Enterovirus type 71 infection in Melbourne

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Between November 1972 and May 1973, 60 strains of a new enterovirus were isolated from 49 patients investigated at Fairfield Hospital for Communicable Diseases, Melbourne. Of these patients 39 were admitted to hospital with aseptic meningitis (which was accompanied by a rash in 6), 5 others had rash alone, 4 had acute respiratory tract infections, and 1 had infective polyneuritis. A representative strain from this outbreak had the physicochemical properties of an enterovirus but could not be identified with antisera prepared against the prototype polio, coxsackie, and echo viruses. Studies, performed in association with the WHO Collaborating Centre for Virus Reference and Research, Houston, TX, USA, showed the outbreak to be due to enterovirus 71. Most of the epidemic strains required sodium deoxycholate treatment before neutralization could be demonstrated.

During each summer many patients with aseptic meningitis are admitted to the Fairfield Hospital for Communicable Diseases, Melbourne. Throat swabs, cerebrospinal fluid, and faecal specimens are obtained and inoculated into a variety of cell cultures, and an attempt is made to identify all isolates so that the etiology and epidemiology of each outbreak can be studied. In the summer of 1972–73, enteroviruses were isolated from over 200 patients with aseptic meningitis. Most of these strains were readily typable but an agent was isolated from 39 patients that could not be identified with existing prototype reagents. The same agent was also recovered from 10 patients with other diseases. This report describes the isolation and identification of this agent.

MATERIALS AND METHODS

Virus isolation and identification

The specimens that were collected and the cells into which they were inoculated are shown in Table 1. The methods used for the collection and treatment of specimens in cell cultures and for the identification of enteroviruses were as previously described (1).

Mouse pathogenicity tests

Selected tissue culture fluids were inoculated both intracerebrally (0.02 ml) and intraperitoneally (0.03 ml) into a litter of newborn mice. The mice were examined daily and sacrificed when they became ill. If the mice were not ill after 14 days they were sacrificed and a 20% suspension of brain and torso (in Hank's buffered saline solution) was then inoculated into another litter of mice; these were observed daily and sacrificed when ill or after 14 days.

Mouse neutralization tests

Equal volumes of mouse-adapted virus (containing approximately 100 ID_{50}) and diluted test serum were incubated at 25°C for 1 h, then inoculated intracerebrally into a litter of newborn mice (0.03 ml per mouse). The mice were observed daily for signs of illness.

Immunodiffusion

The methods for antigen preparation and immunodiffusion were as previously described (3).

Disaggregation of virus suspensions

The technique described by Gwaltney & Calhoun (4) was followed, with the exception that the concentration of sodium deoxycholate was reduced to 0.5%.

Buoyant density

One ml of infected tissue culture supernatant was layered on top of a 3.5-ml, 30-60% (in phosphate

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Table 1. Specimens collected and cell cultures inoculated

Illness	Specimen collected	Cell cultures inoculated
aseptic meningitis	throat swab, cerebrospinal fluid, and faeces	MK, [a] MEK-3, [b] Bo [c]
vesicular rash	throat swab, faeces, and vesicle fluid or vesicle swab	MK, MEK-3, Bo, HEL [d]
respiratory illness	throat swab	MK, Hela, HEL

[a] MK = Primary cynomolgus monkey kidney.
[b] MEK-3 = Heteroploid monkey embryonic kidney (1).
[c] Bo = Borrie heteroploid human epithelial cells (2).
[d] HEL = Diploid human embryonic lung fibroblasts.

buffered saline, PBS, pH 7.4) linear cesium chloride gradient and centrifuged at 246 000 *g* for 24 h at 4°C in a model L2-65B Beckman-Spinco ultracentrifuge using an SW56Ti rotor. Ten fractions were collected from the bottom of each tube and the density determined with a refractometer (American Optical Co., type 10402). Each fraction was then diluted 20-fold with PBS, and the virus was then pelleted and resuspended in PBS to the original volume, and examined by immune electron microscopy.

Immune electron microscopy

In order to deposit any particulate material, rabbit antiserum to one of the epidemic strains was diluted 1 : 100 with PBS and centrifuged at 1 000 *g* for 30 min. Supernatant fluid (0.2 ml) was mixed with 0.8 ml of a 1 : 3 dilution of tissue culture supernatant fluid or a 1 : 5 dilution of a virus-rich cesium chloride fraction (5). Reactant mixtures were incubated at 37°C for 1 h and at 4°C overnight, and were then centrifuged at 27 000 *g* for 90 min. The supernatant was carefully removed and the pellet gently resuspended in 2 drops of PBS. A microdrop sample was applied to a 400-mesh Parlodion-carbon-coated grid and was allowed to adsorb for 5 min. The excess fluid was removed with the edge of a filter paper disk. The sample was then stained with 4% phosphotungstic acid (pH 7.4) for 4 min and examined immediately in a Philips EM 300 electron microscope at 80 kV and a plate magnification of 57 000. All grids were coded before examination and four intact grid squares were examined on each.

Antisera

The enterovirus antisera that were used in tube neutralization and immunodiffusion studies are listed in Table 2.

Viruses

The following prototype enteroviruses were used: echo 1-9, 11-22, 24-27, 29-33; coxsackie B1-6, A2, 3, 7, 9, 10, 12-18, 20, 20A, 20B, 21, 24; polio 1, 2, 3.

A local isolate of echo virus type 23 was used because the prototype strain was not available. The origin of the epidemic strains chosen for further study are shown in Table 3.

RESULTS

Epidemiology

During late 1972 and early 1973, 60 strains of an agent that produced an enterovirus-like cytopathic effect were isolated from 49 patients admitted to hospital in Melbourne. The first strains were recovered in November 1972 and the numbers isolated increased each month, reaching a peak in February 1973. No further isolations have been made since May 1973 (Table 4).

The 49 patients were aged from 3 months to 25 years; most were less than 10 years old and 5 were less than 1 year old (Table 5). Twenty-six of the patients were males and 23 females.

Clinical features

Meningitis. The most common clinical syndrome was aseptic meningitis (Table 6). Thirty-nine patients had typical signs and symptoms of this disease, which was confirmed by lumbar puncture in 38 patients. Lumbar puncture was unsuccessful in the other patient. In 6 children (aged 16 months and 2, 3, 3, 5, and 5 years) with meningitis a rash was also present. This was either a fine erythematous maculopapular rash lasting 24–48 h (4 patients), scattered vesicles on the hands and feet (1 patient), or a mixture of vesicles, macules, and papules (1 patient).

Table 2. Antisera against prototype enteroviruses used in tube neutralization and immunodiffusion tests

Antiserum	Prepared in:	Origin
echo 1-9, 11-27, 29-33	rabbit	FCDH [a]
coxsackie B1-6	,,	,,
coxsackie A7, 9, 21	,,	,,
echo 9, 15 18, coxsackie A16 (local strains)	,,	,,
polio 1, 2, 3	monkey	CSL [b]
echo 1-3, 5, 6, 6', 6'', 7-9, 13-16, 19, 20, 22-26, 29, 30, 31	,,	NIH [c]
coxsackie B1, 3-6	,,	,,
coxsackie A1-10, 12, 14-18, 20, 20A, 20B, 21	,,	,,
echo 4, 11, 12, 17, 18, 21, 27, 32, 33	horse	,,
polio 1-3	,,	,,
coxsackie A11, 13, 19, 22, 24, B2	,,	,,
Lennette & Schmidt intersecting pools	,,	Copenhagen [d]

[a] Fairfield Hospital for Communicable Diseases, Melbourne, Australia.
[b] Commonwealth Serum Laboratories, Melbourne, Australia.
[c] Research Reference Reagents Branch, Collaborative Research Program, National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA.
[d] Statens Seruminstitut, Copenhagen, Denmark.

Table 3. Strains used for cross-reactions and antibody studies

Patient	Age	Sex	Source	Diagnosis
JS	6	M	faeces	aseptic meningitis
AD	3	M	throat swab	aseptic meningitis
KD	17	F	ulcers and vesicles	hand, foot, & mouth disease

Table 4. Monthly isolations of enterovirus 71

	1972		1973				
	Nov	Dec	Jan	Feb	Mar	Apr	May
number of strains	3	5	5	16	12	10	9
number of patients	2	5	5	12	9	7	9

Other. Five patients had rash as the predominant complaint. Two of these (aged 7 and 17 years) had hand, foot, and mouth disease, one (aged 3 months) had a fine, generalized maculopapular rash, one

Table 5. Age distribution of patients from whom enterovirus 71 was recovered

Age (years)	No. of patients
<2	8
2–4	14
5–9	14
10–14	4
15–19	6
20–29	3
⩾30	0

Table 6. Clinical presentation of patients with enterovirus 71 infections

Clinical manifestation	No. of patients
Viral meningitis	
without rash	33
with rash	6
Other	
rash	
hand, foot, and mouth	2
fine maculopapular	1
diffuse erythematous	1
impetigo	1
respiratory tract infection	
whooping cough	2
croup	1
upper respiratory tract infection	1
infective polyneuritis	1

Table 7. Isolations of enterovirus 71 from the 49 patients

Specimen	No. received	No. positive
throat and/or nose swabs	47	40
cerebrospinal fluid	33	0
faeces	24	18
vesicle fluid or swabs	4	2
urine	1	0

(aged 5 years) had a florid diffuse erythematous rash, and one (aged 9 months) had impetigo. Of the remaining patients, 4 had respiratory illnesses comprising 2 children with clinical whooping cough (without bacteriological confirmation), 1 child with croup, and 1 with a mild upper respiratory tract infection. The remaining patient, a 4-year-old, had infective polyneuritis.

Virus isolation

Although the agent was isolated from 87% of the throat swabs and 75% of the faecal specimens submitted, it was never recovered from the cerebrospinal fluid. On two occasions the agent was recovered from vesicle fluid (Table 7). Primary isolation occurred most readily in heteroploid monkey embryonic kidney (MEK-3) cells and in primary cynomolgus monkey kidney (MK) cells, although a few isolations were also made in HeLa, Borrie (Bo), and diploid human embryonic lung fibroblast (HEL) cells. On subsequent passage to MK, MEK-3, HeLa, Bo, heteroploid human epithelial (HEP 2), and HEL cells, most strains grew best in MEK-3, secondary cultures giving titres of $10^{3.5}-10^{5}$ $TCID_{50}$/0.1 ml. About 10% of the strains passaged quite rapidly into HeLa or Bo cells.

Characterization of the JS virus

Isolation. One of the strains chosen for further study, JS virus (see Table 3), was isolated in primary MK but not in MEK-3 or Bo cells. On passage, the agent grew best in MEK-3 and HeLa cells producing complete cytopathogenic effect (CPE) in 7 and 9 days, respectively. In MK only 50% destruction was apparent after 9 days and no CPE was observed in Bo, HEP 2, or HEL cells. After multiple passages in MEK-3, including purification by terminal dilution, the strain was able to produce CPE in all 6 tissues. Each of a litter of newborn mice, inoculated with purified JS virus grown in MEK-3, developed flaccid paralysis after 3–5 days.

Identification. Even after deoxycholate treatment and purification by terminal dilution, JS virus was not neutralized by 20 units of antibody against any prototype polio, coxsackie, or echo virus. In a tube neutralization test in MEK-3 cells using untreated virus, the strain had a homologous titre of 1 : 25. However, when the virus was treated with deoxycholate, the same serum gave a titre of 1 : 12 800. If tested in HeLa or Bo cells, high titres were obtained using either treated or untreated virus.

A minor " one-way " cross was observed between the prototype coxsackie virus A14 and anti-JS serum by tube neutralization (Table 8). A 1 : 25 dilution of the anti-JS serum did not neutralize 32–320 $TCID_{50}$ of the other prototype enteroviruses that grow in cell culture.

A 1 : 25 dilution of the rabbit antiserum to JS virus neutralized approximately 100 ID_{50} of JS virus in newborn mice.

Three epidemic strains, JS, AD, and KD (see Table 3), were concentrated and used in standard immunodiffusion tests. No antisera to the prototype polio, coxsackie, or echo viruses reacted with JS, AD, or KD concentrates, whereas antisera to either JS or AD viruses reacted with all 3 viruses to give immunoprecipitates indicating immunological identity.

Table 8. Cross-neutralization test with coxsackie A14 and JS virus and their homologous antisera

Virus	Reciprocal of antibody titre			
	antiserum to coxsackie A14 (G14)			antiserum to JS (rabbit)
	horse	monkey	rabbit	
Coxsackie A14 (G14)	800	>1600	>1600	100 (25)[a]
JS	<25	<25	<25	400

[a] The figure in parentheses is the value 1 day later.

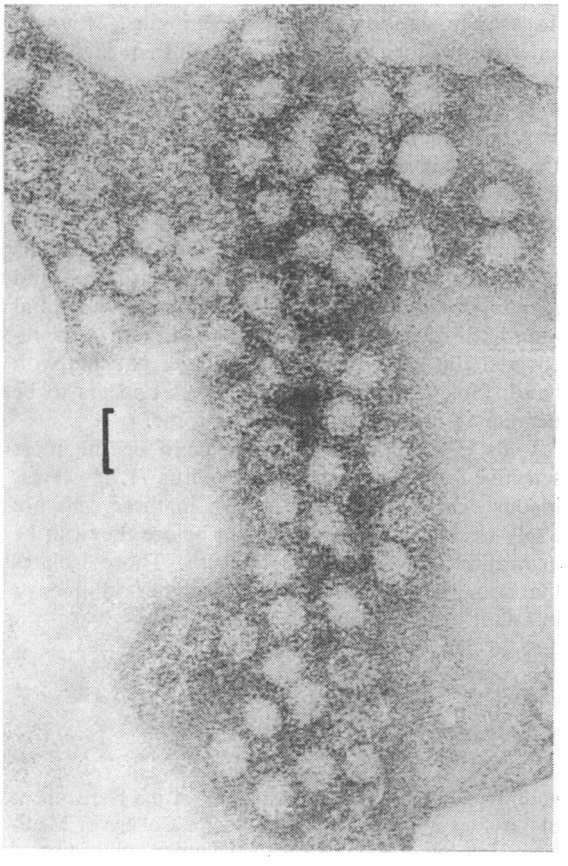

Fig. 1. An immune complex of enterovirus 71 with specific antiserum. The particles are evenly coated with easily visible antibody. The bar represents 50 nm.

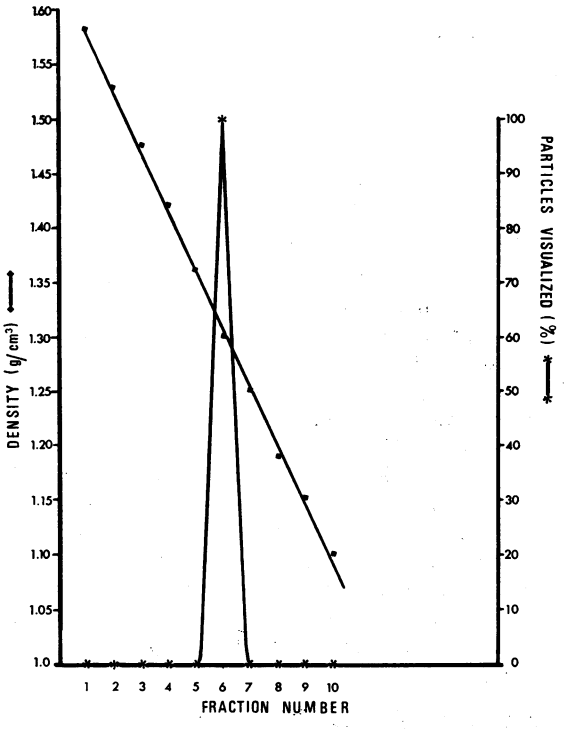

Fig. 2. Determination of buoyant density of enterovirus 71. Particle distribution was measured by immune electron microscopy and expressed as a percentage of the total number of particles counted.

Other properties of the JS strain. The replication of this strain was not inhibited by 100 μmol/litre 5-iododeoxyuridine. It was resistant to treatment with 20% diethyl ether for 18 h at 4°C and to pH 3 for 1 h at 37°C and was stable at 50°C for 1 h in the presence of 1 mol/litre magnesium chloride. On examination by electron microscopy the JS strain was found to have cubic symmetry and a capsomeric substructure, and was 28–30 nm in diameter (Fig. 1). The buoyant density of the virus was determined by isopycnic centrifugation on a cesium chloride density gradient. A total of 780 particles were counted, all of which were in fraction 6, which had a mean density of 1.305 g/cm³ (Fig. 2). JS virus did not agglutinate human " O " erythrocytes at 25°C.

Results of tests performed at the WHO Collaborating Centre for Virus Reference and Research. The JS and AD strains and their homologous antisera were forwarded to the WHO Collaborating Centre for Virus Reference and Research, Houston, Texas,

USA, where the strains were tested against antisera to all the polio, coxsackie, and echo viruses, and also to the recently recognized enteroviruses 68, 69, 70, and 71. It was found that a 1 : 6 dilution of a hamster serum made against the enterovirus 71 prototype neutralized the AD strain and temporarily suppressed the growth of the JS strain. No other antisera neutralized these 2 strains (R. Mirkovic, personal communication).

Further characterization of the JS strain. Tube neutralization tests were performed using strains JS and AD, prototype enterovirus 71 (BrCr), and their respective antisera. The viruses were treated with sodium deoxycholate prior to neutralization and the test was assayed in MEK-3 cells. The results are summarized in Table 9.

In a standard immunodiffusion test when either JS or BrCr antigens were placed in the central well and their respective antisera in alternate outer wells, a line of identity resulted. Similarly, when antisera to either JS or BrCr viruses were placed in the central well and the two antigens BrCr and JS alternately in the outer wells, immunological identity was demonstrated.

Identification of the epidemic strains

All 60 strains were neutralized by a 1 : 25 dilution of anti-JS serum. The few strains that could be adapted to growth in HeLa or Bo cells were readily neutralized. Those that grew well only in MEK-3 required monodispersion before neutralization could be achieved.

Table 9. Cross-neutralization with enterovirus 71 strains

Virus	Antiserum to:		
	BrCr	JS	AD
BrCr	400	12 800	6 400
JS	50	12 800	200
AD	800	>25 600	12 800

DISCUSSION

Enterovirus 71 has recently been recognized as a new enterovirus type (R. Mirkovic, personal communication). The prototype, BrCr, was one of 20 immunologically related strains isolated between 1969 and 1972 in California from patients with central nervous system disease (6). An extensive epidemic of aseptic meningitis and/or hand, foot, and mouth disease caused by enterovirus 71 occurred in southern Sweden in 1973 and several sporadic isolations were made in Sweden in 1967 and 1972 (7).

The Melbourne outbreak was confined to the summer months of 1972–73, and the commonest disease association was aseptic meningitis. Although enterovirus 71 was isolated with high frequency from throat swabs and faecal specimens obtained from these patients, it was never recovered from the cerebrospinal fluid, a finding also recorded in the USA and similar to that observed with poliovirus. Rash was another common sign, particularly in children aged less than 5 years.

The virus was isolated from vesicle specimens obtained from 2 local patients, one with rash and aseptic meningitis, the other with hand, foot, and mouth disease. As the Swedish workers also recovered the virus from vesicles from patients with hand, foot, and mouth disease there appears to be definite association with this syndrome.

Cells of monkey origin appear to be the most sensitive for the growth of enterovirus 71. However, in our experience, strains grown in these cells are likely to require monodispersion before they can be neutralized by homotypic antisera. Those isolates that could be adapted to growth in other cell lines or in infant mice were readily neutralized.

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RÉSUMÉ

INFECTION PAR L'ENTÉROVIRUS TYPE 71 À MELBOURNE

A la fin de 1972 et au début de 1973, on a isolé de 49 malades de Melbourne, Australie, 60 souches d'un entérovirus non typable avec les immunsérums des prototypes de poliovirus, coxsackievirus ou échovirus. Les principaux signes cliniques observés chez ces malades étaient une méningite aseptique, des éruptions, et des infections des voies respiratoires supérieures. La plupart des patients étaient âgés de moins de 10 ans.

Le virus a été facilement isolé des prélèvements au niveau de la gorge et des échantillons de selles, mais n'a pu être mis en évidence dans aucun des 33 échantillons de liquide céphalorachidien examinés. Il a été isolé dans du liquide des vésicules dans les cas d'éruptions vésiculeuses.

La plupart des isolements ont été réalisés en culture primaire de cellules rénales de cynomolgus ou dans une lignée continue de rein embryonnaire de singe. Dans les passages ultérieurs, la plupart des souches ont poussé rapidement en cellules de rein embryonnaire de singe et quelques-unes ont également poussé en cellules Hela et Borrie.

Une souche représentative s'est révélée avoir les propriétés physicochimiques d'un entérovirus, n'agglutinait pas les érythrocytes humains à 25°C et provoquait une paralysie flasque chez les souris nouveau-nées. Dans les épreuves de neutralisation en tube, un immunsérum de lapin préparé contre cette souche a donné un titre d'anticorps homologue de 1/25 en présence de virus non traité, et de 1/12800 après traitement du virus par le désoxycholate de sodium. A part une réaction croisée « en un seul sens » avec le prototype de coxsackie A14, le sérum n'a neutralisé aucun des prototypes de poliovirus, coxsackievirus ou échovirus qui poussaient en culture cellulaire.

Des souches épidémiques représentatives ont été neutralisées par un immunsérum préparé contre le prototype de l'entérovirus 71 souche BrCr. Des épreuves de neutralisation croisée et d'immunodiffusion effectuées au Fairfield Hospital à l'aide de BrCr, de souches épidémiques et de leurs immunsérums respectifs ont confirmé leur identité.

La plupart des souches épidémiques ne poussaient bien qu'en cellules de rein embryonnaire de singe, et nécessitaient un traitement par le désoxycholate de sodium avant qu'une neutralisation par l'immunsérum homotypique puisse être mise en évidence. Celles qui ont pu être adaptées à la culture dans d'autres cellules étaient facilement neutralisées.

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