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Isolation of a New Enterovirus (38506)

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(Introduced by W. Kaufmann)

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In 1972, 12 virus isolates which may represent a new type of enterovirus were obtained from 11 patients. All agents have the characteristics of the Coxsackievirus group A, but neutralization tests revealed no antigenic relationship to any of the known Coxsackievirus serotypes or to the other enteroviruses. Serologic tests, however, indicated that the isolates are related to one another. This paper describes certain characteristics of the virus, which we have designated S/Albany 1/72, S being the initial of the surname of the first patient from whom the virus was isolated.

All 11 patients were children, 5-wk to 7-yr of age. Four resided in Rockland County; the others lived in geographically separated counties on Long Island and in upstate New York. A clinical diagnosis of meningitis was made in nine patients and of encephalitis in one. The 11th case had manifestations of hand, foot, and mouth disease. The dates of onset were from July to November.

Materials and Methods. The patients' specimens were submitted for virus isolation in glycerol (feces) or frozen (throat and mouth swabs, cerebrospinal fluid). Clotted bloods were received for antibody assay. Methods for preparing fecal specimens and immune sera and techniques for neutralization tests in mice and tissue cultures have been described (1). Cultures of primary rhesus monkey kidney (PMK), a continuous line of rhesus monkey kidney cells (LLC MK_2), and human embryonic lung (HEL) cells were prepared in this laboratory and maintained in Eagle's minimum essential medium with fetal bovine serum, 2% for PMK and LLC MK_2 cells and 5% for HEL cells. All media contained, per ml, 100 units of penicillin, 100 μg of streptomycin, and 50 units of mycostatin. The white mice were of the Nya:NYLAR* strain. The techniques for inoculation of mice and tissue cultures have been published previously (2). Virus and antibody were assayed by the 50% endpoint method of Reed and Muench (3). Antibody titers are expressed as the reciprocal of the dilution which protected 50% of the animals.

Studies of physical and chemical characteristics were performed with virus grown in tissue culture. Ether sensitivity was determined by treating the virus with 20% ether at 4° overnight. Heat inactivation was studied at 56° in a waterbath for 30 min. Cationic stabilization was tested by the method of Wallis and Melnick (4). Nucleic acid type was determined by Salzman's procedure (5) as modified by Hamparian, Ketler, and Hilleman (6, 7). The direct method, using 5-bromo-2′-deoxyuridine (BUDR) as inhibitor of deoxyribonucleic acid (DNA) virus synthesis, was employed.

LLC MK_2 cells grown on microplates were maintained in media with the various additives (Table IV). The S/Albany 1/72 virus, vaccinia virus as the DNA virus control, and polio type 2 YSK virus as the ribonucleic acid (RNA) virus control, were assayed in tenfold serial dilutions in these plates using four wells per virus dilution. Each well contained 0.15 ml of medium. The inoculum was 0.025 ml.

For electron microscopy, HEL cell cultures infected with the S/Albany 1/72 virus and uninfected controls were fixed by adding equal amounts of cold 3% glutaraldehyde in 0.2 *M* *s*-collidine to the maintenance medium. The cells were scraped from the glass and incubated at 4° for 2 hr. Suspensions were centrifuged at 2000 *g* for 30 min, and the sediment was suspended in fresh glutaraldehyde in *s*-collidine and incubated at 4° for 20 min. Cells were then washed with cold 0.2 *M* *s*-collidine buffer, pH 7.5, three times for 20 min each. The pooled specimens were centrifuged at 2000 *g* for 10 min, and the sediment was preembedded in agar, postfixed in osmium tetroxide, dehydrated in a graded series of ethyl alcohol followed by propylene

oxide, and finally embedded in Epon mixture. Thin sections were cut on a Sorvall MT-2 ultramicrotome, double stained with uranyl acetate and lead citrate, and photographed in a Zeiss electron microscope.

Results. The agents were isolated from fecal material of 10 patients; from one of these the same agent was isolated from a throat swab. Virus was recovered from a mouth swab of the 11th patient, who had symptoms of hand, foot, and mouth disease. From 2 children with meningitis, a second virus was isolated: Coxsackie B5 virus from the cerebrospinal fluid of one patient, and Coxsackie A4 virus from the fecal specimen of the other.

On primary isolation, the agents were cytopathic for monkey kidney cells. Three of the isolates also caused cytopathogenicity in HEL cells. None was pathogenic for adult or 1-day-old mice. All isolates obtained in monkey kidney cells became cytopathic for HEL cells on further passage and caused death of 1-day-old mice. These mice showed signs typical of Coxsackievirus group A infection, including flaccid paralysis of the extremities and diffuse myositis of the striated muscle as seen in histologic preparations. The unknown isolates caused an enterovirus-like cytopathic effect, which usually first appeared 2 days after inoculation and which, after 2 or 3 additional days, involved the entire cell sheet. The viruses grew in PMK cell culture only to low titers not exceeding $10^{5.5}$ $TCID_{50}$/ml, and in mice to approximately $10^{7.3}$ PD_{50} (paralytic dose)/g of tissue.

Neutralization tests were performed in monkey kidney tissue cultures and/or in newborn mice with reference sera to poliovirus types 1, 2, and 3; Coxsackievirus group A, types 1–24, and group B, types 1–6; echovirus types 1–7, 9, 11–27, 29–33; Berlan (8); and Chu 37 (9) (Table I). None of these sera neutralized the isolates tested. Six of the isolates had shown such low-level growth and cytopathogenicity in cell cultures that neutralization tests in cell cultures could not be performed.

Immune sera were prepared with three of the isolates. S/Albany 1/72 strain had a homologous titer of 7600 in mouse tests. In mouse neutralization tests at a dilution of 1:5

TABLE I. RESULTS OF NEUTRALIZATION TESTS PERFORMED IN MONKEY KIDNEY TISSUE CULTURE AND IN NEWBORN MICE.[a]

	Immune sera to							
		Coxsackie						
Virus	Polio 1–3	A1-24[b]	B1-6	Echo 1–7, 9, 11–27, 29–33	Berlan and Chu 37[b]	S/Albany 1/72[b]	S/Albany 2/72[b]	S/Albany 3/72[b]
S/Albany 1/72	—	—	—	—	—[c]	7600	6300	2160
S/Albany 2/72	—	—[d]	—	—	—[d]	4080	12100	3900
S/Albany 3/72	ND	—	ND	ND	ND	9350	6900	1960
S/Albany 4/72	—	—[c]	—	—	—[c]	+	ND	ND
S/Albany 5/72	—	—	—	—	ND	+	ND	ND
S/Albany 6/72	ND	—	ND	ND	ND	+	ND	ND
S/Albany 7/72	ND	—	ND	ND	ND	+	ND	ND
S/Albany 8/72	—	ND	—	—	ND	+	ND	ND
S/Albany 9/72	ND	ND	ND	ND	ND	+	ND	ND
S/Albany 10/72	ND	ND	ND	ND	ND	+	ND	ND
S/Albany 11/72	ND	ND	ND	ND	ND	+	ND	ND
Coxsackie A1–24						—[e]		

[a] Test doses, $10^{2.3}$–$10^{2.6}$ $TCID_{50}$ or PD_{50}. Titers are reciprocals of highest dilutions protecting 50% of animals; compiled by Reed and Muench method (3).

[b] Mouse neutralization test only unless otherwise noted.

[c] Mouse and tissue culture neutralization tests.

[d] Tissue culture neutralization test only.

[e] See Results.

— No neutralization; +: Neutralization; ND: not done.

TABLE II. RESULTS OF MOUSE NEUTRALIZATION TESTS ON SERA OF EIGHT PATIENTS.

Patient	Date of onset 1972	Serum: Date collected 1972	Serum: Day post onset	Titer	Test virus	Test dose PD_{50}
A	7.29	8.2	4	4500	S/Albany 2/72[a]	63
7 yr, F		8.31	33	1200		
B	7.31	8.3	3	2200	S/Albany 4/72[a]	178
5 yr, F		8.22	22	3500		
C	10.4	10.7	3	126	S/Albany 9/72[a]	158
4 yr, M		11.13	40	126		
D	not	12.23		347	S/Albany 6/72[a]	48
5 wk, F	given	3.27.73		417		
E	8.30	9.1	2	515	S/Albany 1/72[b]	508
6 yr, M						
F	9.13	9.18	5	52	S/Albany 1/72[b]	508
2 yr, M						
G	9.19	9.20	1	87	S/Albany 1/72[b]	320
7 yr, M						
H	11.8	11.13	5	840	S/Albany 1/72[b]	508
2 mo, F						

[a] Patient's isolate.
[b] Representative strain.

TABLE III. SOME PHYSICAL AND CHEMICAL PROPERTIES OF S/ALBANY 1/72 VIRUS.

Treatment	Log_{10} of titer in $TCID_{50}$/ml
Filtration through Millipore filter:	
100-nm	5.0
50-nm	5.0
25-nm	2.5
Ether:	
Before treatment	5.5
After 18 hr, 4°	5.5
After 18 hr, 4° with ether	5.5
Acid (4 hr at room temperature):	
pH 7	5.5
pH 3	5.0
Heat	
Before treatment	6.5
After 30 min, at 56°	0
After 30 min, at 56° with *M* $MgCl_2$	6.0

TABLE IV. STUDY OF NUCLEIC ACID TYPE OF S/ALBANY 1/72.

Additive to maintenance medium of virus-infected cell cultures	Virus titer log_{10} $TCID_{50}$/ml: Controls: RNA (Polio 2 YSK)	Controls: DNA (Vaccinia)	S/Albany 1/72
None	6.0	4.5	2.5
10^{-5} *M* BUDR	5.5	1.7	2.5
10^{-5} *M* BUDR + 10^{-4} *M* thymidine	5.7	4.5	2.5

it failed to neutralize approximately 100 test doses of each of the 24 types of Coxsackievirus group A. All three immune sera neutralized all three isolates (Table I).

Paired bloods were collected from four of the patients during the acute and convalescent phase, and complement-fixation or

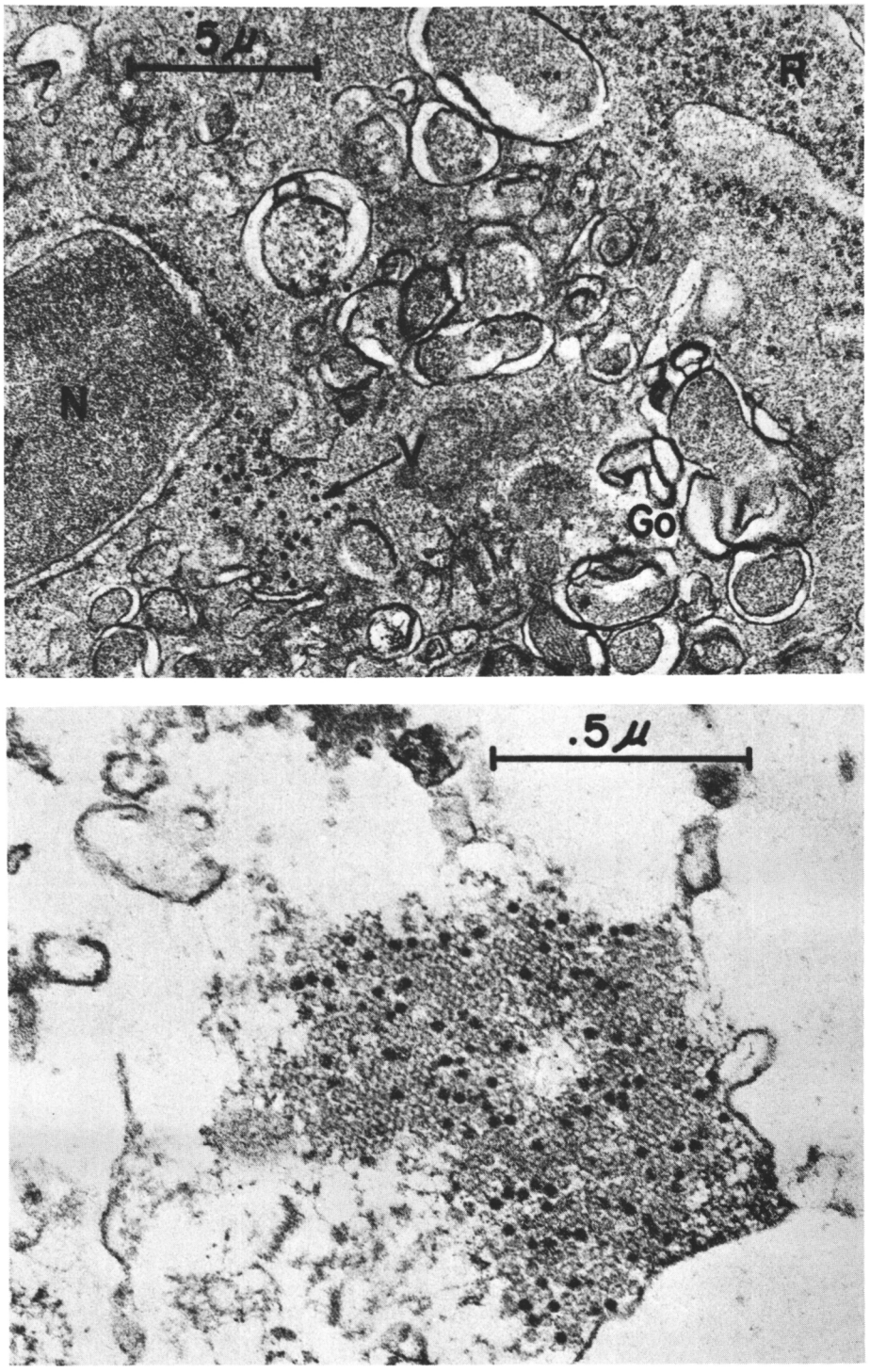

FIG. 1. *Upper:* Virus particles (V) free in cytoplasm adjacent to nucleus (N) of intact cell. Compare virions with ribosomes (R). Dilated Golgi cisternae (GO) X54,000. *Lower:* Lattice of virus particles, many empty, in cytoplasm of disintegrating necrotic cell. X72,000.

hemagglutination-inhibition tests were performed. No antibody conversion was detected with the following antigens: herpes simplex, mumps, Eastern and Western equine encephalomyelitis, St. Louis encephalitis, Powassan, California encephalitis, and Cache Valley of the Bunyamwera group. The results of mouse neutralization tests on these four pairs of bloods and on single bloods from four additional patients are shown in Table II. All patients had antibody, but significant rises were not observed. The levels of antibody were significantly higher than those observed in a study of the incidence of antibody to the S/Albany 1/72 virus in 30 healthy residents of New York State whose sera were submitted for premarital and preemployment tests. Half of these persons were 16–26 yr of age; the rest were 32–64 yr of age. Eight (26%) had antibody; their titers with test doses of 178 or 346 PD_{50} were 4 (3x), 10 (1x), 13 (1x), 20 (2x), and 100 (1x). Six of the individuals with antibody were 18–26 yr of age; the others were 36- and 44-yr old.

Studies of the isolates' physical and chemical characteristics show the virus to be less than 25 nm in size, ether-resistant, acid-resistant, heat-labile, and stabilized by magnesium cations (Table III). Lack of suppression of virus replication by the addition of BUDR (Table IV) suggests that it may be an RNA virus. Electron microscopy showed intracytoplasmic, optically dense, spherical particles. In some sections they were embedded in a lattice of empty structures of approximately the same size (Fig. 1). Criteria for identification were the average diameter of approximately 21 nm and the absence of envelopes of host-cell origin.

Discussion. Widespread occurrence of a new enteric virus was observed in New York State in 1972. Ten of the eleven patients studied had infections of the central nervous system. Since neutralizing antibody was already present in the acute phase at moderate to high titers, significant increases were not observed in the convalescent phase. This type of immune response is analogous to the early appearance of neutralizing antibody in arbovirus infections. In patient D (Table II), the stable level of antibody from age 5 wk to 4 mo is compatible with a congenital, perinatal, or postnatal infection. The laboratory studies indicate that the agent has the characteristics of the Coxsackievirus group A. It shows only limited growth in cell cultures.

Since the preparation of this manuscript, three additional isolates antigenically related to the S/Albany 1/72 virus have been detected among a number of unidentified viruses referred to the State laboratory for identification by the Erie County virus laboratory (Dr. Almen Barron) and the Strong Memorial Hospital in Rochester (Dr. Piero Balduzzi). These data, in addition to the antibody findings in a small sample of the healthy population, suggest widespread occurrence of this new agent in New York State.

Summary. During 1972 an apparently new enterovirus with characteristics of the Coxsackievirus group A was isolated from 11 residents of New York State, 10 of whom had central nervous system disease. The representative strain S/Albany 1/72 has the physical and chemical properties of an enterovirus and causes the pathology typical for Coxsackievirus group A in 1-day-old mice. Cross-neutralization tests indicated that the virus is distinct from currently recognized enteric viruses of man. Antibody levels to the representative strain were moderate to high in patients and were low to moderate in 26% of healthy individuals tested.

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