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Preliminary Characterisation of Torovirus-Like Particles of Humans: Comparison With Berne Virus of Horses and Breda Virus of Calves

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Pleomorphic virus-like particles have been observed by electron microscopy in the faeces of children and adults with diarrhoea. Some of these particles were approximately 100 nm in diameter and had a "fringe" of closely applied peplomers approximately 10 nm long; they closely resembled Berne virus of horses and Breda virus of calves, the two representatives of a newly proposed family called the Toroviridae. In one sample a toroidal nucleoprotein-like structure was observed within the particles. For two samples a buoyant density of 1.14 g/ml was determined by centrifugation through a sucrose density gradient. One sample possessed a haemagglutinin for rat erythrocytes. The serological relationship between these different viruses was observed by immune electron microscopy, haemagglutination inhibition, and serum neutralisation. The role of these virus-like particles as candidate pathogens of humans is discussed.

Key words: viral gastroenteritis, coronavirus-like particles, diarrhoea

INTRODUCTION

In 1975 pleomorphic particles resembling coronaviruses were detected in samples of human faeces by electron microscopy, [Mathan et al, 1975; Caul et al, 1977; Caul et al, 1975]. These coronavirus-like particles (CVLPs), were discussed by Macnaughton and Davies [1981], who concluded that they formed two groups consisting of human enteric coronaviruses (HECVs) and CVLPs. The HECVs were considered to be true members of the coronaviridae whereas the CVLPs representing the other "group" of particles evaded definition. It has been suggested that some CVLPs might be cell membrane fragments from the host gut wall [Caul et al, 1977], mycoplasmas [Mortensen et al, 1985], vesicles from eucaryotic cells [Dourmashkin et al, 1980], or viruses [Moscovici et al, 1980; Beards et al, 1984; Mortensen et al,

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1985]. In further prospective studies CVLPs were often reported to be not only present in diarrhoeic stools, but also in samples from apparently healthy patients [Schnagl et al 1978; Sitbon, 1985]. So it would appear that CVLPs represent a heterologous group of particles, some of which may be artifacts but others could be viruses.

In 1984 a new family of viruses, the Toroviridae, was proposed by Horzinek and Weiss. The members consist of Berne virus (BEV) isolated from a horse in 1972 [Weiss et al, 1983] and Breda virus (BRV) isolated from calves [Woode et al, 1982]. Related viruses may exist in other hosts [Weiss et al, 1984].

Although Berne virus was first isolated in 1972 from a rectal swab from a horse in Berne, Switzerland, it was not described until 1983 [Weiss et al, 1983]. The virus was found to be unrelated to known viruses, although it superficially resembled bovine coronaviruses by electron microscopy. Purified virions had a density of 1.16 g/ml in sucrose and by negative staining electron microscopy appeared as spherical particles 120–140 nm in diameter, with peplomers approximately 20 nm long and an internal structure measuring about 23 nm across and 104 nm long, with possible helical symmetry [Weiss et al, 1983].

In 1982 a previously unknown virus was isolated from diarrhoeic calves in Breda, Iowa USA and is referred to as Breda virus (BRV) [Woode et al, 1982]. This virus was shown to be infectious by mouth in gnotobiotic and conventionally reared calves, causing diarrhoea; virus was shed for 2–3 days.

Virus particles were seen in two forms: roughly spherical particles measuring 69 nm (± 7 nm by 75 ± 9) with peplomers 7.6–9.5 nm long; and kidney-shaped particles measuring 120 (± 15 nm) by 32 (± 8 nm) nm with similarly sized peplomers. The virus agglutinated rat erythrocytes and was distinct antigenically from other bovine viruses, including bovine coronavirus, parvovirus, rotavirus, and parainfluenza virus 3 and reovirus 3. Propagation of Breda virus has been attempted in several tissue culture systems, but so far without success. Two serogroups have been recognised by haemagglutination inhibition and ELISA [Woode et al, 1893].

We have reported previously the existence of Breda virus-like particles in stool samples from children [Beards et al, 1984]. In these patients no other viral or bacterial pathogen was isolated. Although insufficient samples containing torovirus-like particles have subsequently been found for any association with human disease to be established, the specimens did come from patients with diarrhoea. The particles were pleomorphic and approximately 100 nm in diameter with peplomers 7–9 nm long. Some particles were seen with a similar structure, but only 50 nm in diameter. We report here our further investigations of these fringed particles in stool samples and have compared the physical, morphological, and serological properties of these particles to those of Breda and Berne viruses. We suggest that some CVLPs seen in samples of human faeces may be examples of this newly described group of viruses.

MATERIALS AND METHODS

Electron Microscopy

Extracts were prepared from samples of faeces to give a final concentration of 10–20% v/v in either phosphate buffered saline (PBS) containing antibiotics (penicillin 200 units/ml, streptomycin 20 μg/ml, and fungizone 5 μg/ml) or in Tris-HCl (0.01 M), pH 7.2, with the same antibiotics. The extracts were clarified by centrifugation

at 1,000 × g for 10 minutes. Tissue culture fluids from embryonic mule skin cells (EMS cells) showing cytopathic effect from infection with Berne virus were frozen and thawed 3 times and centrifuged at 1,000 × g for 10 minutes. Electron microscope grids were prepared from the supernatants using a method described previously [Flewett et al, 1974]. All grids were examined on a Jeol CX100 electron microscope or a Philips 200 electron microscope at an accelerating voltage of 80 KV.

Virus

Breda virus was a generous gift from Professor G. Woode and was received as samples of calves faeces and caecal contents from gnotobiotic calves inoculated orally with Breda I or Breda II.

Berne virus was a gift from Professor M. Horzinek and Dr. M. Weiss and was received as the 138th passage supernatant from an EMS cell culture. This material was further passaged in EMS cells that had been grown in Dulbecco's minimal essential medium supplemented with L-glutamine with 10% fetal calf serum and antibiotics. Subconfluent EMS cells were inoculated and the virus infected supernatant was harvested after 1–2 days incubation.

Human faecal specimens found to contain fringed, torovirus-like particles were from children and adults with diarrhoea admitted to East Birmingham Hospital or received by the Regional Virus Laboratory. All samples except one "1690" (which also contained rotaviruses) were negative for other pathogens by EM, cell culture, and bacterial culture and ELISA for rotavirus and adenovirus.

Antisera

Antisera to Breda I and II were provided by Professor G. Woode. These were convalescent sera from artificially infected gnotobiotic calves (BI:SB219 and BII: GC21) and a hyperimmune serum produced by intramuscular inoculation of a previously infected gnotobiotic calf using Breda II virus purified from its own faeces (GC76).

Antisera to Breda I and Breda II viruses were produced in this laboratory by intramuscular inoculation of guinea pigs with purified virus (BI:GP420 and BII: GP475).

A rabbit antiserum to Berne virus was provided by Dr M. Weiss and a guinea pig antiserum was produced in this laboratory by repeated intramuscular inoculation. (BeV:GP443).

Convalescent sera from humans in whose faeces fringed particles had been observed were provided by Mme. F. Lamouliatte. The reactivity of these sera has been described previously [Beards et al, 1984].

Immune Electron Microscopy

Serum was added to clarified faecal extracts and tissue culture supernatants to give a final concentration of 1/10 or 1/100. Samples were incubated overnight at 4 °C and then centrifuged for 10 minutes at 10,000 × g. The deposits were then examined by electron microscopy after staining with 2% w/v potassium phosphotungstate, pH 7, (PTA) or 2% ammonium molybdate.

Virus Purification

BEV, BRV, and human particles were purified by the same procedure. To clarified faecal extracts or tissue culture supernatants saturated ammonium sulphate

was added slowly to 50% saturation. The continuously stirred mixtures were held at 4°C overnight. The resultant precipitates were then centrifuged at 10,000 × g for 30 minutes, redissolved in 2–5 ml of 0.01 M Tris HCl, pH 7.2, with 0.001 M EDTA and 0.15 M NaCl (TES buffer), and dialysed at 4°C overnight against the same buffer. They were further purified by centrifugation through pre-formed linear sucrose density gradients of 15–60% w/w solutions of sucrose in TES buffer, which had been allowed to diffuse overnight at 4°C. The dialysed ammonium sulphate precipitates (5 ml) were carefully layered on top of pre-formed sucrose gradients. Centrifugation was at either 100,000 × g for 2 hours or 50,000 × g for 4 hours. After centrifugation the gradients were harvested in 0.2 ml volumes. The density of each fraction and thus the buoyant density of the viruses was calculated using the formula:-

$p = 2.732 \times RI - 2.6425$ at 20°C

The refractive index of the buffer alone was substracted from each measurement.

Antiserum Production

Peak gradient fractions from the purification procedure described above were mixed with an equal volume of Freund's incomplete adjuvant and the animals were inoculated intramuscularly. For BRV only one inoculation was required to produce a hyperimmune antiserum when tested by ELISA (manuscript in preparation). For BEV, five inoculations were required.

Haemagglutination and HAI

Haemagglutinin was titrated using serial two-fold dilutions of clarified faecal extracts in PBS in V-well microtitre plates. 25 μl of 0.5% rat erythrocytes diluted in PBS with 1% w/v bovine serum albumin, was added to 25 μl of each dilution of antigen. After thorough mixing the plate was held at room temperature, about 20°C, for 90 minutes and read. The endpoint was taken as the dilution of extract showing complete haemagglutination.

For haemagglutination inhibition tests faecal samples were diluted to 4 haemagglutinating units, (1 haemagglutinating unit being the highest dilution showing complete haemagglutination). The test sera were then mixed with an equal volume of packed rat erythrocytes and, after centrifugation, diluted serially in PBS, from 1/4 onwards. To each serum dilution 25 μl of Breda virus antigen containing 4HA units were added. The mixture was then left at room temperature for 1 hour. To each well 25 μl of 0.5% rat erythrocytes were added and the plate incubated for 90 minutes at room temperature and read.

Neutralisation Tests

Bovine convalescent, human convalescent and rabbit and bovine hyperimmune antisera were tested for neutralising activity against BEV grown in EMS cells in microtitre plates. Serial dilutions of serum were pre-incubated with 100 TCD50 of BEV for 1 hour at 37°C, in a microtitre plate, before adding the EMS cells. Cells were observed for cytopathic effect for 1–5 days.

RESULTS

Electron Microscopy

Figure 1 shows the morphology of BRV particles. Usually, roughly spherical particles were seen with a characteristic electron dense center and "fringe" of short peplomers. Occasionally kindey shaped particles were also seen.

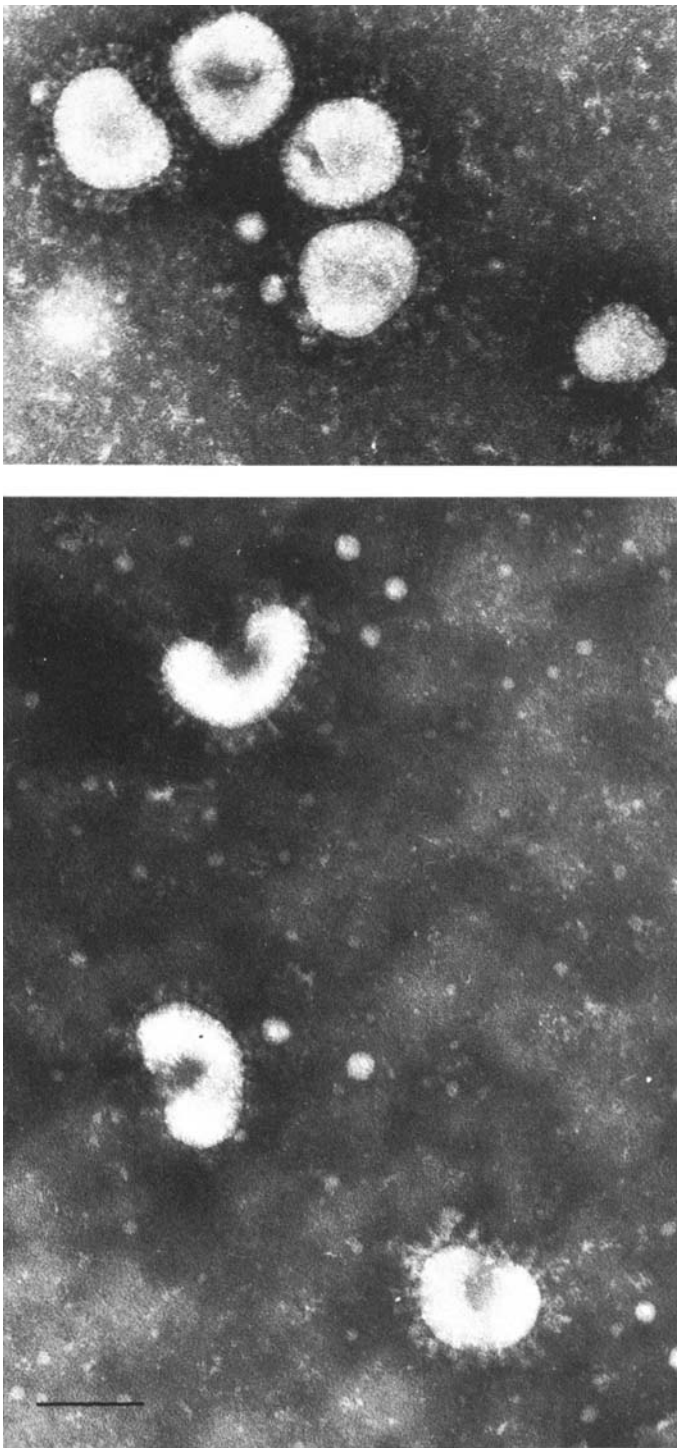

Fig. 1. Breda virus particles in gnotobiotic calf faeces. Roughly spherical (top) or kidney-shaped particles (bottom) are shown. PTA. Bar equals 100 nm.

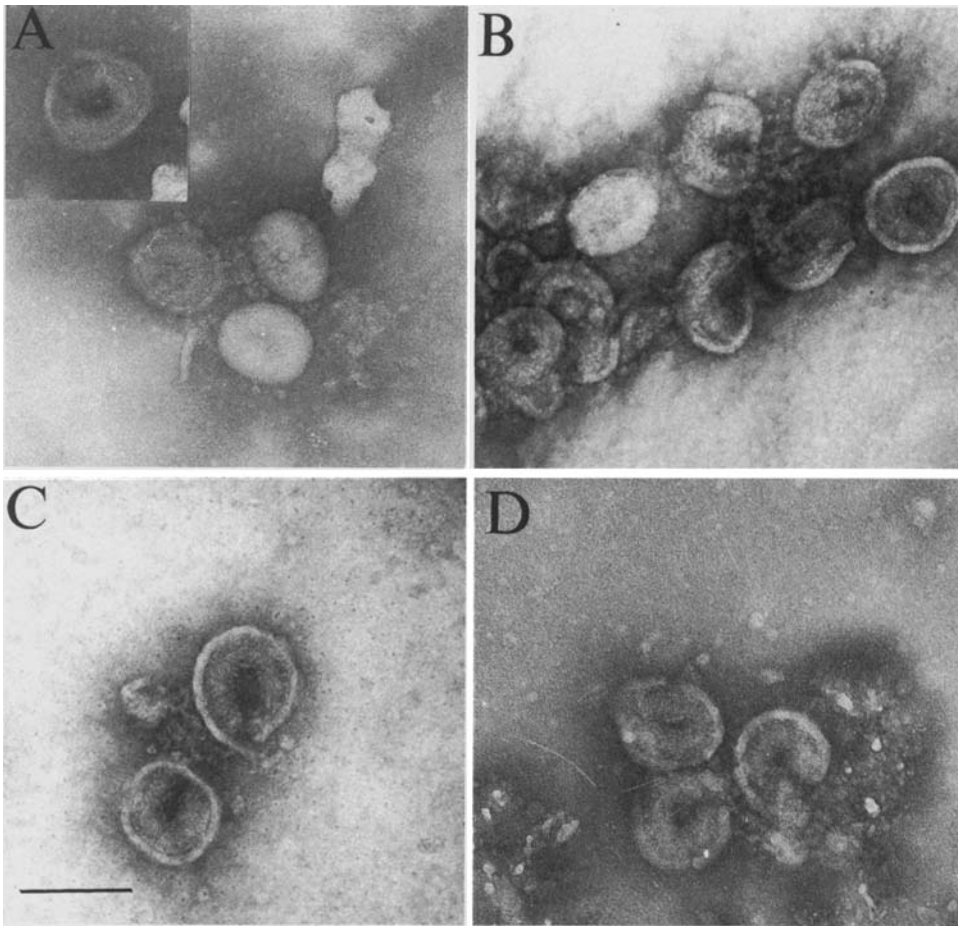

Fig. 2. Berne virus particles propagated in embryonic mule skin cells. A torus is visible within most particles and some peplomers may be present. A and D) amm. molybdate; B and C) PTA. Bar equals 100 nm.

Figure 2 shows the appearance of BEV virus propagated in EMS cells. Peplomers, though present on some particles, were rarely seen. In many preparations a toroidal ribonucleoprotein-like structure was clearly seen within the viral envelope.

Figure 3 (A–D) shows the appearance of torovirus-like particles seen in stool samples from children. Both round and kidney shaped particles were observed, and in one sample (Fig. 3D) a toroidal structure could be seen within the viral envelope.

Table I lists the relative sizes of the viruses and the components for BRV, BEV, and the torovirus-like particles from humans.

Immune Electron Microscopy

Table II shows the results obtained for each sample and serum tested by immune electron microscopy. In most cases electron microscope grids were prepared by one of us and given as coded samples to two others to assess.

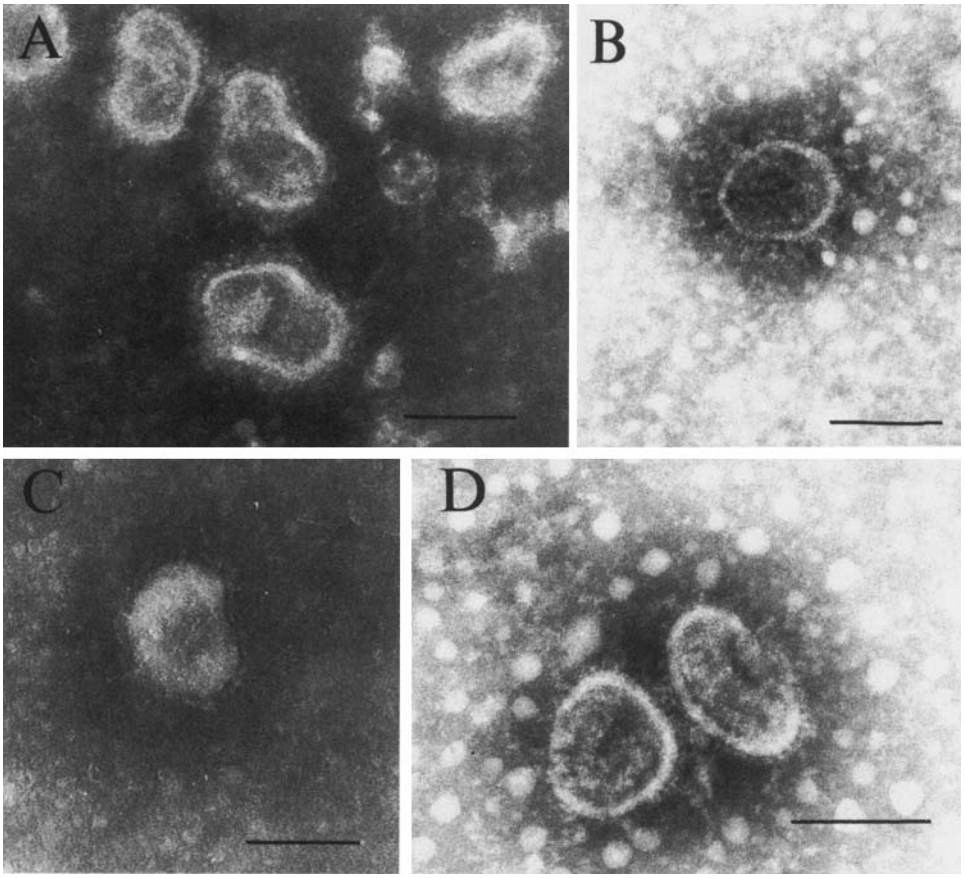

Fig. 3. Torovirus-like particles from human faeces. Peplomers of around 9 nm are present on all particles and an internal torus can be seen in D. PTA. Bar equals 100 nm.

TABLE I. Relative Sizes (in Nanometres) of the Components of BRV, BEV, and Human Particles (1690)

	BEV	BRV	Human
Particle	120–140	120–140	100–120
Membrane	11	10	6–7.2
Peplomers	20	7.6–9.5	7–9
RNP diameter	23	NT[a]	24
RNP helix period	4.5	NT[a]	4.8

[a]NT, not measured.

Figure 4 shows the appearance of immune complexes formed by BRV and particles from human faeces.

Buoyant Densities in Sucrose

For BRV and human sample 1690, peak haemagglutination titres were found to coincide with virus containing fractions as seen by electron microscopy, though some diffusion of haemagglutinin did occur with some remaining at the top of the gradient

TABLE II. Immune Electron Microscopy of BRV, BEV, and Three Samples of Human Faeces Containing Torovirus-Like Particles

	Virus[a]					
				Human		
Antiserum	BRV1	BRV2	BEV	5486	5814	1690
BRVI convalescent	+++	++	+++	−	−	+
BRVII convalescent	+++	+++	+	+++	+++	+++
BRVII hyperimmune	+++	+++	++	+++	+++	+++
BEV hyperimmune	++	+	+++	NT	NT	NT
Human A acute	−	−	NT	NT	NT	NT
Human A conv	++	−	NT	NT	NT	NT
Human B acute	−	+	NT	NT	NT	NT
Human B conv	−	+++	NT	NT	NT	NT
Case C	+++	+	NT	NT	NT	NT
Case D	++	++	NT	NT	NT	NT
GC-ve[b]	−	−	−	−	−	−

[a]Human antisera are from children whose faeces contained torovirus-like particles [Beards, 1984]; +, 2–3 particles clumped per field; ++, 2–3 aggregates per field; +++, many particles agglutinated per field or many clumps; NT, not tested.
[b]Gnotobiotic calf serum rotavirus infected.

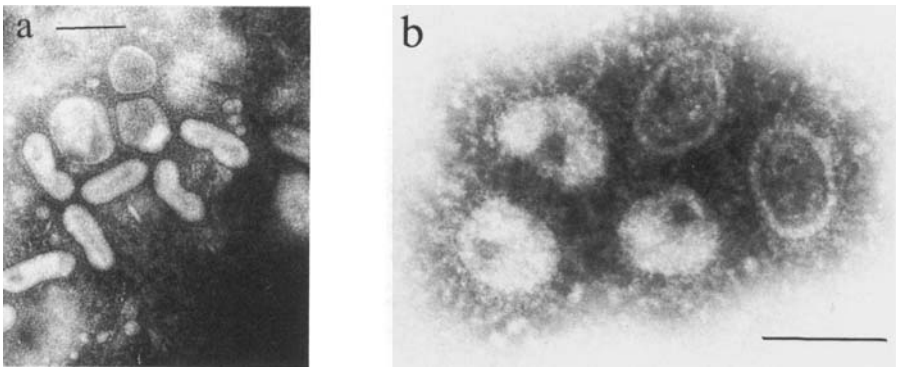

Fig. 4. a) Immune complex formed by BRV and convalescent human serum B (Table II). b) Human torovirus-like particles agglutinated by antiserum to Berne virus. PTA. Bar equals 100 nm.

and in fractions on either side of the peak virus fractions shown to contain virus particles by electron microscopy. The buoyant densities in sucrose were BRV I and II, 1.14 g/ml, BEV 1.16 g/ml and 1690 and 86/1, (two human samples), 1.14 g/ml. The higher density found for BEV may be because after passages most particles had no peplomers attached. Purified BEV from the 1.16 g/ml fraction is shown in Figure 5.

Serology

Of the specimens from children found to contain torovirus-like particles by electron microscopy only one "1690" contained a haemagglutinin for rat erythrocytes. The haemagglutination inhibition test using 4HA units of this human sample gave similar results to those obtained using a sample of calf faeces containing 4HA units Breda II virus (Table II).

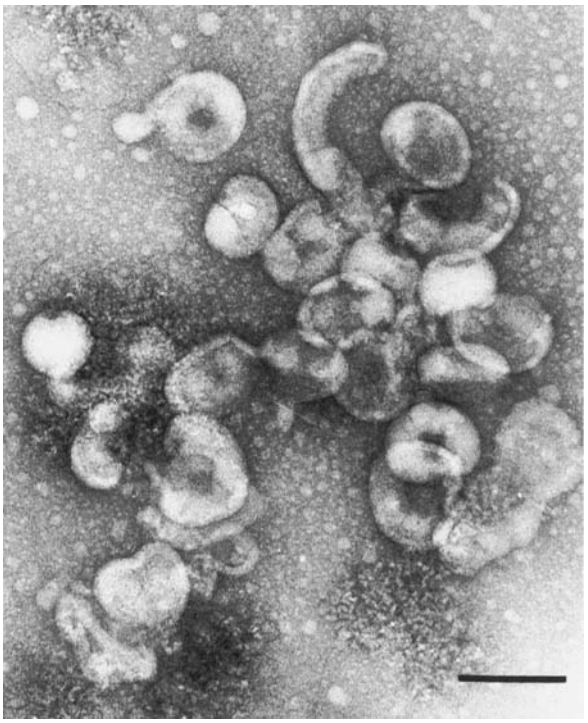

Fig. 5. Purified Berne virus from a 1.16 g/ml sucrose density gradient fraction. Note how the toroidal ribonucleoprotein remains bound to the viral envelope even when the virions are disrupted. Amm. molybdate. Bar equals 100 nm.

Of the specimens received for routine investigation of diarrhoea in human, 49/404 (12%) were found to have HA titres for rat erythrocytes greater than 1/50. None of these reactions were inhibited by antiserum to Breda virus. Furthermore, none contained fringed particles when examined by electron microscopy. No haemagglutinin could be detected in tissue culture supernatants containing Berne virus. In order to confirm this finding, an experiment was performed to investigate whether erythrocytes of different species could absorb infectivity of preparations of Berne virus. To 1 ml of infected tissue culture supernatant 100 μl of packed erythrocytes were added. The mixture was kept for 90 minutes at room temperature and then centrifuged to remove the erythrocytes. The supernatant was then assayed for infectivity. There was no significant difference whether the supernatants had been absorbed with PB (control), chick, human O, sheep, rat, or guinea pig erythrocytes.

DISCUSSION

We have detected Coronavirus-like particles in samples of human faeces. Some of these particles may be related to the recently discovered toroviruses. We have tested this hypothesis here.

The morphological evidence is the most convincing. In one sample it was not only possible to detect appropriately sized peplomers, but a toroidal nuclearprotein-like structure was clearly visible (Fig. 3D). The diameter and period of this helical

structure was close to that previously reported for Berne virus [Horzinek et al, 1984, 1985]. This structure was not seen when the grids were examined at first, but was visible in many particles when the grids were examined again some days later. These particles disintegrate rapidly during storage (this is also the case with human enteric coronaviruses), and the best preparations were those made on the day the sample was received at the laboratory. However, examination of Berne and Breda virus after prolonged storage revealed particles resembling "fringed particles" seen in many samples on which the electron microscopist has not been able to make a definitive comment.

The results of testing by immune electron microscopy extend our earlier findings [Beards et al, 1984] and demonstrate a serological relationship between Berne, Breda, and human torovirus-like particles. It is both interesting and unusual that a common antigenic determinant appears to exist on the peplomers (or some other membrane-bound protein) of toroviruses. For BEV and BRV this determinant could possibly be on the 80–120 Kd-glycoprotein or the 22-Kd non-glycosylated protein [Horzinek et al, 1985]. However, the 20-Kd nucleoscapsid protein does possess common antigens when immune-precipitated with heterologous antisera [Horzinek and Weiss, 1984].

Haemagglutination of rat erythrocytes proved to be an unsatisfactory method for the detection of human torovirus-like particles because rat erythrocyte haemagglutinin was frequently present in samples of human faeces. Sample 1690, however, gave an almost identical serological result to that obtained for Breda II virus (Table III). We can not exclude the possibility that this was a human infection by this bovine virus.

As reported previously, no haemagglutinin was detected in preparations of Berne virus [Weiss et al, 1983]. It has already been suggested that Berne virus may have mutated on passage and lost its haemagglutinin [Horzinek and Weiss, 1984]. Our results of testing Breda viruses I and II by haemagglutination inhibition confirm the results of Woode et al [1982,1985] that these viruses represent two serogroups. A reciprocal four-fold difference in haemagglutination inhibition titres was consistently obtained. Furthermore, convalescent sera to Breda viruses I and II showed no neutralising activity for Berne virus, suggesting that Berne and Breda viruses are serotypically distinct. Hyperimmune sera to Breda I and II however, did neutralise Berne virus, (Table III), so it may be that the convalescent sera were taken before detectable neutralising antibody levels had been reached.

A reproducible difference in the buoyant densities in sucrose of the different viruses were detected. Berne virus was consistently denser (1.16 g/ml) than Breda virus or two human samples (1.14 g/ml), and this may be because most of the Berne virus particles had no peplomers attached. Analysis of the gradient fractions by ELISA revealed two peaks (1.07 g/ml and 1.16 g/ml) when older samples of Breda virus were purified [D.W.G. Brown et al, manuscript in preparation]. The density of the first peak corresponds to the density of the 22-Kd glycosylated protein, which has previously been shown to have a density of 1.07 g/ml in sucrose [Horzinek et al, 1985].

Pleomorphic fringed particles have been observed by electron microscopy in samples of human faeces for many years. There are usually interpreted either as host cell fragments produced naturally by the constantly regenerating gut wall, or as mycoplasmas. These particles vary considerably in size and can be seen in other specimens such as amniotic fluid [T.H.F., personal observation]. The presence of

TABLE III. Haemagglutination Inhibition Tests on BRV I BRV II and Human Sample 1690 and Serum Neutralisations of Berne Virus

Serum	Breda I	Breda II	1690[a]	Neutralisations Berne[b]
GC76	640	5,120	>640	320
GC21	40	160	160	< 10
SB219	160	40	40	< 10
GP420pre	< 10	< 10	NT[c]	< 10
GP420post	1,280	80	NT	640
GP475pre	< 10	lt10	NT	< 3
GP475post	320	2,560	NT	800
GP443pre	< 10	< 10	NT	< 10
GP443post	< 10	< 10	NT	10,000

[a]Human faecal specimen containing torovirus-like particles.
[b]Serum neutralisation results.
[c]NT, not tested.

these objects makes the interpretation of fringed particles difficult. But here we have compared some features of torovirus-like particles from children with diarrhoea with those of Breda and Berne viruses. We suggest that a report of the presence of human torovirus-like particles in a sample might be based on the following:

Particles: 1) of mean diameter 100 nm; 2) bearing peplomers approximately 10 nm long; 3) with an internal toroidal structure; 4) of 1.14–1.16 g/ml buoyant density in sucrose; 5) which are agglutinated by antisera to Berne or Breda virus, by immune electron microscopy.

We also suggest that at least three of these criteria be met before further investigations are made. But we would be most grateful to receive samples, or electron microscope grids from colleagues.

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