tested by neutralization using antiserum pools including the types expected in steps 1 and 2.

4. Strains still unidentified are tested by complement fixation for the adenovirus group antigen and for ECHO type 4.

5. Strains untypable by the above procedures are studied individually by special procedures as research problems.

Summary

At the Enterovirus Unit laboratory, Communicable Disease Center, 984 enterovirus isolates, including almost all enterovirus types that can be isolated in rhesus monkey kidney cell tissue cultures, were tested in HEp-2 cell tissue cultures. Three hundred and sixty-four of 365 poliovirus strains and 185 of 205 Coxsackie B strains produced cytopathic effect in two passages. None of 414 Coxsackie A9 and A16 and ECHO strains demonstrated cytopathogenicity in HEp-2 cells.

Routine use of HEp-2 cells for grouping enteroviruses has enabled the laboratory to reduce significantly the number of neutralization tests necessary for final identification of enteroviruses.

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Pollution Control in the Androscoggin

The States of Maine and New Hampshire have recently agreed on a time schedule to control pollution in the Androscoggin River. To establish quality requirements which municipalities and industries must meet, representatives of these States will recommend classification of the river's tributaries to the 1965 sessions of their State legislatures and classification of the river itself to the 1967 sessions.

The interstate agreement resulted from a series of meetings between representatives of the Department of Health, Education, and Welfare and representatives of the two States and the New England Interstate Water Pollution Commission. The meetings grew out of a conference on pollution on the interstate waters of the Androscoggin, called by the Department on February 5, 1963, under enforcement provisions of the Federal Water Pollution Control Act.

Occupational Health Notes

Conjunctivitis From Ultraviolet Lamps

A Los Angeles family of three experienced severe eye pains after visiting a pet store using unshielded ultraviolet germicidal lamps to reduce incidence of disease in the animals. The family visited the store for about 1½ hours in the afternoon and woke up about 1 a.m. the following morning with eye pains. The store used six lamps located about 7½ feet high on the walls. The wife of the owner of the store told investigators she also had eye pains occasionally. The lamps have since been shielded.

Previously, employees of the city animal shelter developed symptoms typical of conjunctivitis induced by ultraviolet lamps. Shielding of the lamps eliminated the problem.

Artificial Snow

The possible hazards of using artificial snow in aerosol cans for decorating at Christmastime were investigated in California. A propellant, consisting essentially of Freon, and an organic chlorinated solvent constitute the bulk of the contents of the can. Although these substances are of low toxicity, high concentrations in unventilated areas may be hazardous. The solid ingredients, essentially soap with a small amount of plastic stiffener, comprise about 10 percent of the contents. The investigators concluded that no hazard should result as long as the directions on the can are followed.

Drycleaning Hazards

In Michigan one known serious accident has occurred in the past year in connection with coin-operated drycleaning machines. Two children were overcome by perchlorethylene vapors when they were left in an automobile for a few minutes with a 6-pound wool pile rug which had just been taken out of a coin-type machine and which had not been dried properly. The children were revived and apparently suffered no permanent injury.

Leakage of solvent from coin-type machines is not uncommon, according to Michigan occupational health authorities. In many cases, all the solvent in the machine was found to have leaked but was held within a safe ventilated area by the dyke and drain tank required by the State's installation standards.

Because of the time required to survey and license drycleaning installations, an amendment was submitted to the legislature changing the fee schedule for licensing. In 1962 the legislature passed the amendment and approved a budget which provided for addition of three organic solvent inspectors to the division staff.

Mercury Poisoning in Homes

Mercury poisoning was diagnosed in three children of a family in eastern Colorado. The father had brought home about 400 grams of mercury, and, over 2 or 3 months, about half had been spilled on the floor. Urine samples showed 500–800 micrograms of mercury per liter for all family members. Air samples taken about 3 feet above the floor throughout the house contained about 0.08 to 0.12 milligram of mercury per cubic meter. Cleaning operations have so far lowered the concentration to 0.04 milligram—not low enough to permit the family to return to the house.

In a survey conducted by the division of occupational and radiological health of Colorado, many persons remembered that at some time mercury had been brought into their homes for children to play with, either by the children themselves or by parents. In two neighborhoods, young children had canvassed the area asking for broken thermometers and were collecting the mercury in a bottle. As a result of such findings, statewide warnings were issued alerting parents to keep mercury out of their homes.

Filmstrips

Sets of three filmstrips, "Public Health Aspects of the Occupational Environment," "Occupational Diseases," and "Alcoholism in Industry," are available for loan from the Division of Occupational Health, Public Health Service, to universities, official agencies, and other interested groups. The filmstrips are especially useful in the general orientation to occupational health of public health workers and medical, engineering, and nursing students.