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An epidemic associated with echovirus type 18

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SUMMARY

During the period October 1968 to March 1969 echovirus type 18 was isolated from 83 patients investigated at Fairfield Hospital for Communicable Diseases, Melbourne. The illnesses most commonly associated with these isolations were aseptic meningitis, and fever with rash.

We believe that this is the first report of an epidemic due to echovirus type 18 and the first occasion on which this virus has been shown to produce disease in adults.

INTRODUCTION

Echovirus type 18 (hereafter referred to as echo 18) is an infrequently encountered virus which has rarely been associated with clinical disease.

The prototype strain 'Metcalf' was isolated by Ramos-Alvarez & Sabin (1958) from a rectal swab obtained from an 8-month-old child with diarrhoea. Eichenwald, Ababio, Arky & Hartman (1958) demonstrated infection in 17 infants investigated during an epidemic of diarrhoea in a hospital nursery, by isolation of the virus from faecal specimens and detecting simultaneous antibody rises. Echo 18 has also been isolated from the faeces of a 5-month old child who died after a short febrile illness (Sabin, Krumbiegel & Wigand, 1958), from multiple sites in a 3-week-old child with fever and rash (Medearis & Kramer, 1959) and from the C.S.F. and faeces of a 12-year-old boy with aseptic meningitis (Eckert, Barron & Karzon, 1960). The latter two patients developed rises in homologous antibody titre.

In August 1968 a virus was isolated from the cerebrospinal fluid of a 7-year-old boy with aseptic meningitis admitted to Fairfield Hospital for Communicable Diseases, Melbourne. Preliminary studies suggested that this strain, designated R.B., was an enterovirus but it was not typable by procedures routinely used in this laboratory. Further study showed it to be a variant of echo 18.

From October 1968 to March 1969 strains similar to the R.B. virus were obtained from an additional 83 patients investigated at this hospital.

This communication gives an account of the procedures used in the isolation and identification of these strains.

MATERIALS AND METHODS

Specimens

Throat swabs

Throat swabs were collected into screw-capped bottles containing 2–3 ml. of 'virus transport medium' consisting of serum-free Eagle's Medium (B.M.E.) and antibiotics (penicillin, 100 i.u./ml.; streptomycin, 100 μg./ml.; neomycin, 40 units/ml.; amphotericin B, 20 μg./ml.). On arrival at the laboratory the bottles were agitated on a mechanical shaker for 10 min., the fluid transferred to a centrifuge tube and spun at 1200 rev./min. (400g) for 10 min. and the resulting supernatant fluid inoculated into tissue cultures.

Cerebro-spinal fluid

Cerebro-spinal fluid (C.S.F.) was collected into dry sterile tubes and cultured without treatment. Specimens which were obviously bloodstained were centrifuged at 1200 rev./min. (400g) for 10 min. and the supernatant fluid used for inoculation.

Throat swabs and C.S.F.'s taken at night or on weekends were stored at 4° C. and processed within 48 hr.

Faeces

About 10 g. of faeces were collected into sterile screw-capped jars and stored at −20° C. The specimens were subsequently thawed and 20% suspensions prepared using transport medium as diluent. After centrifugation at 2000 rev./min. (1400g) for 15 min. the supernatant fluid was collected and re-spun for 20 min. at 10,000 rev./min. (26,000g) in a 'multi-speed head' of an International PR2 refrigerated centrifuge. The resulting supernatant fluid was decanted and its pH adjusted to 7·2–7·4 by the addition of 4% hydrochloric acid or 5% sodium bicarbonate.

Serum

Serum samples obtained during the acute and convalescent phases of the patient's illness were stored at −20° C. until required.

Cell cultures

Cell cultures were prepared in screw-capped, flat-bottomed, glass phials (72 × 20 mm.)* and incubated stationary at 36° C. until the growth was almost confluent.

A summary of the cells and culture media used is shown in Table 1.

The diploid human embryonic fibroblasts were established from the lungs of a locally obtained embryo. MEK-3, a heteroploid epithelial cell line, was derived from kidneys of a cynomolgus monkey foetus and has been maintained in this laboratory since 1963.

* Suppliers: Johnsen and Jorgensen, London, England.

Table 1. *Cells and culture media*

Designation	Cell type	Growth medium*		Maintenance medium†	
		Medium	Serum	Medium	Serum
MK	Primary cynomolgus monkey kidney	199‡	2% foetal calf	Eagle's	2% foetal calf
HEL	Diploid human embryonic lung fibroblasts	Eagle's§	5% calf	Eagle's	2% foetal calf
HeLa	Heteroploid human epithelial cells	Eagle's	5% calf	Eagle's	2% foetal calf
HEp-2	Heteroploid human epithelial cells	199	5% calf	Eagle's	2% foetal calf
RK13	Heteroploid rabbit kidney epithelial cells	199	5% calf	199	1% foetal calf
BSC-1	Heteroploid cercopithecus monkey epithelial cells	199	5% calf	Eagle's	2% foetal calf
MEK-3	Heteroploid cynomolgus monkey embryonic kidney	199	5% calf	Eagle's	2% foetal calf

* All contain penicillin 100 i.u./ml., streptomycin 100 μg./ml.
† All contain penicillin 100 i.u./ml., streptomycin 100 μg/ml., and neomycin 40 units/ml.
‡ Obtained from Commonwealth Serum Laboratories, Melbourne, Australia.
§ Basal Medium Eagle's, diploid, obtained from Grand Island Biological Co., Grand Island, New York, U.S.A.

Isolation of viruses

All specimens were inoculated into duplicate screw-capped phials of MK and HeLa tissue. Throat swabs from patients presenting with a rash were also inoculated into HEL, BSC-1 and RK13 tissue in an attempt to isolate rubella virus.

Immediately before inoculation the growth medium was poured off and the inoculum added, together with sufficient maintenance medium to bring the final volume of fluid to 2 ml.

For throat swabs, 0·2 ml. of material was used, whereas each C.S.F. specimen was distributed equally among the phials.

Undiluted faecal extracts were dispensed into HeLa cells (0·2 ml.) and monkey kidney (0·5–0·7 ml.), which were rotated on a roller drum at room temperature for 1 hr. The inoculum was then poured off, the tissues washed once with maintenance medium and a further 2 ml. of this medium added. When cytotoxic faecal extracts were encountered, further cultures were seeded with one-third to one-half the original inoculum and, if the cells were again destroyed, a blind passage was made in a final endeavour to recover a virus.

All cultures were incubated at 34° C. on roller drums rotating at 10 rev./hr. After 3–4 days they were examined using the low-power ($\times$ 100) of a Leitz inverted microscope. If a cytopathic effect (C.P.E.) were seen the phials were re-examined daily. In the remaining cultures the medium was decanted, fresh medium added and incubation continued. This procedure was repeated twice weekly for 3 weeks, after which, if no changes had occurred, the cultures were discarded. No blind passages were made unless cell disintegration took place before this time.

Identification of enteroviruses

Cultures showing a C.P.E. were incubated until 50-100% destruction of cells occurred. The culture medium was decanted, centrifuged at 1500 rev./min. (800*g*) for 15 min. and the supernatant tissue culture fluid (T.C.F.) was tested as follows:

Haemagglutination

Human group O erythrocytes washed three times and resuspended in phosphate buffered saline, pH 7·0, were used as a 0·5% suspension. One drop (0·03 ml.) each of undiluted T.C.F. and red cell suspension were mixed in a polystyrene microtitre U plate* with controls in which maintenance medium was substituted for T.C.F. The test was incubated at room temperature (25° C.) and read when the cells in the controls had settled to a compact button (30–60 min.).

Tissue specificity

One drop (0·03 ml.) of T.C.F. was seeded into duplicate phials of MK, MEK-3, HeLa, HEp-2 and HEL cells. The cultures were incubated at 34° C. on a roller drum and examined daily for C.P.E.

Newborn mouse inoculation

A litter of eight newborn mice was inoculated intracerebrally with 0·05 ml. and intraperitoneally with 0·05 ml. of T.C.F. The inoculated animals were observed for 3 weeks. No histopathology or blind passages were carried out.

Serotyping by neutralization

The method used followed that described by Lennette & Schmidt (1969), with the following exceptions:

(1) The test was assayed in the cells showing maximum sensitivity in the tissue specificity test.

(2) The virus–serum mixtures were incubated for 1 hr. at room temperature.

(3) Duplicate cell cultures were each inoculated with 0·1 ml. of virus–serum mixture.

If the tissue specificity of the strain were typical of a particular group of viruses, the antisera thus suggested were used. In all other cases the viruses were tested against pools of high-titre antisera and then against the individual sera of the pool which produced neutralization.

Neutralizing antibody estimations

Neutralizing antibody titrations were performed with serial fourfold dilutions of serum (beginning at 1/4) and 100 TCD50 of virus, controls being set up as for serotyping. Acute and convalescent serum samples were tested simultaneously.

* Cooke Engineering, Alexandria, Va., U.S.A.; catalogue no. 220/24A.

Preparation of rabbit antiserum

The R.B. strain, which had been purified by three terminal dilutions, was cultured in MEK-3 monolayers in 6 oz. flat-sided glass bottles each containing 20 ml. of maintenance medium. When cell destruction was complete, the contents of the bottles were frozen and thawed three times. The material was clarified by centrifugation at 2000 rev./min. (1400*g*) for 15 min. and the virus sedimented by centrifugation at 50,000 rev./min. (150,000*g*) for 3½ hr. in a Spinco Model L2 ultracentrifuge. The pellet was resuspended in Hanks's Balanced Salt Solution to one-tenth the original volume.

Before inoculation, blood was taken from a 4-month-old rabbit and the serum stored at −20° C. The rabbit was injected intravenously with 1 ml. of the virus concentrate weekly for 5 weeks. A blood sample was obtained 7 days after the last injection and the serum tested for the presence of homologous neutralizing antibody. Because the antibody level was unsatisfactory, the rabbit was boosted on the 9th and 13th weeks and exsanguinated 1 week after the final injection.

Preparation of monodispersed cultures

Monodispersed virus suspensions were prepared by (1) filtration (Schmidt & Lennette, 1970) and (2) deoxycholate treatment (Gwaltney & Calhoun, 1970).

Physical and chemical properties

Acid lability, stability to ether and heat, nucleic acid determination

The methods followed those described by Schieble, Fox & Lennette (1967). However, in the determination of nucleic acid, *Herpes hominis* Type 1 was used as the DNA-containing virus, and 5-iodo-deoxyuridine was substituted for 5-bromo-deoxyuridine.

Antisera to prototype viruses

The following enterovirus antisera were used:

Rabbit antisera (Fairfield Hospital, Melbourne): Echo 1–9, 11–33, Coxsackie A7, A9, A21, B1–B6.

Monkey antisera (Commonwealth Serum Laboratories, Melbourne): Polio 1, 2, 3.

Monkey antisera (National Institutes of Health, U.S.A.): Echo 1–9, 11–31; Coxsackie A1–A18, A 20, A 21, A 22, A 24; B1–B6; Polio 1, 2, 3.

Viruses

The prototype viruses, obtained from the National Institutes of Health (U.S.A.), used in neutralization tests, were:

Echo 1–9, 11–33, except Echo 23 (local strain used).

Coxsackie B1–B6.

Coxsackie A2, A3, A7, A9, A10, A12–A18, A20, A20A, A20B, A21, A24.

Polio 1, 2, 3.

The epidemic strains used for cross-reactions and antibody studies and their origin are shown in Table 2.

Table 2. *Strains used for cross-reactions and antibody studies*

Patient	Age	Sex	Source	Diagnosis
R.B.	7	M	C.S.F.	Aseptic meningitis
K.H.	4	F	C.S.F.	Aseptic meningitis, rubelliform rash
A.M.	2	M	T.S.	Pharyngitis, rubelliform rash
J.G.	28	M	T.S.	Aseptic meningitis
M.H.	28	F	T.S.	Atypical pneumonia, pharyngitis, rubelliform rash
T.F.	2	F	T.S.	Rubelliform rash

T.S. = Throat swab.

Table 3. *Age distribution of patients from whom echovirus 18 was grown*

	Age (years)						
	0–4	5–9	10–14	15–19	20–29	30–39	⩾ 40
No. of patients	19	25	13	8	9	9	0

Table 4. *Isolation rate of echo 18 from various specimens*

Illness	T.S.	C.S.F.	F.
Aseptic meningitis	23/65*	29/52	36/43
Others	15/18	—	4/4

* Numerator = number of strains isolated; denominator = number of specimens tested.

RESULTS

Echo 18 epidemic

In the epidemic period October 1968 to March 1969, echo 18 was isolated from 83 patients.

The age distribution of these patients is shown in Table 3.

Sixty-five patients (78%) had aseptic meningitis with or without exanthem, while in 15 the major clinical feature was a rubelliform rash. The other three patients had gastroenteritis, bronchitis and polymyositis respectively.

During the outbreak 107 strains of echo 18 were isolated from 83 patients, primary isolation occurred in MK on 106 occasions and in BSC-1 in one.

Throat swabs and C.S.F. samples were obtained from the 65 patients with aseptic meningitis soon after their admission to hospital. Echo 18 was isolated from 23 (35%) of the throat swabs and 29 (56%) of the 52 C.S.F.'s available for virus studies (see Table 4). In addition, faecal specimens were obtained from 43 of this group usually within 5 days of admission. The virus was recovered from 36 (84%).

Throat swabs were submitted from all 18 patients with diseases other than aseptic meningitis. Echo 18 was isolated from 15 (84%). In one patient both echo 18 and rubella virus were isolated.

Faecal specimens were obtained from only four of this group and the virus was isolated on each occasion.

Table 5. *Results of cross-neutralization tests*

Virus	Antiserum to echo 18		Antiserum to R.B.
	Rabbit*	Horse†	Rabbit
Echo 18 (Metcalf)	**3200**‡	**3200**	400
R.B.	200	50	**200**

* Prepared at Fairfield Hospital against the Metcalf strain.
† W.H.O. reference antiserum. ‡ Reciprocal of antibody titre.

Characterization of the R.B. *virus*

Isolation

The first strain (R.B.) was isolated in primary MK cell culture. A typical enterovirus C.P.E. appeared after 7 days incubation and progressed to 75% destruction of the cell sheet in 10 days. When passaged, the C.P.E. in MEK-3 resembled that seen in primary MK but was more rapid and complete, while HEL showed a fine granular disintegration which was slow to appear and rarely proceeded to completion. No C.P.E. was seen in HeLa or HEp-2 cells. Tissue culture fluid from MEK-3 monolayers had infectivity titres in the order of $10^{5 \cdot 0}/0 \cdot 1$ ml., whereas those in MK and HEL were 10- to 100-fold lower.

Other properties

The virus was stable at pH 3, resistant to ether and heating (50° C. for 1 hr.), and was unaffected by treatment with 5-iodo-deoxyuridine. It did not agglutinate human erythrocytes and was not pathogenic in newborn mice.

Serology

The R.B. virus was not neutralized by any of the rabbit or monkey prototype enterovirus antisera.

A rabbit antiserum prepared against this virus had an homologous titre of only 1/200. Eight units of this serum were used in neutralization tests against all the prototype enteroviruses which will grow in tissue culture and echo 18 alone was neutralized.

Cross-neutralization tests using R.B. and the prototype echo 18 (Metcalf) and their homologous sera are shown in Table 5.

The rabbit serum prepared against R.B. neutralized both viruses to similar titres while the two prototype antisera required 16–64 antibody units to neutralize R.B. virus.

Variation in strains

Tissue specificity

In contrast to the prototype, most strains grew more readily in MEK-3 than in MK or HEL. Considerable variation was observed in the tissue specificity of the strains isolated during the epidemic. The tissue specificity of 'Metcalf' and four epidemic strains is shown in Table 6.

Table 6. *Tissue specificity of some selected echo 18 strains*

Virus	Tissue		
	MK	MEK	HEL
Echo 18 (Metcalf)	+ + + + 6*	+ + 7	+ + + + 3
R.B.	+ 7	+ + + + 7	+ + 7
K.H.	+ 7	+ 7	+ 7
A.M.	+ + + + 7	+ + + + 5	+ + 7
J.G.	+ + + + 5	+ + + + 4	+ + + + 2

* Day on which C.P.E. recorded. Degree of C.P.E.: + + + +, 100%; + + +, 75%; + +, 50%; +, 25%.

Table 7. *Neutralization tests with various antisera*

Virus	Antiserum to echo 18		Antiserum to R.B.
	Rabbit*	Horse†	Rabbit
Echo 18 (Metcalf)	**3200**‡	**3200**	400
R.B.	200	50	**200**
K.H.	800	800	3200
A.M.	—	50	200
J.G.	6400	6400	6400

* Prepared at Fairfield Hospital against the Metcalf strain.
† W.H.O. Reference antiserum. ‡ Reciprocal of antibody titre.

Table 8. *Reactions with patients' sera*

Patient	Serum specimen days after onset of rash	Neutralizing antibody to virus				
		T.F.	R.B.	Metcalf	J.G.	K.H.
T.F.	1	< 4	< 4	8	8	< 4
	4	< 4	< 4	256	64	32
M.H.	1	< 4	< 4	< 4	< 4	< 4
	8	< 4	< 4	128	2000	2000

Neither the prototype virus nor any of the epidemic strains produced C.P.E. in HeLa or HEp-2 cells.

Sensitivity to antisera

Strains which showed differences in tissue specificity also varied in their reactions in neutralization tests with antisera made to the prototype and R.B. viruses.

All strains were neutralized by anti-R.B. serum at its homologous titre. With the prototype antiserum some isolates reacted like the prototype virus, while others required up to 64 units to produce neutralization.

Patients' sera

Strain variation was also reflected in the differences in the patients' serological responses. Paired specimens obtained from two patients (T.F., M.H.) were tested against the prototype virus and four epidemic strains (Table 8).

Sera from both patients failed to neutralize strains T.F. and R.B. but there were significant rises in titre to two other epidemic strains (J.G and K.H.) and to the prototype strain.

Removal of viral aggregates by filtration and deoxycholate treatment

To determine whether the low titres of various sera to the R.B. virus were due to aggregation of the virus, the prototype echo 18 and R.B. were (1) filtered and (2) treated with deoxycholate and the neutralization tests repeated. In neither case was there an increase in antibody titres.

DISCUSSION

Although there have been scattered reports of the isolation of echo 18, no major outbreaks attributable to this virus appear to have been documented.

Before the recent epidemic the only strains of echo 18 which we have encountered were obtained from a group of apparently healthy children surveyed during a study of the incidence of viral infections in childhood (unpublished data). From 1958–64 specimens were obtained at least once a month from a group of children at a Melbourne creche (average attendance 50). In 1958 echo 18 was isolated from 13 children, in 1961 from 2 and in 1964 from 13, but no association with illness was apparent. All strains were recovered during the months of August to November.

The recent epidemic in Melbourne extended over a period of 6 months, October 1968 to March 1969, and echo 18 was isolated from 83 patients investigated at this hospital. Although the majority of strains were from children, 18 were from patients between the ages of 20 and 40 years. This appears to be the first occasion that the virus has been implicated in an epidemic in adults.

Sixty-five patients were admitted to hospital with aseptic meningitis. Echo 18 was recovered from the C.S.F. of 29 and from the throat or faeces of the remainder. As the virus was recovered from the C.S.F. it is reasonable to suggest a causal relationship between the isolations and the disease.

Echo 18 was isolated from 15 patients with a febrile illness and rubelliform rash, and the epidemiology is suggestive of an association with the illness.

In patients with aseptic meningitis, echo 18 was isolated more frequently from faecal specimens than from throat swabs or C.S.F.'s. Similar findings have been reported in epidemics due to echo 4 (Karzon *et al.* 1961; Ishii, Matsunaga, Onishi & Kono, 1968), echo 6 (Winkelstein, Karzon, Barron & Hayner, 1957), and echo 30 (Torphy, Ray, Thompson & Fox, 1970).

Identification of epidemic strains was difficult because most could not be neutralized by 20 units of antiserum to the prototype virus. Subsequent tests showed that these strains could be neutralized by a larger dose (e.g. 64 units) of the antiserum. Whilst 4 units of antiserum is sufficient for typing most strains, Kamitsuka, Soergel & Wenner (1961) have shown that some require 20–200 units and Wenner (1962) has suggested routinely using 50–100 units for the identification of enteroviruses.

Strain variation among the epidemic strains was shown by the variation in titre of neutralization tests performed with sera from patients and hyperimmune animals. Similar strain variation during an epidemic due to echo 6 was noted by Karzon, Pollock & Barron (1959).

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REFERENCES

ECKERT, G. L., BARRON, A. L. & KARZON, D. T. (1960). Aseptic meningitis due to ECHO virus type 18. *American Journal of Diseases of Children* **99**, 1.

EICHENWALD, H. F., ABABIO, A., ARKY, A. M. & HARTMAN, A. P. (1958). Epidemic diarrhoea in premature and older infants caused by ECHO virus type 18. *Journal of the American Medical Association* **166**, 1563.

GWALTNEY, J. M. & CALHOUN, A. M. (1970). Viral aggregation resulting in the failure to correctly identify an unknown rhinovirus. *Applied Microbiology* **20**, 390.

ISHII, K., MATSUNAGA, Y., ONISHI, E. & KONO, R. (1968). Epidemiological and virological studies of echovirus type 4 meningitis in Japan, 1964. *Japanese Journal of Medical Science and Biology* **21**, 11.

KAMITSUKA, P. S., SOERGEL, M. E. & WENNER, H. A. (1961). Production and standardization of ECHO reference antisera. 1. For 25 prototypic ECHO viruses. *American Journal of Hygiene* **74**, 7.

KARZON, D. T., ECKERT, G. L., BARRON, A. L., HAYNER, N. S. & WINKELSTEIN, W. (1961). Aseptic meningitis epidemic due to ECHO 4 virus. *American Journal of Diseases of Children* **101**, 610.

KARZON, D. T., POLLOCK, B. F. & BARRON, A. L. (1959). Phase variation in ECHO virus type 6. *Virology* **9**, 564.

LENNETTE, E. H. & SCHMIDT, N. J. (1969). *Diagnostic Procedures for Viral and Rickettsial Infections*, 4th ed., p. 573. New York: American Public Health Association.

MEDEARIS, D. N. & KRAMER, R. A. (1959). Exanthem associated with ECHO virus type 18 viremia. *Journal of Pediatrics* **55**, 367.

RAMOS-ALVAREZ, M. & SABIN, A. B. (1958). Enteropathogenic viruses and bacteria. *Journal of the American Medical Association* **167**, 147.

SABIN, A. B., KRUMBIEGEL, E. R. & WIGAND, R. (1958). ECHO type 9 virus disease. *American Journal of Diseases of Children* **96**, 197.

SCHIEBLE, J. H., FOX, V. L. & LENNETTE, E. H. (1967). A probable new human picornavirus associated with respiratory disease. *American Journal of Epidemiology* **85**, 297.

SCHMIDT, N. J. & LENNETTE, E. H. (1970). Antigenic variants of Coxsackievirus type A 24. *American Journal of Epidemiology* **91**, 99.

TORPHY, D. E., RAY, C. G., THOMPSON, R. S. & FOX, J. P. (1970). An epidemic of aseptic meningitis due to ECHO virus type 30: Epidemiologic features and clinical and laboratory findings. *American Journal of Public Health* **60**, 1447.

WENNER, H. A. (1962). Problems in working with enteroviruses. *Annals of the New York Academy of Sciences* **101**, 343.

WINKELSTEIN, W., KARZON, D. T., BARRON, A. L. & HAYNER, N. S. (1957). Epidemiologic observations on an outbreak of aseptic meningitis due to ECHO virus type 6. *American Journal of Public Health* **47**, 741.