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Received November 2, 1960. P.S.E.B.M., 1961, v106.

Distinctive Cytopathology of ECHO Viruses Types 22 and 23.*† (26430)

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The cytopathology of the enteroviruses in rhesus monkey kidney tissue culture has been described(1). All of the enteroviruses studied (poliovirus 1-3, Coxsackie B1-B5, A9, ECHO 1-14) manifested a characteristic cytopathic effect (CPE) in fixed and stained preparations. Only ECHO virus type 10 (Reovirus type 1) was found to have features which differed from the other enteroviruses. In the present paper, the study was extended to ECHO serotypes 15-24. Types 22 and 23 were found to have distinctive CPE. The cytopathology of the remainder was similar to that previously described for the members of the enterovirus group.

Materials and methods. Tissue cultures. Rhesus monkey kidney coverslip cultures were prepared as described previously(1). Cultures were maintained in 1.8 ml of a medium consisting of 0.5% lactalbumin hydrolysate, 0.1% yeast extract (Difco), 0.2% bovine albumin, Fraction V (Armour and Co.) in a base of Earle's saline with 200 units penicillin, 200 μg streptomycin per ml and inoculated with 0.2 ml undiluted virus§ suspension. Titers of ECHO 22 and 23 were 5.1 and 5.3 log TCD_{50}/0.1 ml respectively. At selected intervals, coverslips were removed and fixed for staining. HeLa cell cultures grown in a medium containing calf serum were also used(3).

Histologic technic. Hematoxylin and eosin stain as modified by Reissig was used routinely(4). Feulgen stain was also used(5). Cultures were fluorochromed with acridine orange at pH 6.0(6) and examined under the fluorescence microscope. The fluorescent equipment consisted of a Reichert 'Lux X' high intensity mercury vapor arc lamp, 2 BG12 ultraviolet filters and a modified model

* Aided by a grant from the Nat. Cancer Inst., Nat. Inst. of Health.

† Presented in part at annual meeting of Soc. of Am. Bact., Philadelphia, May 1960.

‡ Markle Scholar in Medical Science.

§ ECHO virus prototypes were obtained through the courtesy of members of the Committee on Enteroviruses (2).

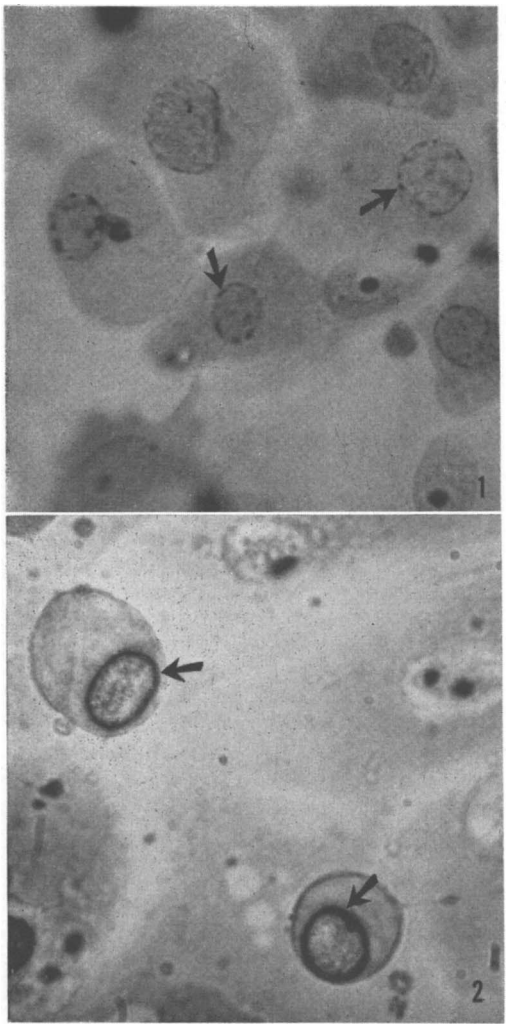

FIG. 1. Coverslip monkey kidney cultures infected with ECHO 22 and stained with hematoxylin-eosin. Cells show rounding loss of nucleoli with peripheral beading of chromatin. Photographed under oil immersion, ×970.

FIG. 2. Coverslip monkey kidney tissue culture infected with ECHO 22 and stained with hematoxylin-eosin. Two cells show 'empty' nuclei with prominent nuclear membranes. Photographed under oil immersion, ×970.

No. 414 American Optical binocular microscope with 35 mm camera attachment. With this equipment, observations under oil immersion (970×) were satisfactory.

Results. Examination of infected cultures stained with hematoxylin and eosin revealed that, with the exception of types 22 and 23, the CPE of ECHO viruses types 15-24 was characteristic of the enterovirus group(1). The typical enterovirus cytopathic changes consisted of rounding of cells, formation of a round, eosinophilic cytoplasmic mass and enlargement of eosinophilic nuclear granules. The cytoplasmic mass indented or appeared to cause folding of the nucleus. Formation of basophilic cytoplasmic granules was observed in late stages of infection. The changes occurring in the cytoplasm of cells infected with ECHO 22 and 23 were similar to those seen with other enteroviruses. The major differentiating features occurred in the nuclei. Since the cytopathic changes were the same for the prototype ECHO 22 and 23 strains, ECHO 22 prototype was selected for more detailed examination. Studies with ECHO 22 revealed a sequence of changes appearing in the following order: 1) *Rounding of cells and appearance of an eosinophilic cytoplasmic mass with no nuclear changes.* The nuclei remained oval or round and the cytoplasmic mass was present in all subsequent stages. 2) *Decrease in staining intensity of the nucleolus.* In this stage, the nucleolus stained very faintly giving a faded appearance. 3) *Disappearance of nucleolus without changes in chromatin.* 4) *Beading of nuclear chromatin either diffusely or peripherally.* (Fig. 1). In this stage, the chromatin of the nucleus appeared to be in dense clumps or 'beads' which were either arranged around the inner surface of the nuclear membrane or scattered diffusely throughout the nucleus. 5) *Disappearance of chromatin leaving an 'empty' appearing nucleus.* (Fig. 2). In this stage, the nucleus appeared to be devoid of any chromatin and the nuclear membrane was smooth and stained intensely. There was, however, in some nuclei, a very faintly staining, fine reticular network.

At selected intervals, 100 cells in stained preparations were examined and the number of these cells manifesting CPE was tabulated (Fig. 3). Throughout the period studied, relatively few cells showed CPE. Changes in cells appeared at 8 hours and the number of these cells increased through 12 hours and then decreased. The number of cells involved did not exceed 28% of total number of cells in the culture.

Nuclear changes at the various stages of infection have been summarized in Table I.

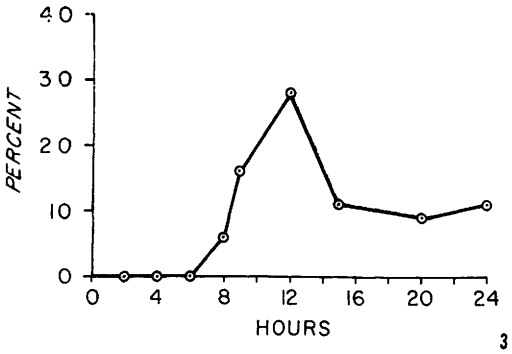

FIG. 3. Involvement of cells in fixed and stained monkey kidney tissue culture inoculated with ECHO 22. Percent = No. of cells manifesting CPE/100 cells.

The numbers were obtained by examining 100 cells manifesting CPE at times indicated. Rounded cells with an eosinophilic cytoplasmic mass and no nuclear changes were most numerous at 8 hours. Nuclei, which showed decrease in staining intensity or 'fading' of the nucleoli, sharply increased in number from 8 hours through 9 and 12 hours and then decreased. Nuclei with absent nucleoli but with no chromatin changes gradually increased in number from 8 hours through 20 hours and then decreased. Nuclei with absent nucleoli and with beaded chromatin appeared at 9 hours in small numbers, gradually increased through 20 hours and then showed a slight decrease. Cells with so-called 'empty' nuclei showed a steady increase from 9 hours through 24 hours. All cells showing CPE were rounded and had an eosinophilic cytoplasmic mass.

In wet preparations of cultures, cytopathic changes, which consisted of rounding and increased refractility of cells, generally appeared first at the edges of the monolayer and later spread throughout the culture. Neutralization tests and virus titrations were easier to read microscopically when the cultures were incubated on a roller drum. In fixed and stained HeLa cell cultures, ECHO 22 manifested a CPE similar to that seen in fixed and stained monkey kidney tissue culture.

Acridine orange fluorescence stain. Uninoculated cultures stained with acridine orange and examined under the fluorescence microscope showed a bright green nucleus indicative of DNA content and bright orange or orange-red cytoplasm and nucleoli indicating RNA content(6,7). In cells infected with ECHO 22 or 23, the thickened nuclear membranes fluoresced a bright green and the chromatin also appeared as bright green clumps. These structures were also Feulgen positive. Cytoplasmic masses were greenish and peripheral cytoplasm red or orange-red. In the 'empty' nuclei, there were fine reticular networks which fluoresced a faint gray-green and were Feulgen negative. There were no Feulgen positive structures in the so-called 'empty' nuclei.

Additional strains of ECHO 22. ECHO 22 has only recently been classified as a distinct serotype in the ECHO virus group(8). The role of this virus in human illness has not been defined. The prototype Harris strain was isolated in Ohio from a case of diarrhea by Dr. A. B. Sabin and his colleagues. ECHO 22 was isolated in Buffalo during a study of well infants in 1957 and also from clinical specimens submitted for virus diagnosis in succeeding years. The history of the Buffalo ECHO 22 strains has been summarized in Table II. The cytopathic effect of these strains in monkey kidney tissue culture revealed the same cellular changes as noted for the prototype Harris strain.

Discussion. The distinctive cytopathic changes resulting from infection with ECHO viruses types 22 and 23 were confined to the nucleus. The nucleus was round or oval and

TABLE I. Sequence of Nuclear Changes with ECHO 22.

Hr	No change	Faded nucleoli	No nucleolus	Beaded chromatin	'Empty'
0	0	0	0	0	0
2	0	0	0	0	0
4	0	0	0	0	0
6	0	0	0	0	0
8	83*	12	5	0	0
9	36	45	11	4	4
12	38	47	9	3	3
15	31	26	16	11	16
20	29	15	22	19	15
24	18	23	16	15	28

* No. of cells with nuclear changes, based upon count of 100 cells manifesting CPE.

TABLE II. History of ECHO 22 Strains.

Year	Strain	Clinical history	Age	Specimen
1957	Johnson	Well infant	2 mo	Rectal swab
1957	Host	" "	6 "	" "
1957	Morrison	" "	9 "	" "
1957	Borowiec	" "	15 "	" "
1957	Estelle	Diarrhea & jaundice	1 "	" "
1958	Hubbard	Diarrhea	5 wk	Throat swab
1960	Albaralla	"	13 mo	Stool

in the late stages, the nuclear membrane was prominent. The nucleoli and chromatin gradually disappeared leaving the so-called 'empty' nucleus. In contrast, cells infected with other enteroviruses showed indented or folded nuclei and unaltered nuclear membranes. The nucleoli and chromatin persisted until the late stages and nuclear granules became enlarged and more eosinophilic. Basophilic cytoplasmic granules observed in classical enterovirus type infection were absent in cultures infected with ECHO virus type 22 or 23.

In the experiments described, the multiplicity of infection was not controlled and the sequence of cellular changes was made on the basis of relative numbers of cells showing changes. The number of cells which appeared infected on the basis of cytopathology at any one time was found to be less than 30%. After 12 hours incubation total number of cells involved decreased (Fig. 3). The data to explain this phenomenon are not available from the present experiments.

The CPE in cultures infected with ECHO virus type 10 was distinguishable from either the typical enterovirus CPE or the changes described above for ECHO 22 and 23(1,9, 10,11). The main differentiating feature was the appearance of globular or filamentous eosinophilic material in the cytoplasm around or adjacent to the nucleus. ECHO 10 has been reclassified as Reovirus type 1(11) and one of the contributing features was the characteristic cytopathic effect in cell cultures. The use of cytopathology in establishment of the Reovirus group suggests that further studies of the physical and biologic characteristics of ECHO 22 and 23 should be undertaken to establish the relationships of these serotypes to the ECHO virus group and other enteroviruses.

The distinct cytopathic effect of viruses in tissue culture has proved to be a useful tool in identification of viruses isolated from the human alimentary tract. In our laboratory, using CPE as a guide, 7 strains of ECHO 22 were detected and confirmed serologically. Four strains were isolated from healthy infants and the remaining 3 were recovered from infants with diarrhea. ECHO 22 was isolated in this study only from very young children. Other viruses, such as adenoviruses, have been excluded from routine enterovirus serologic tests because of their cytopathology. Conversely, unknown agents have been assigned to the enterovirus antibody screening program when their cytopathology was found to resemble that of the enteroviruses.

At present there are no *positive* criteria for inclusion of an unknown virus into the enterovirus group. The results of this study indicate the use of cytopathology as a classification tool and it might be suggested that new agents considered for addition to the enterovirus group, particularly ECHO viruses, be examined from this point of view.

Summary. The cytopathology of ECHO 22 and 23 was compared with other enteroviruses and found to be distinctive. The important differentiating features appeared in the nucleus. An eventual disappearance of the nucleolus and nuclear chromatin and a thickening and increase in staining intensity of the nuclear membrane resulted in appearance of an 'empty' nucleus. Seven isolates of ECHO 22 were found to have similar cytopathic changes. It is suggested that detailed study of the cytopathic effect of viruses in tissue culture may play an important role in relating new agents to the enterovirus group.

The authors wish to acknowledge the technical assistance of Miss Johanne G. Taggert.

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Received January 3, 1961. P.S.E.B.M., 1961, v106.

Binding of Calcium to Bence-Jones Proteins.* (26431)

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Bence-Jones proteins appear in the urine of approximately half of the patients who have multiple myeloma. The fact that these abnormal proteins of small molecular weight are barely detectable in blood signifies that they are efficiently and rapidly transferred from plasma to urine, and in some cases several grams may be excreted per day. Because patients with multiple myeloma frequently have bone lesions, the calcium binding properties of their pathological proteins are of interest. Prior investigations have revealed no special affinity of serum myeloma globulins for calcium(1,2), but the calcium ion binding properties of urinary Bence-Jones proteins have not previously been reported.

Materials and methods. In the present study, Bence-Jones proteins were obtained from the urine of 7 patients† having multiple myeloma by precipitation in ⅔ saturated ammonium sulfate at 4°C. The precipitates were dialyzed exhaustively against distilled water, and the resulting solutions were filtered into flasks and lyophylized. One of the proteins, that from patient C. P., used in these investigations was further purified by column chromatography of a solution of the lyophylized powder on DEAE-SF‡. Just prior to measurements of calcium binding, the lyophylized proteins were dissolved in water, then electrodialyzed in a collodion cell for 24 to 48 hours against flowing distilled water. Standardized calcium hydroxide was then added to effect the desired pH, followed by addition of calcium chloride to reach the desired Ca^{++} concentration. Total calcium values were derived from the measured volumes added. Calcium ion activities were determined by potentiometric measurements using a permselective sulfonated polystyrene-collodion membrane as described by Carr(3). Protein concentrations were determined by weight after drying at 105°C.

Ultracentrifugal analysis of all of the proteins confirmed their classification as Bence-Jones proteins on the basis of sedimentation constants of approximately 3S. Double diffusion in agar gel plates gave precipitin bands in combination with rabbit anti gamma globulin. Electrophoresis by moving boundary and starch gel zone methods indicated varying degrees of heterogeneity with the exception of one homogeneous Bence-Jones protein, C.P., which also was eluted by increasing phosphate, decreasing pH gradient as a

* This investigation was supported in part by a grant from Am. Cancer Soc. and in part by a grant from Nat. Cancer Inst., N.I.H.

† Bone lesions observed by roentgenography: osteolytic lesions, osteoporosis, and vertebral compression in one patient; osteolytic lesions and vertebral compression in one patient; only osteolytic lesions in one patient; only osteoporosis in one patient; and no demonstrable abnormalities in 3 patients.

‡ DEAE-SF: diethylaminoethylcellulose.