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Virological Studies on Acute Respiratory Disease in Young Adults. I. Isolation of ECHO 28.* (26749)

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The JH and 2060 viruses were originally isolated from cases of mild acute respiratory disease and have been reported to induce this type of illness in human volunteers(1,2,3,4). Recent laboratory investigations have failed to detect any significant antigenic differences between these 2 viruses(5,6), which have now been designated ECHO 28 by the Committee on ECHO Viruses(6). No additional isolations of ECHO 28 have been reported and its incidence has been deduced entirely by serological methods(5,7). The present report describes the isolation of 8 strains of ECHO 28 from young adults with naturally occurring mild acute respiratory infections.

Materials and methods. Study population. One hundred and one freshmen and sophomore medical students, 5 of whom were women, were enrolled as volunteers in this project. The range in age was from 20 to 31 years. At approximately 6-week intervals throughout the academic year the total group was sampled as described below and served as asymptomatic controls. Each individual reported to the laboratory at the very first signs of acute respiratory disease, when additional samples were taken. They returned 2 to 3 weeks later for convalescent specimens.

Samples. Separate dry throat and nose swabs were taken in duplicate. One set was placed in 0.5% bovine serum albumin in phosphate buffered saline for virus isolation. The second nose and throat swabs were placed in individual tubes of broth for bacteriologic studies. A portion of the samples for viral study were inoculated into tissue cultures on the same day, and the remainder was frozen at –40°C. Serum specimens were also obtained and stored at –20°C.

Tissue cultures. The H.Ep. 2 line was carried in our laboratory on Eagle's medium (MEM) with 10% calf serum. Cultures used for virus isolation were maintained on Eagle's medium with 5% inactivated chicken serum and were incubated in stationary slanted racks at 37°C for at least 14 days after inoculation. Monkey kidney tissue cultures were prepared from cells that had been grown in bottles, trypsinized and frozen according to the method of Stulberg *et al.*(8). The cell suspension, stored at –90°C, was quickly thawed and diluted in growth medium containing 0.5% lactalbumin, 2% calf serum and 0.2% SV-5 rabbit antiserum to provide cultures with islands rather than a complete monolayer of cells. These cultures were maintained on 50% Medium 199 and

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50% Eagle's medium with 0.2% SV-5 antiserum.

Human kidney cultures were prepared from cell suspensions preserved in the same manner as the monkey kidney cells. These cultures were grown on medium described by Hsiung(9) and maintained on 50% Medium 199, 50% Eagle's medium with 1% calf serum. The maintenance medium for both monkey kidney and human kidney contained .03% sodium bicarbonate. To all media were added 100 units/ml penicillin, 100 mg/ml streptomycin and 1 mg/ml amphotericin B. After inoculation with specimens, both the monkey kidney and the human kidney cultures were incubated at 33°C on roller drums. Tissue cultures were examined 3 times a week and maintenance medium was changed twice a week. Monkey kidney cultures were tested for the presence of hemadsorbing viruses by the method of Chanock *et al.*(10) at 3 to 5 day intervals during the 3-week incubation period.

Titration of viruses. The JH and 2060 viruses were obtained from Dr. W. J. Mogabgab, Tulane University, New Orleans, La. Titrations were carried out by inoculating into each of 2 tubes of appropriate tissue culture 0.1 ml of 10-fold falling dilutions of virus prepared in 0.5% bovine serum albumin in phosphate buffered saline. The endpoint was considered as the highest dilution producing a cytopathic effect involving at least 20% of the cell culture within one week following incubation.

Neutralization tests were carried out by a modification of the method described by Mogabgab and Holmes(11). Approximately 100 $TCID_{50}$ of virus was mixed in equal volume with dilutions of serum, previously inactivated at 56°C for 30 minutes. After remaining at room temperature for 1 hour, the mixtures were inoculated into each of 2 secondary monkey kidney tissue cultures which were incubated in stationary racks at 33°C. The tubes were examined daily for the presence of cytopathic effect. The final readings usually were made 4 to 5 days after inoculation. In all neutralization tests a known guinea pig antiserum control was included and the virus was titrated. Neutralization titers are the reciprocal of the highest dilution which prevented a cytopathic effect of more than 1 or 2 small foci.

TABLE I. Isolation of Viruses from Mild Acute Respiratory Disease.

Week of (1960)	No. of samples		No. viruses isolated	No. identified as ECHO 28
	ARD	Routine		
Oct. 3	17	—	2	1
" 10	10	74	4*	2
" 17	14	—	6	4
" 24	5	—	2	1
" 31	3	—	1	—
Total	49	74	15	8

* Includes 1 unidentified virus from routine sampling.

Preparation of animal antisera. Antisera to JH, 2060 and the prototype virus isolated in this study were prepared in guinea pigs by intramuscular inoculation of 1 ml of undiluted human kidney tissue culture fluid. The inoculations were repeated at weekly intervals over a period of 6 weeks. The animals were bled 10 to 14 days after final inoculation.

Results. The isolation of viruses from samples obtained during a 5-week period beginning Oct. 3, 1960 is shown in Table I. The first routine sampling of the volunteer group as asymptomatic controls was scheduled for the second week of this period. All of the students reported except for 17 who had been sampled during illness in the previous week. During the week of routine sampling 10 students reported with acute respiratory infections and 74 were well. In the following 3 weeks, 22 of the latter reported with respiratory infections. Most of these illnesses were mild, with symptoms of coryza, nasal congestion and sore throat persisting for about one week. However, one student was subsequently hospitalized with a diagnosis of streptococcal pneumonia and 2 others developed infectious mononucleosis.

Fifteen viruses were isolated, 9 in both human and monkey kidney cultures, 6 in human kidney cultures only and none in H.Ep. 2 cell cultures. Eight of the 15 were identified as ECHO 28 by neutralization tests with

TABLE II. Neutralizing Antibody Titers to 3 Strains of ECHO 28 Virus in Paired Sera of Students from Whom This Virus Was Isolated.

Student No.	Neutralization titers: 3/Chicago/60 Acute	3/Chicago/60 Conv.	JH Acute	JH Conv.	2060 Acute	2060 Conv.
118	8	64	8	64	8	128
120	<8	8	<8	8	<8	8
124	<8	>64	16	>64	8	32
150	16	>128	16	>128	16	>128
140	16	>64	8	32	8	32
147	<8	32	8	32	16	32
142	<8	32	8	32	8	32
149	8	>64	16	64	16	64

JH guinea pig antiserum.† One of these strains was selected as a prototype and designated 3/Chicago/60. Neutralizing antibody titers were determined with this virus as well as JH and 2060 on the acute and convalescent sera from the 49 students with acute respiratory infections. Antibody rises occurred only in sera of persons from whom ECHO 28 virus was isolated. Seven of the 8 showed 4-fold or greater increases in titer (Table II) and titers to the 3 strains were very similar. Cross neutralization tests with guinea pig antisera and the 3 strains of ECHO 28 (Table III) failed to reveal significant antigenic differences.

Of 7 viruses listed as unidentified in Table I, one was isolated in both human kidney and monkey kidney cultures. Six grew only in human kidney cultures. One of the latter was isolated from an asymptomatic student during the week of routine sampling. All grew more slowly than ECHO 28, although the cytopathic effect was similar. These viruses were not neutralized by guinea pig antisera to ECHO 28 or to HGP(13) and are still under investigation.

Discussion. Isolation of 8 strains of ECHO 28 virus and demonstration of significant increases in antibody in 7 of the 8 persons from whom the viruses were isolated implicates this agent in mild acute respiratory infections in young adults. Since serological tests failed to reveal any further cases of infection by ECHO 28 it appears that the method used for isolating this virus was quite efficient. Previous reports have established the importance of incubating cultures on roller drums in a medium with a low bicarbonate concentration (5,6,7,12). Our results indicate that a lower temperature of incubation (33°C) as used by Tyrrell *et al.*(13) for HGP virus is also suitable for isolation of ECHO 28. This virus also grew well in secondary human kidney cultures.

All serologic data presented here indicate that the 3 strains of ECHO 28 virus; JH, 2060, and the newly isolated prototype 3/Chicago/60, are antigenically very similar. Although tests with hyperimmune animal sera might not be expected to reveal small antigenic differences, tests with convalescent serum from infected humans should show a higher homologous titer. This was not observed.

The isolation of 7 unidentified viruses suggests a multiple etiology of mild acute respiratory infections during this period.

Summary. Fourteen viruses were isolated from 49 volunteers presenting with symptoms of mild acute respiratory disease during a 5-week period. A 28.6% isolation rate was thus obtained. Samples from 74 asymptomatic students obtained during this period yielded only one still unidentified virus. Eight viruses were identified as ECHO 28. Paired sera of 7 of the 8 subjects from whom this virus was isolated showed a significant increase in neutralizing antibody titer. None of the other subjects with acute respiratory infections demonstrated this antibody response. No antigenic differences among the ECHO 28 strains were detected by neutralization tests employing the newly isolated prototype strain 3/Chicago/60, JH or 2060 with human acute and convalescent sera or with hyperimmune guinea pig sera. Other viruses, as yet unidentified, were also isolated

TABLE III. Cross Neutralization Tests with 3 Strains of ECHO 28 and Their Respective Guinea Pig Antisera.

Guinea pig antisera	Neutralization titers: 3/Chicago/60	JH	2060
3/Chicago/60	*512*	256	256
JH	512	*256*	512
2060	32	32	*64*

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at the time ECHO 28 was prevalent.

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Separation of Brain Proteins by Starch Gel Electrophoresis.* (26750)

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The complex nature of the mixture of proteins occurring in most tissues has rendered their identification and characterization exceedingly difficult. Analysis by conventional Tiselius technics and paper electrophoresis procedures have not yielded definitive results. The use of agar gel and starch gel (1,2) as supporting media for electrophoresis studies provides some superiority over paper in that more fractions have been visualized, and both have been successfully employed for studies of serum proteins. However, the applicability of these procedures to the study of tissue proteins has been limited. Voskobolnokov(3) reported on the separation of liver proteins using agar gel and was able to identify 13 separate moieties. Karcher, van Sande and Lowenthal(4) have reported on the separation of central nervous system proteins on agar gel in various pathological conditions. They characterized the proteins in a manner analagous to serum proteins and demonstrated 7 protein bands in normal white matter and 8 in normal brain stem, using a densitometer to quantitate their material. Eariler studies using paper electrophoresis(5) had inadequately resolved brain proteins although Palladin and Poliakova(6) reported the presence of 7-8 fractions in the central nervous system of the cat using this technic.

In this study, starch gel was used as the supporting medium, and the vertical electrophoresis procedure(7) was adopted. This yielded a satisfactory separation of the protein moiety, which appeared superior to that reported by the other methods mentioned. While this work was in progress Bailey and Heald(8) reported on use of horizontal starch-gel electrophoresis for separation of central nervous system proteins. They achieved some satisfactory delineation of the proteins.

Methods. Adult rats were sacrificed by fracturing the spinal column with the aid of a heavy metal bar, and the brain was extir-

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