

Diagnostic electron microscopy of faeces

I The viral flora of the faeces as seen by electron microscopy

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SYNOPSIS A method is described for examining viruses in faeces by direct electron microscopy using negative staining. The particles found in a group of patients with gastroenteritis and a group with other conditions are compared. Small particles in the range of sizes covering parvoviruses and enteroviruses were found about as frequently in each group.

Many of these were probably bacteriophages. Many bacteriophages with tails of various sizes and lengths were found. Adenoviruses were found in five of eight patients from whom they were isolated. Reovirus- (or orbivirus-) like particles were found associated with some gastroenteritis patients.

Groups of workers in Bethesda, Md, USA, and in Bristol, England (Kapikian, Wyatt, Dolin, Thornhill, Kalica, and Chanock, 1972; Paver, Caul, Ashley, and Clark, 1973) have reported the finding of small virus particles, agglutinable by some human sera, in extracts of faeces from volunteers fed with bacterium-free filtrates of faeces from patients with acute gastroenteritis. Estimates of the sizes of these particles have varied between 22 and 27 nm diameter.

We thought it might be worth while to find out, without using antisera possibly relevant to any particular virus, what virus-like particles one might expect to see by electron microscopy in faeces from persons of different ages admitted to hospital with various ailments. In particular, we studied faeces from young children admitted with acute gastroenteritis. Such illnesses are common. When recognized bacterial pathogens, eg, Shigellae and type-specific *Escherichia coli*, are responsible for epidemics they are easily isolated from almost every case. But at some times of the year, as at the time of writing, in Birmingham, these pathogens can be isolated from a small proportion only of patients with gastroenteritis. Attempts at virus isolation from these patients by conventional means have often given us the embarrassing result that the only faeces yielding an enterovirus have also contained a pathogenic bacterium and the rest have yielded nothing.

Man appears to be remarkable in being the only well investigated vertebrate species, or insect, from which pathogenic parvoviruses have not been iso-

lated; the Bethesda and Bristol experiments have given the only indication that parvoviruses might be important. The method described below was therefore designed to deposit all particles smaller than bacteria, down to 20 nm diameter, so that they could be examined in the electron microscope.

Methods

Suspensions of faeces, about 10-20% (v/v), were centrifuged first in an MSE Superminor at 3000 rev/min for 10 minutes and then in a Spinco 40 angle head rotor at 7000 rev/min for 30 minutes to deposit bacteria and debris. Volumes, each of 3 to 5 ml, of the supernatants were then centrifuged at 50 000 rev/min for one hour in swing-out tubes in an MSE 50 or Spinco 50L rotor. The deposits were resuspended in 0.2 ml distilled water.

For electron microscopy, grids bearing carbon or formvar membranes were touched to a droplet of the suspension in distilled water and allowed almost to dry; then dipped thrice into distilled water, being blotted from the edge after each dip; then dipped into 2% potassium phosphotungstate, pH 5.5, blotted again, and when dry examined in a Philips EM200 electron microscope. For immunoelectron microscopy, 4 parts by volume of clarified faecal suspensions, or washed virus particles derived from infected tissue cultures, were mixed with 1 part of serum, and allowed to stand at room temperature for two hours (about 18°) and for 18 hr at 4°. The mixtures were centrifuged at 50 000 rev/min for

half an hour in 5 ml swing-out tubes. The deposits were resuspended in distilled water and negatively stained for electron microscopy.

Patients

Samples of faeces from 245 patients were examined, some diagnosed clinically as having acute gastroenteritis, some with other conditions.

Results

THE TYPES AND SIZES OF VIRUS-LIKE PARTICLES

An adult's intestinal tract contains about 10^{14} bacteria (Williams, 1973) and even a young child's must contain 10^{13} . Bacteria have their own virus infections and so one expects to find many bacteriophage particles in faeces. We have found most of the morphological varieties illustrated by Bradley (1967). Particles with tails were easily identified as bacteriophages—some long, some short (figs 1, 2), some in great number (fig 3). Often the heads had been penetrated by negative stain and amid the debris they were not immediately recognized on the fluorescent screen (fig 1). Filamentous phages were not recognizable as such. Phages are very numerous in human faeces and these pictures illustrate but a few of those seen. Filamentous objects were extremely numerous in almost all our preparations, mostly being flagella, or pieces of flagella, or pili, which had not been deposited with the bacteria in the first centrifugation. An occasional lysed bacterial shell was seen with phages attached (fig 4). These resemble coliphage T1. Substructure could not easily be made out upon them.

ISOMETRIC PARTICLES

We were looking particularly for particles of parvovirus size and appearance. These were numerous in some preparations (fig 1) and often could be seen to be clearly hexagonal in outline (fig 5). There was no means of knowing whether these were bacterial or human viruses; in the crudely purified preparations used, dimers on the axes of five-fold symmetry, as occur on bacteriophage Φ X174, would probably not have been detected. We suspected that small particles in some preparations had dimers, but could not be sure.

Thirty-three per cent of all faeces from patients with gastroenteritis contained these particles; they were found in 27% of faeces from patients with other diagnoses. The difference is probably not significant, and the finding of these particles is not diagnostic.

Enteroviruses cannot be recognized with certainty, but they have a circular rather than hexagonal out-

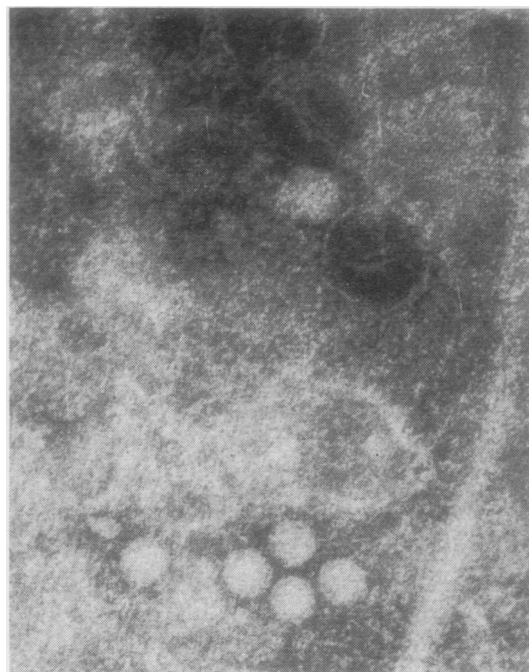


Fig 1 Particles resembling parvoviruses 22 nm diameter. Phage particles with tails are also present (arrows). A segment of bacterial flagellum crosses the picture. $\times 270\ 000$.

Figures 1-10 are of particles centrifuged from faeces. All are negatively stained with phosphotungstate.

line, and in faeces from one patient from whom an Echo 11 virus was isolated a group of particles enclosed in a membrane was seen (fig 6). This appearance is very similar to that of polioviruses in tissue culture fluid, illustrated by Horne and Nagington (1959). It may well be that by its agglutination by a specific antiserum, a particular enterovirus might be recognized in faeces by electron microscopy, but without this aid the evidence so far indicates that the finding of particles in this size range is not diagnostic of enterovirus infection.

Isometric particles in the range 30-60 nm were sometimes seen (fig 7). They did not appear to be specifically associated with any disease.

Adenoviruses are easily recognizable. Icosahedral phages, eg, of *Pseudomonas* species, are known but have tails. We have been able to detect adenovirus particles in five of eight specimens of faeces from which they were isolated and in three from which adenovirus could not be isolated. On two occasions, both adenoviruses and reovirus-like particles were found together in faeces of children with gastroenteritis (fig 8). Even the fragments of disintegrated

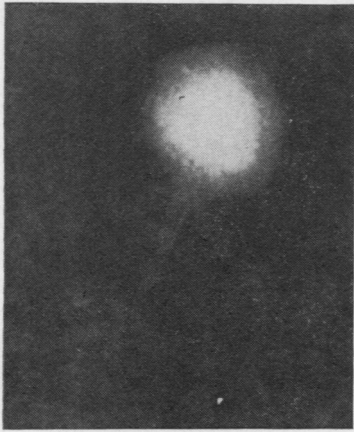


Fig 2a

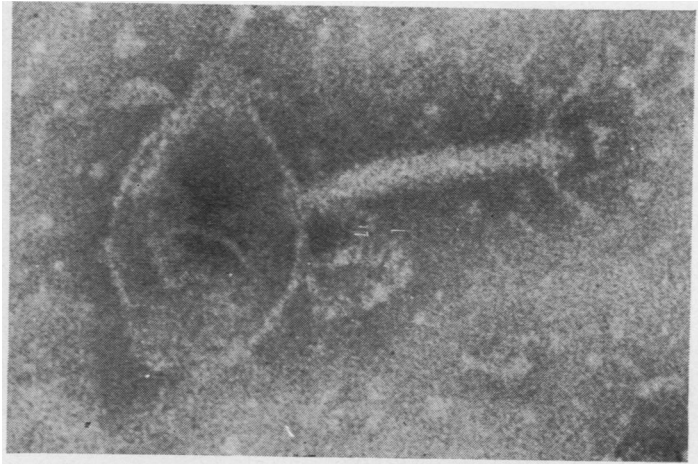


Fig 2b

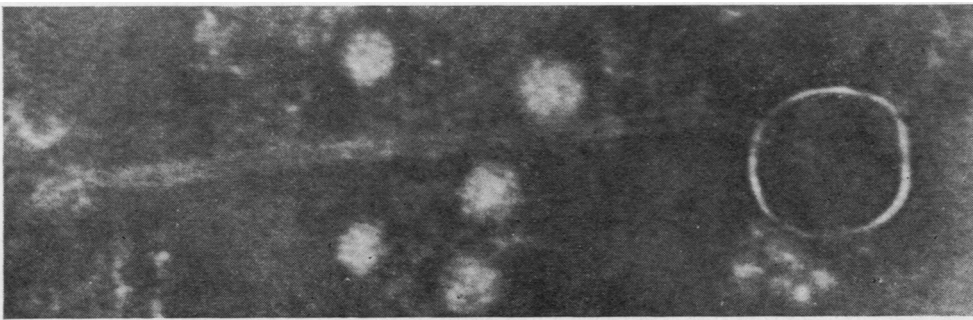


Fig 2c

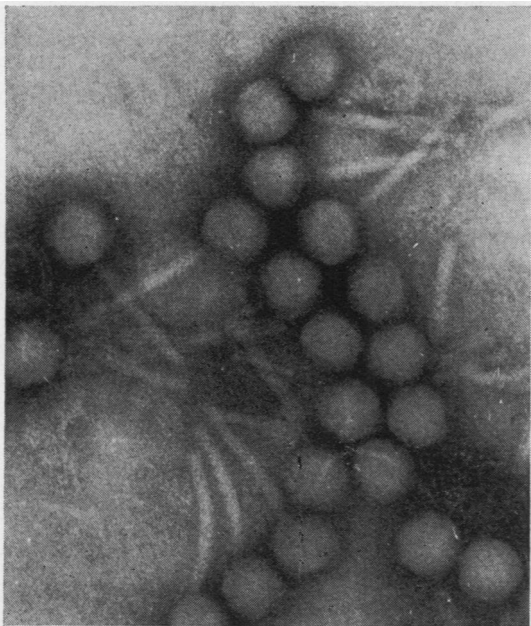


Fig 3

Fig 2 a, b, c. *Phage particles with short, medium, and long tails. All at $\times 296\ 000$.*

Fig 3 *A group of phage particles with icosahedral heads and parallel tails. $\times 114\ 000$.*

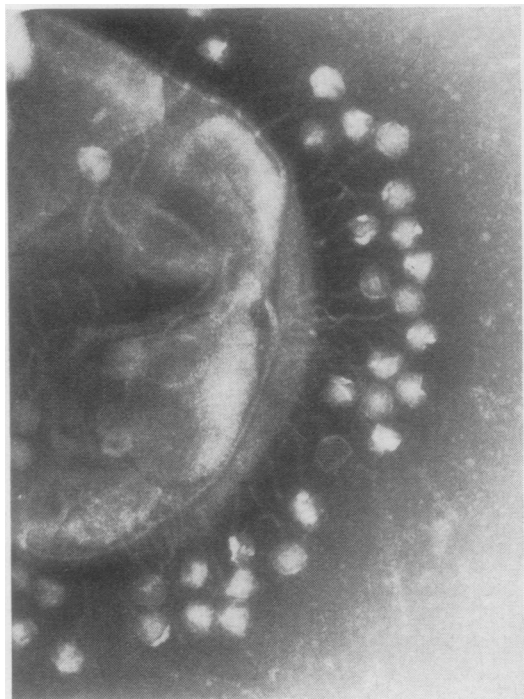


Fig 4

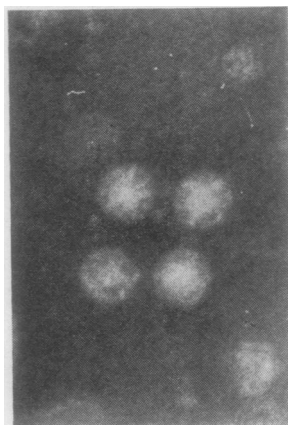


Fig 5

Fig 4 An empty bacterial cell wall to which phage particles are attached by their tails. These particles are the size and shape of coliphage T1. $\times 88\ 000$.

Fig 5 A group of particles, 25 nm diameter, with a hexagonal outline visible on two. $\times 296\ 000$.

Figures 1-10 are of particles centrifuged from faeces. All are negatively stained with phosphotungstate.

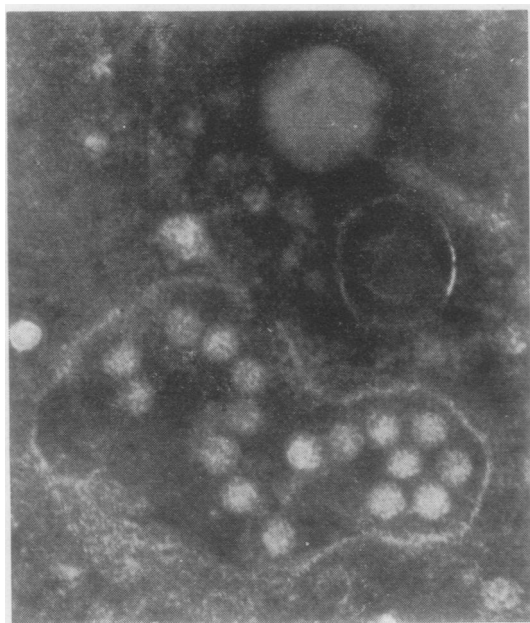


Fig 6

Fig 6 A group of particles surrounded by a membrane, 28 nm diameter. Echo 11 was isolated from the faeces, probably enterovirus (see Horne and Nagington, 1959). $\times 185\ 000$.



Fig 7

Fig 7 Large isometric particles, 40 nm diameter, some with hexagonal outline. $\times 185\ 000$.



Fig 8 Adenoviruses, bacterial flagella, and reovirus-like particles, of which the outer capsid layer is missing. $\times 190\ 000$.

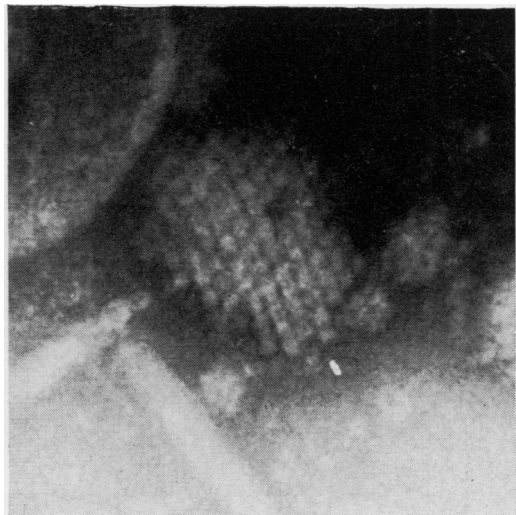


Fig 9 A group of adenovirus capsid subunits. The pattern is characteristic. Adenovirus was isolated from the faeces. $\times 222\ 000$.

capsids are recognizable (fig 9); adenovirus was isolated from this sample. Some of the particles in the range 20-30 nm might have been adeno-associated viruses, but such particles were not more often found in faeces containing adenoviruses than in the rest.

Reovirus-like particles

These were frequently found in the faeces of children with acute gastroenteritis, but with two exceptions not in faeces of patients in other categories. Their prevalence, morphology, and clinical significance will be described in the accompanying paper (Flewett, Davies, Bryden, and Robertson, 1974b).

Discussion

Electron microscopy of faeces for the presence of viruses—a technique long neglected—has recently become important (Paver *et al*, 1973; Kapikian, Gerin, Wyatt, Thornhill, and Chanock, 1973; Bishop, Davidson, Holmes, and Ruck, 1973, 1974; Feinstone, Kapikian, and Purcell, 1973; Woode, Bridger, Hall, and Dennis, 1974; Flewett, Bryden, and Davies, 1974a). This paper illustrates the variety of small particles, almost certainly viruses, which may be found in faeces. Some may be identified by agglutinating them with specific antibody, of which the strands can be seen by electron microscopy. The particles may be seen invested with a globulin 'fuzz', a condition which obtains when antibody is in gross excess, or linked together by strands of globulin, visible in the electron microscope as threads. But unless one can isolate a virus and raise antisera in hyperimmunized animals it is usually difficult to be sure of the specificity of the antibody agglutinating the particles. Of the particles we have found in faeces, only the adenoviruses and reovirus-like particles are clearly agents infecting human and not bacterial or mycotic cells (Flewett *et al*, 1974a). For the reovirus-like particles, the observations of Bishop *et al* (1973) provide valuable evidence of actual infection of gut epithelium. A double-stranded RNA-containing bacteriophage is known, the $\Phi 6$ of *Pseudomonas phaseolicola*, with a polyhedral head 60 nm in diameter (Wood, 1973) though fortunately its morphology is clearly different from that of the particles illustrated in the accompanying paper. Several mycophages containing double-stranded RNA are also known with capsid shells 34-41 nm diameter (Wood, 1973); some of them may, for all we know, be illustrated in our pictures.

It appears, from these results, that direct electron microscopy of faeces is applicable to the diagnosis of adenovirus and reovirus-type infections. The clinical value of detecting adenoviruses in young children hardly seems worth the work involved—especially

as the method does not reveal the serotype. The accompanying paper discusses the value of the method for infection by reovirus-like viruses.

Electron microscopy of faeces will not be widely

applicable as a diagnostic tool until antisera of known specificity are available. Without these small isometric particles all look alike and cannot be distinguished from each other.

II Acute gastroenteritis associated with reovirus-like particles

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SYNOPSIS Virus particles resembling reoviruses or orbiviruses were found in the faeces of 40 of 73 patients under 6 years of age with acute gastroenteritis and in faeces of only two babies among 31 patients under 6 years admitted to hospital with other diagnoses. In morphology the particles resemble orbiviruses more closely than reoviruses, but differ in appearance from the orbiviruses in having a smooth, circular outline with a well marked continuous rim as seen in negatively stained preparations. They appear not to be serologically related to reovirus types 1, 2, or 3 and may be members of a new group.

Acute infectious gastroenteritis of young children is sometimes clearly associated with a bacterial pathogen, either a 'type-specific' strain of *Escherichia coli* or one of the non-lactose fermenters. From most patients, however, no pathogen can be isolated. It is generally presumed that a virus or viruses are responsible, but although viruses of various kinds have occasionally been isolated evidence of a specific viral pathogen has usually been lacking.

We have used the technique of electron microscopy of faeces described in part I (Flewett, Bryden, and Davies, 1974a) to investigate patients with acute gastroenteritis occurring during the last 10 months.

Patients

Seventy-three patients with acute gastroenteritis and 31 with other conditions, all under 6 yr of age, have been studied. Fifty-nine gastroenteritis patients over 6 years of age and 82 other patients were also examined. The patients not suffering from gastroenteritis were admitted with a wide variety of diagnoses, mostly with febrile illnesses—respiratory tract infections, meningitis, hepatitis, etc. All these had been admitted to hospital, most of them to the com-

municable diseases unit of the East Birmingham Hospital under the care of Drs M. E. Barton, E. Carr-Saunders, R. Fothergill, A. M. Geddes, E. E. Hill, or Professor H. V. Morgan. The gastroenteritis patients were of various ages (fig 1). Their illnesses in general consisted of diarrhoea of acute onset, usually with vomiting and fever, sometimes up to 39.5°C (103°F) in the infants and younger children. The duration of the illness was usually short: most children were sent home after seven to 10 days in hospital. About one quarter were admitted in a severely dehydrated state (25% or greater dehydration) and required emergency fluid replacement by intravenous drip.

Methods

Virus suspensions from faeces were prepared for electron microscopy as described by Flewett *et al* (1974a). For immunoelectron microscopy, the virus suspensions were resuspended in 1.5 ml phosphate-buffered saline (PBS) pH 7.2. One drop of serum at various dilutions was added to 0.5 ml of the resuspended virus. After standing one to two hr at room temperature and 4° overnight these suspensions were brought to 5 ml with PBS and were centri-