A CLOSED EPIDEMIC OF ACUTE ASEPTIC MENINGITIS CAUSED BY ECHO VIRUS TYPE 4

PART II. LABORATORY STUDIES

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During June 1960 a closed epidemic of acute aseptic meningitis occurred in the closed community of the Schools for the Deaf and the Blind at Worcester, Cape Province. The epidemiological and clinical features of this 'epidemic' are described in the preceding paper by Wilsen *et al.*[1]

Isolation and Identification of the Aetiological Agent

This report represents the laboratory findings on specimens taken from approximately 40% of patients. The virus isolated from a number of specimens was shown to be ECHO virus type 4.

Outbreaks of acute aseptic meningitis caused by ECHO virus type 4 have been described previously both in South Africa[2] and elsewhere.[3-6]

MATERIALS AND METHODS

1. *Specimens*

Throat swabs, cerebrospinal fluids, rectal swabs, and blood samples were collected in parallel from patients during the first week of the 'epidemic'. In addition 6 specimens of faeces were collected. Follow-up samples of blood were collected approximately 6 weeks later.

2. *Bacteriological Investigations*

At the onset of the 'epidemic' specimens collected from several of the seriously ill patients were submitted for bacteriological investigation.

Blood was cultured in bile-broth and clot cultures were put up for isolation of salmonellae. Rectal swabs and cerebrospinal fluids were cultured for pathogenic bacteria.

3. *Virological Investigation*

(*a*) *Inoculation of mice.* Litters of 6 - 8 suckling mice, less than 24 hours old, were inoculated subcutaneously, intraperitoneally, and intracerebrally with single and pooled specimens of cerebrospinal fluid and suspensions made from rectal and throat swabs.

(*b*) *Inoculation of eggs.* The chorio-allantoic membranes of batches of 6 eleven-day old white Leghorn embryonated eggs were inoculated according to the method of Beveridge and Burnet[6] with cerebrospinal fluid and suspensions of rectal and throat swabs.

(*c*) *Inoculation of tissue cultures.* Suspensions were made from stools, throat, and rectal swabs in 1 - 2 ml. of tissue-culture fluid (0·5% lactalbumin hydrolysate in Hanks balanced salt solution or Parker No. 199), containing antibiotics. After centrifugation, the supernatant fluid was used for inoculating roller tissue-culture tubes, the inoculum being 0·1 - 0·25 ml. per tube. The cerebrospinal fluids were inoculated undiluted, the inoculum being 0·25 - 0·5 ml. per tube. Sera were inoculated undiluted in 0·1 ml. amounts.

Roller-tube cultures of renal epithelium of the vervet monkey (*Cercopithecus aethiops pygerythrus*), HeLa cells, and primary human amnion cells were inoculated with various specimens. The fluid in these cultures was renewed at 3 to 5-day intervals.

Neutralization tests. An estimated dose of 100 TC ID_{50} of the virus isolated was mixed with equal volumes of suitably diluted heat-inactivated antisera to ECHO virus types 1 - 19 and to poliovirus types 1 - 3. As controls in the case of the ECHO virus antisera, the above virus dose

was mixed with equal volumes of heat-inactivated pre-immunization rabbit sera.

After allowing these mixtures to stand for 1 hour, 0·1 ml. of each mixture was inoculated into each of 3 monkey kidney roller tube cultures. Tubes were observed daily for cytopathic effect.

Antibody tests on acute and convalescent sera. Some of the paired acute and convalescent sera were investigated for antibodies by mixing approximately 100 TC ID_{50} of the isolated virus with equal volumes of heat-inactivated acute and convalescent sera. The sera were tested both undiluted and in serial dilution. Mixtures were allowed to stand at room temperature for 1 hour, after which 0·1 ml. of each mixture was inoculated into each of 3 monkey kidney roller tube cultures. Tubes were observed daily for cytopathic effect.

RESULTS

1. *Bacteriological Investigations*

No pathogenic bacteria were isolated from the cultures of blood, blood clot, rectal swabs, and cerebrospinal fluids.

2. *Virological Investigations*

(*a*) *Mice.* No clinical signs of disease were noted during observation periods of 21 and 28 days. The viruses recovered from specimens in tissue culture were inoculated into 24-hour-old suckling mice, but they remained clinically healthy for observation periods of 14 and 21 days. Histological sections of mice, sacrificed on the 10th day after inoculation with active virus, did not show lesions compatible with virus infection.

(*b*) *Eggs.* No deaths occurred amongst the chick embryos inoculated. Chorio-allantoic membranes, examined 48 hours after inoculation, showed no lesions.

(*c*) *Tissue culture.* Virus was isolated from 32 of the 131 specimens inoculated into monkey kidney cell cultures. An analysis of these isolates is shown in Table I.

TABLE I. RESULTS OF TISSUE CULTURE

Specimen	*No. Investigated*	*No. positive for ECHO virus type 4*	*Positive %*
CSF	41	23	56
Throat swabs	32	7	22
Rectal swabs	33	2	6
Stools	5	—	—
Serum	20	—	—
Total:	131	32	24

Of the specimens inoculated into monkey kidney cell cultures, those inoculated with cerebrospinal fluid showed the earliest cytopathic effect. Within 4 - 8 days, 15 of the cerebrospinal fluids gave positive results whereas the remainder took 9 - 16 days. Cultures inoculated with suspensions prepared from throat swabs showed cytopathic changes within 6 - 23 days, whereas when suspensions were made from rectal swabs these changes were apparent in 7 - 15 days.

No cytopathic changes appeared in the HeLa or human amnion cell cultures with one exception. This was a virus isolated in HeLa cells from a specimen of stool. The nature of this virus is being investigated.

Neutralization tests. Neutralization tests carried out on the viruses isolated in monkey kidney cultures showed that these were ECHO virus type 4. In each case ECHO 4 antiserum either protected the monkey kidney cells completely or delayed cytopathic effects for several days. These tubes were set up in parallel with controls using pre-immunization sera.

Antibody tests on acute and convalescent sera. The antibody tests carried out on 10 sets of paired sera showed in 7 out of the 10 cases a low level of antibody, the cytopathic effect in tissue culture being delayed for 24 - 48 hours in comparison with the acute sera controls.

DISCUSSION

A striking feature of this investigation is that over 50% of the cerebrospinal fluids yielded ECHO virus type 4 on inoculation of monkey kidney cell cultures. Such a high isolation rate provides valuable evidence for implicating this virus as the aetiological agent in this 'epidemic' of aseptic meningitis. The specimens of cerebrospinal fluid from which virus was isolated were collected during the first 7 days of the illness.

Sore throat was not a symptomatic feature of the 'epidemic' despite the fact that virus was isolated from 22% of throat swabs examined. The isolation rate was considerably higher from the throat swabs than from the rectal swabs.

Although classified as an enterovirus, spread could be equally well explained by the oro-naso-pharyngeal route as by faecal contamination. This would seem to be supported by the higher virus isolation rate from throat as opposed to rectal swabs. It could, however, be argued that, if faeces and not rectal swabs had been examined, a higher isolation rate would have been obtained. It is possible that the isolation rate from these two sites may vary at different stages of the illness.

No viraemia was demonstrated.

In neutralization tests with the ECHO antisera prepared in rabbits, it is advisable to use pre-immunization sera in virus controls. By this means misinterpretation due to the effect of inhibitors present in rabbit serum and resistant to inactivation at 56°C. for 30 min., is avoided.

Examination of some of the convalescent sera shows a poor antibody response. It is probable that the plaque reduction technique, as suggested by Itoh and Melnick,[5] would be more useful for demonstrating antibodies. These findings of low levels of antibodies are similar to those previously reported by Chin *et al.*[3] and Malherbe *et al.*[2] and may be partly responsible for the high relapse rate.

It is important to carry out laboratory investigations in outbreaks of acute aseptic meningitis. In this manner the nature of the aetiological agent and the local conditions governing its spread may be ascertained. Such knowledge is essential for the ultimate prevention and control of these diseases.

SUMMARY

A closed epidemic of acute aseptic meningitis due to ECHO virus type 4 occurred at Worcester in the Cape Province during June 1960. This report represents the

laboratory findings relating to the 'epidemic'. Of the 131 specimens collected from patients who were ill with the disease and examined virologically, 32 yielded ECHO 4 virus in monkey kidney tissue culture. Of 41 cerebrospinal fluids investigated, 23 yielded ECHO virus type 4. No viraemia was demonstrated. Serum-neutralization tests carried out on paired sera showed a poor antibody response.

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