

## The isolation of an unclassified virus from an outbreak of infantile diarrhoea

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### INTRODUCTION

The role of viruses in human infantile diarrhoea is still obscure. Planned studies by different workers (Ramos-Alvarez & Sabin, 1958; Sommerville, 1958; Joncas & Pavilanis, 1960; Walker *et al.* 1960; Yow, Melnick, Blattner & Rasmussen, 1963) have resulted in conflicting reports and in only two instances have the number of isolations of enteroviruses from the test group been significantly greater than those from the control group. Undoubtedly there are viruses which have escaped detection to date, but the rapid increase in the practice of virology and the gradual improvement in techniques make it certain that many 'new' viruses will be isolated in the next few years. Some of these may be of significance in infantile diarrhoea. Recent reports (Duncan, 1960; Cooney, McLaren & Bauer, 1962; Abrahams, 1963) already indicate the existence of several enteroviruses which cannot be classified as members of the existing subgroups (Polio, Echo and Coxsackie).

These unclassified viruses have a number of characteristics in common, the most notable of which is their inability to produce cytopathic changes in cultures of monkey kidney epithelium. They are non-pathogenic for unweaned mice but are readily cytopathic when inoculated into tissue cultures of human origin.

Investigations into an outbreak of diarrhoea in a Children's Home resulted in the isolation of a virus with characteristics of this latter group and which is serologically distinct from the recognized serotypes of the enterovirus groups.

### MATERIALS AND METHODS

#### *Tissue cultures*

H.Ep II cells and HeLa cells were propagated in four ounce McCartney bottles in a medium consisting of 10% human serum, 5% calf serum, 0.5% lactalbumin hydrolysate and 84.5% Hanks's balanced salt solution. Monkey kidney cultures were obtained from Commonwealth Serum Laboratories, Melbourne, and were maintained in Medium 199 and 0.5% bovine albumin. Cultures of human amnion, human embryonic kidney, rabbit kidney and guinea-pig embryonic kidney were prepared by trypsinization. Chick and mouse embryo fibroblasts were grown from explants and human embryonic fibroblasts were propagated according to the method of Moorhead & Hayflick (1961). All cell cultures except monkey kidney were in a medium of 5% calf serum, 0.5% lactalbumin hydrolysate and 94.5% Hanks's balanced salt solution at the time of inoculation.

*Sources of viruses and antisera*

Antisera to poliovirus types 1-3, Coxsackie B virus types 1-6 and Echo virus types 1-25 were obtained from Microbiological Associates, Bethesda, and antisera to Adenovirus types 1-17 and 19-24 were obtained from Italdiagnostic Ltd. Antisera to Coxsackie A viruses 1-10, 20 (*a*), 20 (*b*) and 21, Echovirus types 26, 27, 28, F.E.B. and 1266 were prepared in this laboratory from prototype strains. Coxsackie A viruses 11-19, 22 and 24 were received from Dr Duxbury of the Commonwealth Serum Laboratories. Viruses Hu39, Hu659, Hu2220 and C-Thai 18 with their homologous antisera were kindly sent by Dr A. Abrahams, Pittsburgh, U.S.A.

*Neutralization tests*

Specific antisera diluted to contain 50 antibody units per 0.1 ml. to homologous virus were mixed with an equal volume of virus suspension diluted to contain 100 ID<sub>50</sub> per 0.1 ml. and allowed to stand at room temperature for 2 hr. Tissue cultures were then inoculated with 0.2 ml. of this mixture. Virus was considered neutralized if no cytopathic effect (C.P.E.) was observed when 75% or more of the cells in the virus control cultures showed degeneration.

*Preparation of antiserum against prototype strain*

One of the fifteen isolates was selected for the preparation of antiserum and determination of physical and biological properties. This virus was designated the 'Mill' strain. It was poorly antigenic in the rabbit and a prolonged course of inoculations was necessary to elicit an antibody response. Two rabbits were each given twelve intravenous inoculations of 5 ml. of tissue culture fluid at weekly intervals and also two intramuscular inoculations of 5 ml. Each rabbit thus received 70 ml. of tissue culture fluid. The rabbit serum had a titre of 64 against 100 ID<sub>50</sub> virus.

*Preparation of tissue-culture fluid for estimation of particle size*

Harvested tissue-culture fluid was clarified at low-speed centrifugation (2000 r.p.m. for 10 min.). Fluorocarbon (10%, v/v) was added and the mixture homogenized in a Waring Blendor for 90 sec. Further clarification was carried out at 2500 r.p.m. for 15 min. The clear supernatant was used for filtration through Millipore membranes of 100, 50 and 10  $\mu$  porosity.

*Handling of specimens*

Faecal specimens were collected by the nursing staff and were stored at  $-20^{\circ}$  C. while bacteriological tests were carried out. When no bacterial pathogens were isolated they were processed in the usual manner by preparing a 10% suspension in cold nutrient broth containing 1000 units of penicillin and 1000 units of streptomycin per ml. and shaking in a McCartney bottle. The resulting supernatant, after three centrifugations at 3500 r.p.m. for 15 min., was used to inoculate tissue cultures.

*Brief description of the outbreak*

An outbreak of diarrhoea occurred in a section of an infants' home, Sydney, in April 1961. All the children in this section were below the age of 2 years. Upper respiratory tract infections and diarrhoea had occurred sporadically for about 6 weeks and at the time of collection of the specimens all children had diarrhoea. Faecal specimens from nineteen children were examined. Seven of the children had had an upper respiratory tract infection within the previous 7 days but the relationship of these symptoms to the virus isolated is not known.

## RESULTS

*Isolation and attempted serological identification of the virus*

Of the nineteen specimens examined, fifteen yielded a virus cytopathic for H.EpII cells but not for monkey kidney epithelium. Cultures of H.EpII cells showed cytopathic effect 3–4 days after inoculation. Passage of the virus into further H.EpII cells produced cytopathic effect in 48 hr. but passage into monkey kidney cultures still caused no degeneration.

It was thought that the virus was most likely to be a Coxsackie A 21 (Coe virus), but antisera to this virus failed to neutralize any of the viruses isolated and the antiserum prepared against the prototype Mill strain failed to neutralize 100 ID 50 Coe virus.

Similarly it was shown that the virus was not related to Coxsackie Group A viruses 11, 13, 15, 18 and 20, which are known to be cytopathic for HeLa and H.EpII cells. A full study was then undertaken in an attempt to serologically identify the virus with members of the enterovirus group. Antisera to Polioviruses 1–3, Coxsackie B 1–6, Coxsackie A 1–10 and Echo viruses 1–28 all failed to neutralize the agent, as did antisera to Rhinoviruses F.E.B. and 1266 (a Rhinovirus isolated in this laboratory and cytopathic for H.EpII cells). An antigen prepared from tissue culture fluids did not fix complement in the presence of five different sera known to contain Adenovirus complement-fixing antibodies, and Adenovirus antisera 1–17 and 19–24 failed to neutralize the virus.

The antiserum prepared against the Mill strain and possessing a homologous titre of 64 failed to neutralize 100 ID 50 of Coxsackie A viruses 12, 14, 16, 17, 19, 22 and 24 when tested in unweaned mice. It was therefore concluded that this virus was not antigenically related to any of the classified enteroviruses and did not possess the common soluble antigen of the adenovirus group.

Dr A. S. Abrahams has isolated a number of viruses with similar properties to the Mill strain and kindly sent four prototype strains and their homologous antisera. Cross-neutralization tests established the Mill virus as serologically indistinguishable from Hu 659, a virus isolated by Abrahams in Pittsburgh. No relationship to the other three viruses, Hu 39, Hu 2220 and C-Thai 18, could be demonstrated (Table 1).

The remaining fourteen strains were also neutralized by Mill and Hu 659 antisera.

Table 1. *Neutralization titres obtained with Mill virus and four strains received from Dr Abrahams*

Antiserum	Virus	
	Mill	Hu 659
Mill	64	64
Hu 39	< 4	< 4
Hu 659	128	128
Hu 2220	< 4	< 4
Thai 18	< 4	< 4

*Cytopathogenicity of Mill virus in different tissue-culture systems*

The Mill virus produced cytopathic changes in two continuous cell lines (HeLa and H.Ep II), human amnion, human embryonic fibroblasts and human embryonic kidney. It failed to produce any effect in monkey kidney, rabbit kidney, embryonic guinea-pig kidney, mouse embryonic fibroblasts and chick embryo fibroblasts.

The cytopathic effect seen in H.Ep II cells was similar to that shown by the cytopathic members of the Coxsackie A group. Small foci of refractile rounded or shrunken cells were first seen in the culture. These foci then spread throughout the culture until all cells were affected. In haematoxylin–eosin stained preparations the nucleus was frequently pyknotic and crescentic in shape and was usually located at the periphery of the shrunken cell.

The behaviour of this virus in human embryonic fibroblasts was unusual in that it was rapidly cytopathic when the growth medium consisted of Hanks's balanced salt solution, lactalbumin hydrolysate and calf serum, but it was not cytopathic when medium 199 was used. Alteration to the pH of medium 199 with dilute acid or alkali and variation in the bicarbonate concentration did not assist the virus to produce cytopathic changes. To date this unusual phenomenon has not been investigated further.

*Inoculation of unweaned mice*

Mill virus after three passages in H.Ep II cells failed to produce any obvious disease in mice inoculated when less than 24 hr. old and observed for 21 days. No lesions could be found in the brain, fatty tissue or skeletal muscle of mice killed at 7 and 14 days. The original faecal extracts from which the viruses were recovered also failed to produce any symptoms in unweaned mice. From these results it was concluded that the virus is not pathogenic for unweaned mice and therefore cannot be classified in the Coxsackie group.

Intraperitoneal inoculation of adult rabbits, guinea-pigs and mice failed to produce any symptoms. No poeks were found following inoculation of the chick embryo chorioallantoic membrane.

*Haemagglutination*

The virus was tested for haemagglutination by the method of Goldfield, Sriongse & Fox (1957) at 37, 22 and 4° C. No haemagglutination could be demonstrated using human O, rabbit, guinea-pig, mouse, fowl, calf or sheep erythrocytes.

*Multiplication at 33° C.*

When cultures of H.EpII cells, inoculated with Mill virus, were maintained at 33° C., both C.P.E. and virus titres were delayed by approximately 48 hr. as compared with cultures given a similar inoculum and maintained at 36° C. This suggests that the virus is not a member of the Rhinovirus group.

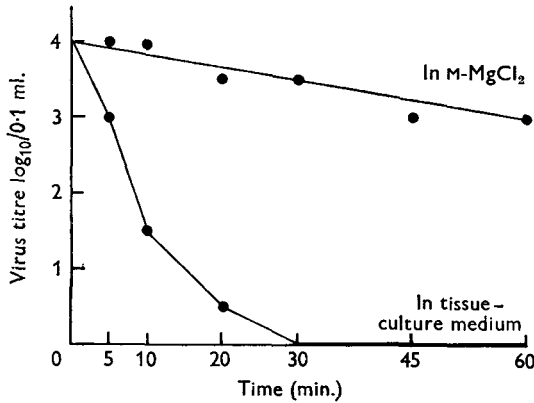


Fig. 1. Effect of heating virus at 50° C.

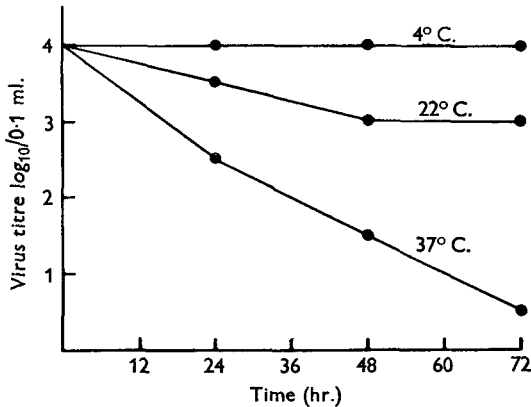


Fig. 2. Effect of temperature on virus survival.

*Resistance to heat and chemicals*

Equal volumes of virus suspension were heated at 50° C. in stoppered tubes for periods of up to one hour. No virus could be recovered after 30 min. at this temperature. Infectivity was retained, however, when the virus was heated at this temperature in the presence of m-MgCl<sub>2</sub> (Fig. 1). No loss of infectivity was found after storage at 4° C. for 72 hr., but 99 % of the virus was destroyed in 72 hr. at 37° C. (Fig. 2).

Resistance to ether is an important characteristic of enteroviruses and this was tested by adding one part of diethyl ether to four parts of virus suspension and gently agitating the mixture for 18 hr. at 4° C. The ether was then removed and the

virus titrated. The titre of the suspension before ether treatment was  $10^{4.5}$  and afterwards  $10^{3.5}$ . The virus could therefore be said to be resistant to ether.

Treatment with HCl was used by Ketler, Hamparian & Hilleman (1962) to distinguish the Coryza-Rhinovirus group from the enteroviruses. The former were inactivated by the acid conditions but the latter survived. Mill virus after standing for 3 hr. at room temperature in HCl at pH 3 was not significantly reduced in titre.

#### *Estimation of particle size*

After treatment with fluorocarbon 113 the suspension was passed through Millipore membranes of 100, 50 and 10  $m\mu$  porosity. The virus was recovered from the first two filtrates but not the third. This gives an approximate size of 30  $m\mu$ , a figure generally accepted as the size of the enteroviruses.

#### *Nature of the virus*

Treatment of the cultures with 5-bromodeoxyuridine in concentrations of 10 and 50  $\mu\text{g./ml.}$  failed to prevent multiplication of the virus in H.EpII cells, suggesting that it is a RNA virus. In parallel experiments vaccinia virus was completely inhibited.

The properties of Mill virus are summarized in Table 2. These properties suggest the virus should be classified in the enterovirus group.

Table 2. *Summary of properties of 'Mill' virus*

1. Size 30  $m\mu$  (approximately).
2. RNA virus.
3. Cytopathic in HeLa, H.EpII and human amnion, embryonic kidney and fibroblasts.
4. Not cytopathic for monkey kidney, rabbit kidney, guinea-pig embryonic kidney and mouse or chick fibroblasts.
5. Serologically distinct from any of the classified enteroviruses or adenoviruses.
6. Not pathogenic for unweaned mice or adult mice, rabbits or guinea-pigs.
7. Does not produce pocks on the chick embryo chorioallantoic membrane.
8. Does not haemagglutinate human 'O' cells.
9. Resistant to ether, HCl at pH 3, and heating to 50° C. for 1 hr. in  $\text{m-MgCl}_2$ .
10. Inactivated at 50° C. for 1 hr. in T.C. medium.
11. Multiplication rate slower at 33° C. than at 36° C.

#### *Antibodies in children from whom the virus was isolated*

It was possible to obtain serum specimens from eight of the children from whom the virus was isolated and from another child in the home from whom an adenovirus only was isolated.

All eight children possessed neutralizing antibody to Mill virus but in low titres (8-32) only. Antibodies were not found in the serum from the other child. No antibodies could be found in a sample of human gamma-globulin.

There is no obvious explanation at present for the poor antibody response in the patients from whom the virus was isolated and also in the rabbits immunized with tissue-culture suspensions. It may be necessary to employ more sensitive methods using plaques to determine antibody titres as has been found in work with the Rhinoviruses.

## SUMMARY

A virus was isolated from fifteen of nineteen children living in an infants' home during an outbreak of diarrhoea. The virus possesses many of the characteristics of the enterovirus group but is serologically distinct from any of the accepted members of this group. It is non-pathogenic for unweaned mice, non-cytopathic in tissue cultures of monkey kidney, but is rapidly cytopathic for tissue-cultured cells of human origin. It is serologically similar to virus Hu 659 isolated by Abrahams in the United States (personal communication).

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