NOVEL DIARRHOEA VIRUSES

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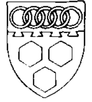
Ciba Foundation Symposium 128



NOVEL DIARRHOEA VIRUSES

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Introduction

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1987 Novel diarrhoea viruses. Wiley, Chichester (Ciba Foundation Symposium 128) p. 1–4

The symposium on 'Acute Diarrhoea in Childhood', held in 1975 here at the Ciba Foundation, heralded a renewal of interest in the aetiology and pathophysiology of acute diarrhoea, particularly in humans, which until early in the 1970s had been a rather neglected disease syndrome. There were several reasons for the renewed interest. Firstly, there was a slowly growing appreciation in developed countries of the overwhelming problem posed by acute diarrhoea in developing countries; that there was a high morbidity, and an unnecessarily high mortality, in young children, with many millions dying each year in the acute stage of the disease. The survivors, as the result of repeated enteric infection, frequently failed to thrive and progressed to malnutrition and eventual death.

Secondly, there had been rapid advances in the study of aetiology, with the hope that further advances lay ahead. New viruses had been identified by a relatively simple technique using the electron microscope; and new pathogenetic mechanisms, such as the production of toxins by enteric bacteria, had begun to be investigated. The realization that a technique as simple as examination of diarrhoeal faeces by electron microscopy could identify viral pathogens revolutionized the diagnosis of viral diarrhoeal disease. Virologists working on human disease were able to draw on considerable knowledge already available to veterinarians.

Thirdly, developing countries had themselves begun to realize the extent of their problems and to want to do something about them. The World Health Organization, with its expressed goal of 'health for all by the year 2000', began to put a major effort into decreasing the morbidity and mortality of children in the developing countries. Primary health care networks were set up by developing countries to educate mothers in recognizing the dangers of diarrhoea and to establish oral rehydration therapy in the home to reduce the mortality in young children. Overall, in both developing and developed countries, there was a shared realization of the size of the problem and of solutions in terms both of aetiology and of therapy. This concurrence of events galvanized laboratory

scientists into taking an interest in the problems of acute diarrhoea in veterinary and in human medicine.

Since 1975 there have been rapid developments in diagnostic techniques (based on enzyme immunoassays), and in protein chemistry, molecular biology and immunology. All have been brought into the overall thrust to solve problems related to acute diarrhoea. There has been an understanding on the part of people working in human medicine of all that veterinary science has to offer, and much fruitful collaboration has been established between veterinary and medical microbiologists. Thus, since 1975, we have attempted to do what Jon Rohde and Rob Northrup then urged us to do, which was to take science 'where the diarrhoea is' (Rohde & Northrup 1976).

In the 1975 symposium the emphasis as far as viruses were concerned was on Norwalk agent and on rotavirus. We mentioned adenoviruses briefly: Tom Flewett pointed out that they were often shed in large numbers in faeces from diarrhoeal children, and that they had the puzzling characteristic of being difficult to grow; but that was as far as our appreciation of the role of adenoviruses went. Astroviruses had already been named, and small round viruses had been described, but there was caution about their relation to diarrhoeal diseases. (There was even the suggestion that, on morphological grounds, many of them could be phages derived from bacterial or yeast gut infections and bore no relation to diarrhoeal disease.)

Coronaviruses were thought to be a possible new pathogen in human medicine. TGE virus (transmissible gastroenteritis virus) was being used as a model of physiological changes, by infecting piglets and studying the electrolyte and fluid fluxes resulting from infection of the pig gut. But it seems that the early promise held out by the study of coronaviruses has been either unfulfilled or held in a state of suspended animation.

The present symposium is a timely one. It is deliberately restricted to the so-called 'novel' diarrhoea viruses and will not be concerned to any great extent with what have become the 'classical' rotaviruses—the group A rotaviruses that were a major topic in the earlier symposium. We shall discuss what are collectively called the 'novel rotaviruses' (non-group A) that have emerged as important causes of diarrhoea in both human and animal medicine since 1975; then the enteric adenoviruses; the small round viruses; and the new group of Berne and Breda viruses, suggested as a new family, the Toroviridae.

The novel rotaviruses were recognized almost simultaneously in animals and in humans, although publication of the observations was delayed. Particles, morphologically identical with the classical rotaviruses, had been seen in animals and humans with acute diarrhoea; however, these rotaviruses would not react in serological tests. It became apparent that they were rotaviruses, but serologically distinct ones; they were characterized by failure to react with group (A) antiserum; they had the further useful characteristic of having genomic patterns markedly different from those of the 'classical', group A

rotaviruses. These new groups of viruses were then thought to cause a less severe disease. This concept was shattered (at least in man) with reports from China of severe watery and life-threatening diarrhoea in adults caused by a non-group A (probable group B) rotavirus. We shall be concerned here with the classification of novel rotaviruses, based on their serology and molecular biology. We shall discuss a major concern, namely how to facilitate the diagnosis of these non-group A infections; we shall examine the epidemiology and pathology of disease, and the potential for reassortment, or cross-infection, or both, between animals and man.

The enteric adenoviruses are now a well recognized group, classed as a subgenus and assigned serotypes 40 and 41. They are an important human pathogen, causing approximately 7–10% of diarrhoeal disease in children admitted to hospital in developed countries. Their recognition is easy but the assignment of their serotype numbers is still difficult. We need to discuss ways of facilitating their diagnosis, with a view to further epidemiological studies, particularly in developing countries, where their importance is not yet known.

The importance of adenoviruses as causes of disease in farm or domestic animals seems to be less than in man. The pathophysiology of adenovirus infection in animals will be discussed. We need studies of the physiological changes produced in the animal gut. Enteric adenoviruses may have a role in chronic infections and may be particularly important in terms of persistent infection and its possible corollary, malnutrition, in the human population.

The role of the small viruses in diarrhoea needs constant reassessment, largely because of the difficulties inherent in their identification and classification. They have been exceedingly elusive in the laboratory; they are generally shed in such small numbers that they are difficult to see and impossible to use as a source of antigen; and in the absence of success in their cultivation in the laboratory, a major barrier to progress has been the lack of development of suitable diagnostic tests. The small viruses loom large in our minds as a result of the catastrophic appearance of lethal parvovirus infection in dogs, associated with destruction of crypt epithelial cells of the small intestine. If a similar disease emerges in the veterinary community at large, or in man, it is likely, on the evidence of what parvovirus does to the dog gut, also be catastrophic.

The Berne and Breda viruses are more recently emerging pathogens. Berne viruses were first identified some years ago in horses in Switzerland and lay quiescent in the literature until it was recognized that a Berne-like virus (called the Breda virus) infected cattle in the USA, and could occasionally infect man (in the UK). We need to explore the complex structure of these viruses and to improve their diagnosis so that epidemiological studies can be extended, both in animal and in human populations.

Pathophysiological changes produced by the new diarrhoea viruses have been investigated in experimental animals, man hardly being suitable for many of these studies. Where it has been possible to compare histological changes it

has been encouraging to note that similar changes occur in animals and man. The physiological changes and the mechanisms by which virus infections produce diarrhoeal symptoms require further study. This area is beyond the scope of most of us at this symposium, but there is a case now for inviting gut physiologists to start looking, in animal models, at mechanisms by which viruses produce their effects in the intestinal tract. Perhaps such studies might locate a therapeutic agent to reduce escalating damage in the gut.

In summary, since the 1975 symposium there have been major changes in our knowledge of the aetiological agents of diarrhoeal disease, and of their importance in both animals and man. The stubborn resistance of these viruses to being coaxed to adapt to cell culture continues. This has held back both the development of diagnostic tests, and the study of viral gene function and control. At the end of this symposium I hope we shall depart with ideas on how to attack these problems or how to enlist help from others who may have experience that would add momentum to the study of the novel diarrhoea viruses and their role in disease.

Reference

Rohde JE, Northrup RS 1976 Taking science where the diarrhoea is. In: Acute diarrhoea in childhood. Elsevier/Excerpta Medica/North-Holland, Amsterdam (Ciba Found Symp 42) p 339–366

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Novel rotaviruses in animals and man

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Abstract. Novel (non-group A) rotaviruses have many of the morphological, biochemical and biological properties described originally for group A rotaviruses but they do not share the same group antigens. By negative-stain electron microscopy, novel rotaviruses have the characteristic rotavirus morphology, although with some novel rotaviruses the characteristic single- and double-shelled particles may not be readily apparent. Comparison of novel rotaviruses in serological tests has revealed the existence of at least six rotavirus serogroups, A to F, with the original rotaviruses belonging to group A. As with group A rotaviruses, viruses from different animal species, including man, can belong to the same serogroup. A further point of difference between novel and group A rotaviruses is their genome profiles, which lack the triplet of segments in the 7-8-9 region of group A rotaviruses. This is a useful diagnostic aid.

Novel rotaviruses have been found in farm animals and man. They can cause enteritis experimentally and infect villus enterocytes. In chickens, turkeys, lambs and pigs the viruses and/or antibody to them are commonly found, in association with either clinical or subclinical infection. In humans one type of novel virus has emerged as a cause of severe diarrhoeal disease in adults. The possible reasons for the relatively recent discovery of the novel rotaviruses are discussed.

1987 Novel diarrhoea viruses. Wiley, Chichester (Ciba Foundation Symposium 128) p. 5–23

After more than a decade of research on rotaviruses, it was sobering to realize that only one section of the rotavirus genus had been studied. Reports of porcine and avian viruses with the morphology of rotaviruses but without the group antigen, by which it was thought all rotaviruses were related, were published between 1979 and 1981 (Debouck & Pensaert 1979, Bridger 1980, Saif et al 1980, McNulty et al 1981). Since then, similar viruses have been identified, often in association with diarrhoea, in several animal species, including man.

The viruses to be considered here have been given different names: novel, atypical, antigenically distinct, antigenically unusual, rotavirus-like, non-group A, and pararotaviruses (which may just refer to viruses classified as group C). The term 'novel' will be used here and the established rotaviruses will be referred to as group A. Individual viruses will be described by an abbreviated cryptogram composed of their serogroup designation, where known, and their strain

identification; for example, B/NIRD–1. Novel rotaviruses from all animal species are included in this chapter; the proposed classification scheme is discussed; and the current prevalence of some serogroups is presented.

Morphology

Both single- and double-shelled particles can occur. Double-shelled particles whose diameter is about 70 nm have the characteristic wheel-like appearance and smooth periphery of group A rotaviruses (Saif et al 1980, McNulty et al 1981, Bridger et al 1982, Rodger et al 1982, Hung et al 1984, Espejo et al 1984, Jashes et al 1986).

Morphological differences are now becoming apparent between group A viruses and some of the novel viruses. Thin-walled, featureless particles, 48 to 52 nm in diameter, which appear to be cores on which the 70 nm particles are built, occur with novel rotaviruses from pigs (Bridger et al 1982, Theil et al 1985) and humans (Hung et al 1983, Jashes et al 1986). Similar cores have been seen with group A rotaviruses but only after chemical treatment. Furthermore, another novel virus from humans requires different conditions for the activation of its RNA polymerase and responds differently to chelating agents, which argues in favour of a virus structure different from that of group A rotaviruses (Jashes et al 1986).

Precise information is not available about the quantities of novel viruses in faeces but both Bridger (1980) and Saif & Theil (1985) commented that the numbers of particles were low compared to group A rotaviruses. Saif & Theil (1985) found that the type of particle seen in faeces after negative staining depended on the virus, and investigators working with different novel rotaviruses have recorded the presence of only double-shelled particles (Jashes et al 1986), predominantly double-shelled particles (Saif et al 1980, McNulty et al 1981, Askaa & Bloch 1984, Espejo et al 1984), or only very few double-shelled particles (Chasey & Banks 1984, Theil et al 1985). However, the type of negative stain can influence the type of particle seen. Both I and Chasey et al (1986) have found that, with the Chinese human virus and with two porcine viruses belonging to group E, ammonium molybdate revealed double-shelled particles better than potassium phosphotungstate.

Antigenicity and classification

Novel rotaviruses lack the group A antigen(s) which failed to distinguish rotaviruses reported before 1979 (Saif et al 1980, McNulty et al 1981, Bridger et al 1982, Bohl et al 1982, Rodger et al 1982). Comparison of novel rotaviruses by tests expected to detect major group antigens revealed that some of them were antigenically distinct from each other and that they could be classified into distinct serogroups. Initially, two groups, B and C, were proposed, with group A

being reserved for the rotaviruses described originally (Pedley et al 1983). Subsequently, an additional serogroup D was established when the avian 132 virus was included (Snodgrass et al 1984). Recently, group E has been established, making a total of five rotavirus serogroups including group A (Pedley et al 1986). An independent comparison of three novel avian rotaviruses proposed four avian serogroups, represented by group A rotaviruses and the three viruses 132, A4 and 555 (McNulty et al 1984a). Recent comparisons of the avian A4 antigen and its antiserum and antiserum to the avian 555 virus with the mammalian viruses B/NIRD-1, C/Cowden and E/DC-9 showed that these two avian viruses did not share group antigens with the mammalian viruses (J. C. Bridger & M. S. McNulty, personal observations). Hence six, and possibly seven, serogroups exist. Clearly, it is now important that as rotaviruses are identified, they should be examined for their relationship to existing serogroups. Antisera and antigens, perhaps in the form of fixed infected cell cultures or tissues, should be exchanged so that the full extent of the variation in group antigens can be quickly assessed.

As with the group A rotaviruses, novel rotaviruses from different animal species can belong to the same serogroup (Table 1). Human viruses identified in Brazil, Australia and the United Kingdom share the group C antigen with some porcine viruses (Bridger et al 1986), and preliminary evidence shows that the Chinese human virus shares the group B antigen with viruses from pigs, sheep and calves (Chen et al 1985, Saif & Theil 1985). It will be interesting to establish whether viruses belonging to other rotavirus groups will be confined to a limited number of animal species or whether, as with group A, viruses from many different animal species share group antigens.

TABLE 1 Animal species from which novel rotaviruses have been indentifed

Serogroup[a]						
A	*B*	*C*	*D*	*E*	*F*	*(G)*[b]
Many species	Pigs Cattle[c] Sheep[c] Humans[e]	Pigs Humans[d]	Chickens	Pigs	Chickens	Chickens

Isolates from rats and turkeys have yet to be grouped serologically.
[a] Represented by A/UK, B/NIRD-1, C/Cowden, D/132, E/DC-9, F/A4 and G/555 rotaviruses.
[b] Preliminary evidence only for the existence of this group (see text).
[c] Snodgrass et al 1984.
[d] Bridger et al 1986.
[e] Chen et al 1985, Saif & Theil 1985.

Nucleic acid

In common with group A rotaviruses, novel rotaviruses have 11 segments of double-stranded RNA with a similar range of M_r, namely 2×10^6 to 0.2×10^6, but the electrophoretic mobilities of some of the segments differ (McNulty et al 1981, Bohl et al 1982, Bridger et al 1982, Rodger et al 1982, Hung et al 1984, Vonderfecht et al 1984, Wang et al 1985, Chasey et al 1986). The four (or five with some avian viruses) segments with the highest molecular weights segregate similarly to those of group A rotaviruses but, so far, all novel rotaviruses lack the triplet of segments in the 7-8-9 region of group A rotaviruses. It will be interesting to see whether this difference continues to be a diagnostic character for novel rotaviruses and how specific the genome profiles are (see below).

Pathogenesis and cross-protection

Novel rotaviruses have been identified in association with enteritis in pigs (Saif et al 1980, Askaa & Bloch 1984, Theil et al 1985, Chasey et al 1986), calves (Chasey & Davies 1984), lambs (Chasey & Banks 1984), rats (Vonderfecht et al 1984), turkeys (Saif et al 1985) and human infants (Rodger et al 1982, Nicolas et al 1983, Pereira et al 1983, Espejo et al 1984) but the most severe natural disease reported so far has been in adults in China, where several large epidemics of a cholera-like illness with dehydration have occurred (Hung et al 1984, Wang et al 1985). In contrast, the avian D/132 virus was found in normal faeces of healthy three-week-old chickens and subsequently four different avian serogroups were detected in broiler chickens without signs of enteritis being recorded (McNulty et al 1981, 1984a).

A range of clinical signs has been reported in animals experimentally infected with novel rotaviruses. With the avian D/132 virus, clinical signs in chickens were mild (McNulty et al 1981) but, in pigs aged 72 hours or less, there was 90% mortality (Bohl et al 1982). Clinical signs included acute diarrhoea which was often watery, anorexia, dehydration, and weight loss in pigs (Askaa & Bloch 1984, Hung et al 1984, Theil et al 1985, Chasey et al 1986). With the B/NIRD–1 virus, all nine infected piglets, aged five to eight days, developed enteritis with abnormal faeces for 5–6 days. To aid recovery, milk was withheld from five, but one piglet died. All inoculated piglets lost or failed to gain weight for between one and six days and the mean time taken to return to weight at inoculation was four days (J. C. Bridger, personal observations). It seems likely that, as with group A rotaviruses (Bridger & Pocock 1986), novel rotaviruses will vary in virulence.

In common with group A rotaviruses, viral antigen has been detected in the villus enterocytes and particles resembling rotaviruses have been seen in damaged enterocytes (McNulty et al 1981, Bohl et al 1982, Askaa & Bloch 1984, Vonderfecht et al 1984, Theil et al 1985, Chasey et al 1986). The viruses can infect more than one animal species, as has been demonstrated with a human virus

which infected rats, bovine viruses which infected lambs and piglets, and an ovine virus which infected piglets (Snodgrass et al 1984, Eiden et al 1985, Saif & Theil 1985). Cross-protection was not observed between porcine group A viruses and B/NIRD–1 (Bridger et al 1982) or porcine group A and two group C viruses (Bohl et al 1982). Furthermore, no cross-protection was observed between two novel viruses belonging to group B, suggesting that there are important antigenic differences between viruses in the same serogroup (Saif & Theil 1985).

Diagnosis

Novel rotaviruses were identified first by a lack of agreement between electron microscopy and serology (Bridger 1980, Saif et al 1980, McNulty et al 1981, Rodger et al 1982, Hung et al 1983). Subsequently, they have been detected by their genome profile, which is different from that of group A viruses (Nicolas et al 1983, Espejo et al 1984). Electron microscopy is unable to distinguish between the groups, unless immuno-electron microscopy is used, but even then Theil et al (1986) found it inferior to genome electropherotyping. With some of the novel viruses, including the porcine B/NIRD–1 virus (J. C. Bridger, personal observation), electron microscopy with negative staining has been a poor method of detection and, in some instances, the examination of thin sections of centrifuged material has been used to detect rotaviruses-like particles (Hung et al 1984, Eiden et al 1985).

Although genome profiles (electropherotypes) indicate the presence of a novel rotavirus, whether they will be specific for serogroups is still unclear. Some investigators have either predicted the existence of serogroups from genome profiles, or suggested that profiles are serogroup specific. Todd & McNulty (1986) have proposed the term 'electropherogroup' and assigned avian viruses to five electropherogroups.

Novel rotaviruses have been assigned to serological groups by indirect immunofluorescence using gut sections and cultures of infected cells. Staining of sections or mucosal smears with reference antisera could be a method of diagnosis but it has not been applied widely. Nor has virus isolation in cell culture been applied, although cell cultures have been infected to a limited extent with several avian and porcine viruses (Bohl et al 1982, McNulty et al 1984a, Theil & Saif 1985, Terrett et al 1985). The only successful serial passage recorded has been with a porcine virus identified in Denmark (Askaa & Bloch 1984). ELISAs (enzyme-linked immunosorbent assays) have been used to detect antigen and antibody in pigs (Debouck et al 1983) and rats (Vonderfecht et al 1985).

Prevalence

Novel rotaviruses seem to be very common in some animals, but less common in man. They have been identified in seven animal species (pigs, chickens, turkeys,

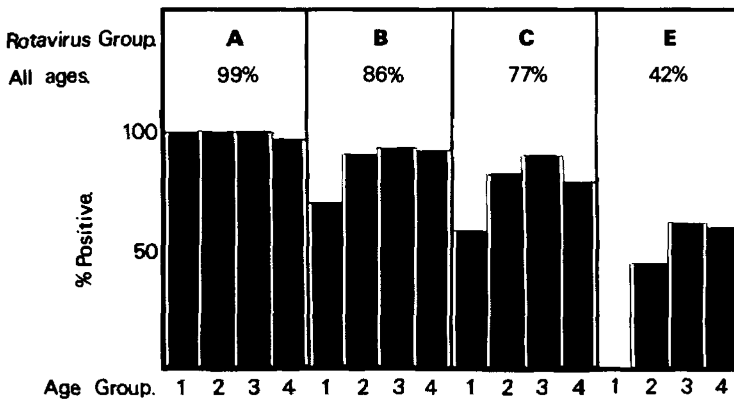

FIG. 1. Prevalence, by indirect immunofluorescence, of antibody to four rotavirus serogroups in 162 porcine sera collected from five farms in England in 1981. The age groups tested were: 1. pigs aged 3–8 weeks; 2. pigs aged 10–12 weeks; 3. pigs aged 15–26 weeks; 4. sows. Compiled from Bridger & Brown (1985) and Chasey et al (1986).

TABLE 2 Prevalence of antibody to serogroups A, B and C in sera from cattle and sheep

Species	*No. of sera tested*	*No. of locations tested*	*Year of sampling*	*Rotavirus serogroup*		
				A	*B*	*C*
Cattle	118[b]	4	1973 to 1983	91[a]	20	24
Sheep	50[c]	5	1981	43	19	0

[a] % of sera positive at 1:20 dilution by indirect immunofluorescence with A/UK, B/NIRD-1 and C/Cowden as antigens.
[b] From cattle of all ages in England and Wales.
[c] From adults in England and Wales.

man, calves, lambs and rats) from more than 14 countries. In chickens, turkeys, lambs and pigs, either the viruses or their antibody, or both, can be very frequent. In chickens in Northern Ireland, antibody to the D/132 virus was found on all of 14 farms tested and, as with turkeys in the USA, novel rotaviruses were more common than group A viruses, both in association with disease and without it (McNulty et al 1984a, 1984b, Saif et al 1985, Theil et al 1986). In lambs, analysis of the genome profiles of rotaviruses from 17 outbreaks of diarrhoea in the United Kingdom in 1983–1984 implicated novel rotaviruses as the cause of the disease (Chasey & Banks 1984). The genome profiles resembled group B profiles, but the prevalence of antibody to groups B and C in ovine sera taken in England and Wales in 1981 was found to be low (Table 2). This suggests either that novel rotaviruses belonging to group B became more common between

TABLE 3 Countries where novel rotoviruses have been identified in man

Country	*Date first reported*	*Serogroup*
Australia	1982	C[a]
Bulgaria	1983	?
France	1983	?
Brazil	1983	C[a]
China	1983	B[b]
United Kingdom	1983	C[a]
Mexico	1984	?
North America	1985	?
Switzerland	1985	?
Italy	1985	?
Ecuador	1985	?
Argentina	1986	?
Chile	1986	?

[a] Bridger et al 1986.
[b] Chen et al 1985, Saif & Theil 1985.

TABLE 4 Prevalence of antibody to rotavirus serogroups A, B and C in human sera and immunoglobulin pools

Source	*No. tested*	*Year of sampling*	*Rotavirus serogroup*		
			A	*B*	*C*[a]
Sera[b]					
Children	15	1982	93[c]	0	0
Adults	38	pre-1984	95	3	11
Immunoglobulin pools[d]	12	pre-1984	100	0	0

[a] Taken from Bridger et al 1986.
[b] Taken in the UK and tested at 1:20 dilution.
[c] % of samples positive by indirect immunofluorescence with A/UK, B/NIRD-1 and C/Cowden as antigens.
[d] Six pools made from sera taken in the UK, two from Canada and one each from Belgium, Switzerland, USA and Japan and tested at 1:100 and 1:500 dilutions.

1981 and 1983–1984, or that the novel rotaviruses circulating in lambs belong to serogroups other than B and C.

In pigs, the viruses or their antibody have been found to be common in England, Belgium and the USA. Antibody to serogroups B, C and E was found frequently in porcine sera collected in England in 1981 (Fig. 1) and, in the USA, antibody to group C was as common as in the UK, although antibody to group B was less frequent (Theil & Saif 1985, Terrett et al 1985). In the United Kingdom,

antibody to groups B and C was present in pigs before 1981, as two serum pools made in 1974 and 1978 contained these antibodies (Pedley et al 1983). In Belgium, in swine, Debouck et al (1983) found pararotavirus (group C) to be enzootic in three herds, often in association with group A rotaviruses in sub-clinical infections. Faecal samples from cases of porcine enteritis in England and Wales did not reflect the high prevalence of antibody to novel rotaviruses; only 5% of rotavirus genome profiles were atypical (Chasey & Davies 1984, Chasey et al 1986). Possible reasons for this are that novel rotaviruses are uncommon in neonatal porcine enteritis, or that the diagnostic tests for them are insensitive. In cattle, neither the viruses nor antibody to serogroups B or C seem to be common at present (Table 2). Only about 1% of rotavirus-positive faecal samples from cases of bovine enteritis in the United Kingdom had atypical genome profiles (Chasey & Davies 1984, Snodgrass et al 1984).

In man, novel rotaviruses have been identified mainly in sporadic cases of enteritis between 1982 to 1986 from Australia, Western and Eastern Europe, North, Central and South America, and China (Table 3). Excluding the Chinese human virus, the average detection rate of viruses whose genome profile resembles that of serogroup C viruses is one sample in about 300 (compiled from the results of seven investigators in seven countries). This low prevalence of virus agrees with the low prevalence of group C antibody in human sera and immunoglobulin pools taken from several regions of the world (Table 4).

Preliminary evidence indicates that the novel rotavirus that has been responsible for very large epidemics of enteritis in adults in China belongs to serogroup B (Chen et al 1985, Saif & Theil 1985). The prevalence of antibody in human sera and immunoglobulin pools from Europe, North America and Japan was therefore examined with the B/NIRD–1 virus as antigen, but was found to be almost nil (Table 4); whereas, in epidemic areas of China and in non-epidemic areas of China, Hong Kong and Australia, 41–53% and 12–24% of adults had antibody to the human Chinese antigen (Hung et al 1985). Interestingly, antibody to the Chinese virus was also found in sera from rats and pigs, but not in sera from cattle, sheep or horses, which endorses the idea that humans, pigs and possibly rats have rotaviruses with the same group antigen. The highest prevalence of antibody in humans has been recorded in North America, with the novel rat virus as antigen (Table 5).

Discussion

Only a narrow section of the rotavirus genus had been studied until the novel non-group A viruses were identified. Their late discovery might suggest that they are rare; this is not so in several animal species, although it may be so in man. The outcome of experimental infections indicates that novel rotaviruses cause enteritis but, as they can occur in healthy and diseased animals, their role in natural disease will require careful assessment. Genome electropherotyping

TABLE 5 Prevalence of antibody to novel rotaviruses in human sera and immunoglobulin pools found by different investigators

Antigen (group/virus)	*Country*	*% positive*
B/porcine[a]	United Kingdom	0–3
B/human[b]	China Hong Kong Australia	12–24
C/porcine[c]	United Kingdom	0–11
?/human[d]	Mexico	42
?/rat[e]	North America	24–88

[a] See Table 4.
[b] Hung et al 1985.
[c] Bridger et al 1986.
[d] Espejo et al 1984.
[e] Eiden et al 1985.

appears to be a good method of diagnosing these viruses but care must be taken to establish whether genome profiles are truly serogroup specific. It has been shown that viruses from different animal species can belong to the same serogroup, so the description of species-specific serogroups should be avoided.

Why have the novel viruses been discovered only recently, after so much research on group A rotaviruses? Some workers have commented that the numbers of particles in faecal preparations are low and that the novel viruses break down readily, making electron microscopy an insensitive technique. However, genome electropherotyping has been applied widely in rotavirus research and, with their distinctive genome profiles, it is surprising that the novel rotaviruses were not found sooner, if they were present. It is possible that in man they are an emerging infection and that the viruses and antibody to them will be identified more frequently in the future, throughout the world. Novel rotaviruses do not appear to be an emerging infection in domestic avian species or in pigs, however, as the viruses or their antibody, or both, are common. Perhaps the majority of novel rotaviruses are not associated with natural disease and thus were not identified when diarrhoeic samples were examined, or perhaps the ubiquity of group A rotaviruses has usually masked their presence. Whatever the reasons for their recent discovery, novel rotaviruses are being increasingly detected.

The lack of antigenic relationships between the rotavirus serogroups indicates that vaccines for each group will be needed if the novel groups are found to be important in disease. It will be interesting to see whether the novel groups are identified more frequently when effective vaccination for group A viruses is commonly practised.

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DISCUSSION

Greenberg: Do the novel rotaviruses replicate in exactly the same cells in the intestine in which the group A viruses replicate? Have co-infection studies been done?

Bridger: I don't know of any experimental co-infections, but the novel rotaviruses do multiply in the same cells, the mature enterocytes on the villi, and Graham Hall will discuss our recent work with a porcine group B rotavirus (p 192–207).

Greenberg: I am interested in syncytia formation in atypical rotavirus infections. How common is it in these infections?

Saif: A syncytia-forming enteric virus was identified in cattle in 1978. The only particles found were coronavirus-like, according to Dr Mebus (Mebus et al 1978), but we re-examined those specimens and put one sample into gnotobiotic calves. We found that it is antigenically and electrophoretically related to group B rotaviruses (Ohio, porcine and bovine), and the pathology (syncytia formation) was confirmed. We are doing further studies to see if this virus cross-protects against Ohio bovine or other group B rotaviruses.

Hall: Syncytia are seen in pigs infected with porcine group B rotaviruses. We can perhaps begin to think of syncytia as pathognomonic for atypical rotaviruses. Or is the evidence, so far, that syncytia are only pathognomonic for group B rotaviruses?

Bridger: So far, we have the novel rat rotavirus which induces syncytia *in vivo*. Also, Dr Askaa's porcine virus was reported to cause syncytia formation and we will be determining its serogroup soon. When we know to which serogroups novel rotaviruses belong, we shall be able to decide whether syncytia are pathognomonic for all novel rotaviruses or just some groups, perhaps group B.

Hall: Has anybody examined the lesions produced by any rotavirus group, other than B?

Bridger: With group C, we have not studied the pathology of intestinal infection.

McNulty: With group D avian rotavirus we saw no syncytia in the intestinal epithelium of experimentally infected chickens.

Bishop: Do we know why syncytia form? Is it evidence of lateral spread of virus?

Greenberg: Usually syncytia form because a virus contains a protein that is able to fuse membranes. We have evidence that group A rotaviruses have the ability to penetrate membranes, but we have been unable to produce syncytia with group A viruses.

Horzinek: Have the novel viruses ever been found in avian species which are not domesticated? In view of the 'split genome' character of their RNA, perhaps migratory birds might have a similar function in producing reassortants, as was found for influenza virus.

Bridger: Not to my knowledge.

McNulty: Non-domesticated avian species have not yet been studied.

Holmes: The problem with accepting the suggestion that the novel rotaviruses have emerged only recently as a human infection is that workers have not really looked for them. The number of people doing even electrophoretic typing of sporadic diarrhoea samples from human adults is small, because it is so hard to persuade patients to provide samples. Perhaps gradually, as more human faecal samples are studied, more non-group A rotaviruses will be

found. In young children with acute diarrhoea, we think they really are rare, but where other mammals or birds or older people are concerned, I doubt whether enough samples have been looked at to provide any conclusions. I have heard of some non-group A virus being found in humans in South Africa with an RNA pattern like that of group A. Can anyone amplify this?

McCrae: We have examined these viruses. They are group A, in fact, in serogrouping reactions, but they appear to have an enlargement of their segment 11 RNA. The normal segment 11 has disappeared from the genome profile, but a new segment has appeared in the segment 5–6 region of the gel. There is another virus, first isolated by David Pocock from cattle at Compton, having a similar genomic aberration. Fortunately, that virus grows in tissue culture and we have grown it up and cloned the altered segment 11. As with the South African isolates, the triplet of segments 7–9 is still present and it is still considered to be diagnostic for group A rotaviruses.

I should add here that I am concerned that people making individual isolations of atypical viruses are stating that because they do not fall into group A (i.e., they don't give an ELISA cross-reaction), they must be a group B or group C or a group X rotavirus, entirely on the basis of the genome profile. While these profiles are useful for showing that a rotavirus is not group A, they cannot show to which group a particular sample belongs. I shall discuss an example of that in my paper.

Kapikian: Dr Bridger, you demonstrated a relatively high prevalence of serum antibody in pigs to the B, C and E groups (your Fig. 1), and you stated that faecal samples did not reflect this prevalence, since only 5% of the rotaviruses detected in pigs with diarrhoea had a genome profile consistent with that of non-group A rotaviruses. An important question is whether or not these 'novel' rotaviruses are merely 'novelties'. Apart from the reports from China of large outbreaks of the non-group A rotaviruses associated with severe gastroenteritis in adults, the relative importance of these agents in other areas appears to be low. This pattern may be similar to that of the echoviruses, which are comprised of many serotypes, but only a few have been shown to be important in causing disease.

Blacklow: On that same theme, has anyone explored mixed experimental infections by novel rotaviruses and group A, in animals? Is there any evidence that novel rotaviruses interfere with infection by group A rotaviruses?

Hall: I am not aware that anyone has looked at the pathology of co-infection.

McNulty: We tried mixed infections of cell cultures with avian group A and group D rotaviruses. Using fluorescein-labelled antisera to group A and rhodamine-labelled antisera to group D, we could not demonstrate co-infection of the same cells (unpublished observation). This may support Dr Blacklow's suggestion of interference.

Blacklow: Was there any reduction in the percentage fluorescence of group A by the presence of group D?

McNulty: I couldn't say that there was.

Kapikian: Has anyone tried reassortment with these viruses? That is, has the 'rescue' been attempted of these non-cultivable atypical rotaviruses by co-infection with cultivable group A rotaviruses under selective pressure, to yield a reassortant with the neutralization specificity of the novel rotavirus, which might then be cultivable, since it might contain genes from the cultivable group A rotavirus also?

Greenberg: I have tried to isolate reassortants between group A avian and mammalian rotaviruses. I chose this system because the avian rotaviruses are adapted to tissue culture and grow to high titre, which makes it likely that one could isolate reassortants if such reassortants were viable. However, group A avian rotaviruses have abnormal electropherotypes and are genetically very distinct from the mammalian group A viruses. I could not isolate any mammalian/avian reassortants. The rate of reassortment between avian and mammalian group A was less than one per 1000 in a co-infection where every cell should have been doubly infected. I think the isolation of reassortants between non-A and A is therefore quite unlikely, since these more closely related viruses (avian and mammalian group A) do not reassort at a detectable frequency.

Saif: There are mixed natural infections with group A and non-A rotaviruses, particularly in swine; we have seen groups B, C and A together in pigs from the same litters, particularly A and C, together in the same animal. We think that the novel rotaviruses may be more prevalent in weanling swine, from the electropherotype data as well as detection by immuno-electron microscopy. The group C rotavirus (= pararotavirus; our early nomenclature; Bohl et al 1982) is found in pigs as early as three or four days of life, and in weanling pigs. Group B is least frequent, and usually seen in the weanling pigs. Group A is still predominant, in all our studies.

We studied the genome profiles (electropherotypes) of porcine and bovine rotaviruses, typical and atypical (Saif & Theil 1985). The profiles of group B viruses from two different species, pig and calf, are fairly similar (Fig. 1).

We have now done cross-challenge studies in gnotobiotic pigs, and in one gnotobiotic calf, using the porcine and bovine group B rotaviruses. They did not protect against one another, although they cross-react by immunofluorescence, immuno-electron microscopy and other serological tests. So there are evidently different serotypes within serogroup B (Saif & Theil 1985).

Snodgrass: When we identified atypical rotaviruses from calves and lambs, Dr Bridger sent us an antiserum to her pig prototype group B virus, and cryostat gut sections containing that virus. By cross-immunofluorescence tests, the pig antiserum did not react with either the calf or lamb virus, but antisera to these viruses prepared in gnotobiotic animals did react with the pig group B virus (Snodgrass et al 1984). Thus there seems to be a one-way cross-relationship. Has anyone found any intermediate groups or subgroups within group B?

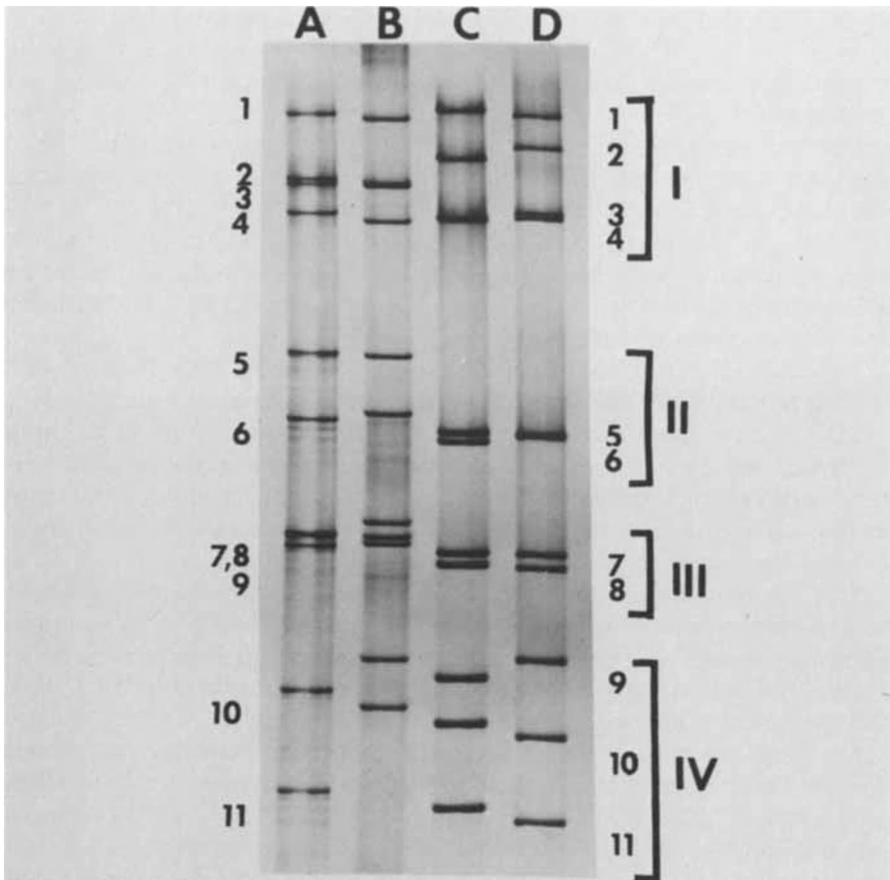

FIG. 1 (*Saif*). Comparison of the double-stranded (ds) RNA electropherotypes of group A and B rotaviruses in the same polyacrylamide gel slab. Migration is from top to bottom. Roman numerals on the right designate ds RNA size classes. Numbers on the left indicate segments of the group A rotavirus genome (lanes A and B). Numbers on the right indicate segments of the group B genome (lanes C and D). *Lanes*: A, bovine group A rotavirus; B, human S2 group A rotavirus; C, porcine group B rotavirus; and D, bovine group B rotavirus. (L.J. Saif & K.W. Theil.)

Bridger: I haven't seen that, except in the example you just mentioned. It may be a question of looking at more isolates and trying to confirm whether this is a common finding.

Saif: We sent you our bovine group B antiserum and it did cross-react with your porcine group B virus.

Bridger: Yes: that was only a one-way cross. With David Snodgrass we did a two-way cross, and there seemed to be a lack of cross-reaction in one direction.

Apart from that, where we have looked, we have always found two-way crosses.

Snodgrass: You confirmed our finding, using our reagents?

Bridger: Yes.

Saif: Our two group B viruses (bovine and porcine) showed two-way cross-reactivity using our gnotobiotic pig and calf specimens in immuno-electron microscopy and cross-fluorescence (Saif & Theil 1985).

Bridger: It is important to do the two-way crosses, and to titrate them to homologous titres, while we are in this phase of trying to assess the properties of these atypical viruses. Then we shall see how widespread the unusual finding of a one-way cross is.

Kapikian: Have you looked at the human group B virus from China to see if there is any cross-reaction with your porcine or calf group B viruses, Dr Saif?

Saif: Yes. Dr Hung Tao sent the Chinese group B virus to Dr M.K. Estes, who made antisera in guinea-pigs. In testing pre- and post-exposure guinea-pig sera, only the hyperimmune antiserum reacted with the pig group B rotavirus, by immuno-electron microscopy and cross-fluorescence (Saif & Theil 1985).

Kapikian: Is there a two-way cross-reaction?

Saif: We didn't have enough of Dr Hung's group B virus to do this. Dr Estes and colleagues have since done the two-way cross, which will be published jointly (Nakata et al 1986).

Bridger: We have published that there is a two-way cross between B/NIRD-1 and the human Chinese virus (Chen et al 1985).

Kapikian: But there is no cross-protection between the calf and porcine viruses, so, as noted before, there probably are different serotypes within group B.

Saif: I think so. Also, Bob Yolken and his group have information on the atypical rat rotavirus, which they found shares antigenic determinants and genome sequence homology with the Chinese group B human rotavirus (Eiden et al 1986).

Greenberg: One way of assessing whether the atypical rotaviruses are likely to spread rapidly through human populations is to see how frequently one finds nosocomial infections of laboratory workers studying these viruses. Have there been laboratory outbreaks? I have worked with Dr Hung Tao's virus and, fortunately, nobody in my lab has come down with the disease. Considering how the group B virus marched through China, I was rather concerned about its spread in the laboratory.

Cubitt: We have run about 100 RNA electropherotypes of rotaviruses from clinical infections in adults in the UK; the only group C infection that we found was in a doctor at the Central Middlesex Hospital. She was extremely ill for about 10 days, but although she had young children, no one else was infected. This suggests that group C virus isn't spreading readily from person to person.

We have also been working with Dr Alexandre Linhares in Brazil on samples

obtained from Amazonian Indians. We have found only two rotaviruses so far on the grids he has sent back. Neither reacted in the ELISA test for group A rotaviruses. Both have atypical RNA profiles. Perhaps the atypical strains are more prevalent in that sort of community.

Bishop: We need to separate the question of how prevalent these novel rotaviruses are from the question of how often they cause disease. To do this we need serological assays that estimate prevalence of serum antibody to groups B and C rotaviruses. It seems likely that group C viruses are widespread in communities but that they seldom cause severe disease requiring admission to hospital. We have identified two children who excreted group C rotaviruses while hospitalized for acute watery diarrhoea. Both had pre-existing disease that may have contributed to the severity of their symptoms. One child was being treated with high dose steroids for fibrosing alveolitis. The other had a three-week history of diarrhoea associated with *Salmonella* infection and possibly also with *Giardia lamblia* infestation.

Chiba: My comment concerns novel rotaviruses in Japan. Japanese investigators have been eager to look for a novel rotavirus in human stools, especially after Professor Hung Tao's report from China. To my knowledge, however, they have not been able to find group B viruses as yet. Recently, a group of investigators has found atypical rotaviruses, presumably of group C, in a considerable number of patients seen at one paediatric clinic during the last two epidemic seasons (M. Ohseto et al, unpublished paper, 34th Annual Meeting of the Society of Japanese Virologists, Fukuoka, October 1986). They could detect 12 such strains, which accounted for about 10% of the rotavirus-positive stools obtained at that clinic. It would be interesting to follow up this particular rotavirus.

Flewett: We have been careful in handling the Chinese virus in our laboratory, especially after Hung Tao's description of the severity of the illness in volunteers. I also didn't want this virus to get into the UK community, where at present we don't have it!

McNulty: We have collected information on the prevalence of group D rotavirus antibody in various species, by indirect immunofluorescence using chick embryo liver cells infected with the prototype group D virus (the 132 chicken rotavirus). We tested sera at a 1:40 dilution from pigs, cattle, sheep, horses, humans and turkeys. The virus is widespread in chickens. We found a high prevalence of antibody (50.3%) in 137 pig sera taken from 17 farms; quite a low prevalence (17.5%) in cattle, and in sheep (6%); and a high prevalence (30%) in horses. We found 7/70 human sera were positive (10%); these sera were obtained from the virus laboratory of a local hospital. The prevalence in turkeys was high (36%). So group D is not merely confined to chickens.

Bourne: Are there any known epidemiological factors that might explain the general prevalence of novel rotaviruses in farm species, but not in man? Or is

Stewart McNulty now suggesting that this isn't so, and if you look hard enough, you do find them in humans as well?

Bishop: Yes. We suspect that they are common in humans, but we require information based on surveys of serum antibodies. We are not sure to what extent infections cross from animals to man. There may be strains highly adapted to particular animals, as with the group A rotaviruses.

McCrae: Given the prevalence of antibody to group B in China and the spread there of that virus into humans at a high level, it seems surprising that in Europe group B antibody is frequent in several animal species, so that the virus is occurring, even if not causing disease; yet there is virtually no evidence for a spread into humans. This may indicate some epidemiological factor which is determining the spread of the group B novel rotavirus across the species barrier.

Mathan: In children with acute gastroenteritis in southern India, about 10% of the stool samples positive for rotavirus by electron microscopy were ELISA-negative, or atypical. In this semi-rural environment, people and animals live under the same roof, and I am sure there is a strong likelihood of cross-infections.

Kapikian: It is quite impressive to hear that 10% of the rotaviruses in your Indian population were electron microscope-positive, yet negative by serological assay (ELISA). This is the highest percentage of atypical rotavirus infection in children with acute gastroenteritis of which I am aware. Could you tell us more about the natural history of these infections? Did they occur in a sharp outbreak, or were they spread out over a longer period of time?

Mathan: These were children with acute gastroenteritis attending the outpatient department of the Vellore hospital over a two-year period. At the same time we found rotaviruses in control children, but none of the controls had electron microscope-positive, ELISA-negative viruses. This was a WHO study. We haven't previously compared electron microscopy and ELISA, but used electron microscopy alone to detect rotavirus.

Bishop: Have you done electropherotypes?

Mathan: No; I have brought the samples to Dr Flewett to do that.

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Nucleic acid-based analyses of non-group A rotaviruses

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Abstract. Simple genome profile studies on polyacrylamide gels allow all non-group A rotaviruses isolated so far to be recognized by the absence of the tight triplet (7–9) of RNA segments seen in all group A viruses. However, reliance solely on genome profile studies for rotavirus grouping can be misleading and, for virus group definition, additional corroborating nucleic acid and serological studies are essential. Terminal fingerprint analysis was the first generation of nucleic acid-based assays that allowed discrimination between the various rotavirus groups. By means of this technique the clear definition of five rotavirus groups (A–E), correlating exactly with those found by a serological assay, has been possible, with preliminary evidence for at least two additional groups. The technical sophistication of fingerprinting techniques prevents their widespread use in epidemiological studies; the development of a second generation of nucleic acid-based assays is therefore under way. These employ molecularly cloned cDNA probes to the genomes of non-group A viruses which can be widely distributed for use in 'dot-blot' screening of faecal samples and, if expressed as protein in *Escherichia coli*, should provide a ready source of viral antigen for use in surveying viral prevalence through the screening of serum antibody levels.

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The non-group A (Pedley et al 1983), atypical or pararotaviruses (Bohl et al 1982) were first recognized in pigs (Bridger et al 1982, Saif et al 1980) and chickens (McNulty et al 1981) almost simultaneously in the United Kingdom and USA. They were morphologically virtually indistinguishable from the 'normal' or group A rotaviruses, and shared their association with acute gastroenteritis, but differed significantly from them when analysed at the protein and nucleic acid levels. At the protein level, non-group A isolates failed to react in a variety of serological assays for detecting the 'group' antigen of rotaviruses previously thought to be present as a component of the inner shell of the virus (Woode et al 1976), irrespective of its animal species of origin. Genome profile studies on these unusual virus isolates have revealed a level of variability in the electrophoretic migration of their genome segments considerably greater than that previously observed; in particular, the characteristic tight triplet of bands (7–9)

was in all cases reduced to a doublet, with the displaced RNA segment migrating either more rapidly or more slowly on polyacrylamide gels, depending on the virus isolate (Bridger et al 1982, Saif et al 1980, McNulty et al 1981). In view of the fact that a significant fraction of infectious gastroenteritis has no known cause, the initial recognition of non-group A rotaviruses prompted a re-examination of many acute gastroenteritis specimens, particularly those falling into the electron microscope-positive, enzyme-linked immunosorbent assay (ELISA)-negative category. As a consequence a considerable number of non-group A isolations have been made from several animal species (Dimitrov et al 1983, Espejo et al 1984, Hung et al 1983, Rodger et al 1982, Snodgrass et al 1984, Vonderfecht et al 1984). Unfortunately, with the exception of the avian isolate made by Dr McNulty and his colleagues (McNulty et al 1981), these non-group A viruses have been refractory to growth in tissue culture, and this has severely curtailed their characterization. The problems with tissue culture growth have also hampered the development of diagnostic reagents and assay procedures for the various types of non-group A isolate. These deficiencies have, in their turn, led to most published reports concentrating solely on the isolate actually made, with attempts to fit it into any coherent framework of interrelationships being based mainly on inference from other published data rather than direct comparative analyses. In collaboration with Dr J. C. Bridger, my group at Warwick University has been involved in comparative serological and nucleic acid analyses of a number of non-group A virus isolates, aimed at establishing experimental criteria by which to compare the relationships of these viruses both with each other and with the 'normal' or group A rotaviruses. Our results indicated that not only were the non-group A isolates all grossly divergent from the group A viruses, but they could themselves be sub-divided into a number of distinct groups according to both serological and nucleic acid criteria. These observations have led us to propose a new classification scheme for rotaviruses, consisting of a number of groups where the members of any one group share a group antigen and nucleic acid sequence homologies (Pedley et al 1983). So far, five groups have been clearly defined, with preliminary indications of the existence of at least two additional groups (Pedley et al 1983, 1986).

The purpose of this chapter is to discuss the various nucleic acid analyses that we have done on non-group A rotaviruses in terms of the results achieved, the advantages and limitations of each technique, and possible priorities for future work. This discussion will be centred on the three main nucleic acid-based techniques that have been applied to non-group A viruses—namely, genome profile analysis, terminal RNA fingerprinting, and hybridization/recombinant DNA-based procedures. Each will be dealt with in turn.

Genome profile studies

Analysis of the overall mobility pattern of genomic RNA segments on

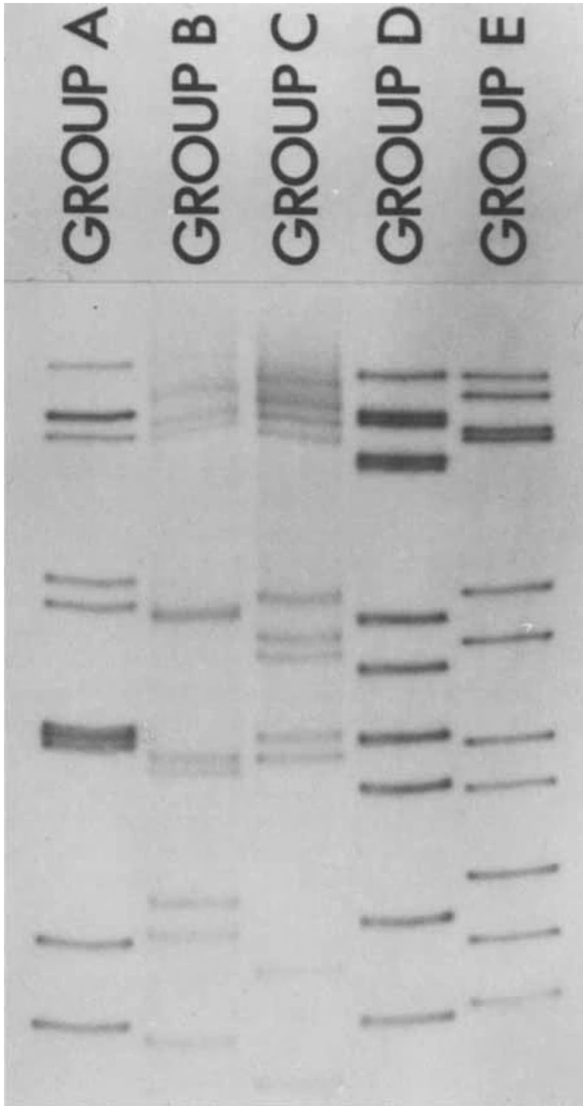

FIG. 1. Genome profile comparison of the type members of the five rotavirus groups defined so far.

polyacrylamide gels is the simplest technique used to study the non-group A rotaviruses. It has the advantage that it is technically straightforward, and has minimal equipment and consumable requirements, allowing it to be applied on a wide scale, even in fairly primitive laboratory facilities. It has proved to be a relatively quick and simple assay that can be used on small amounts of faeces to detect non-group A viruses. The ability to differentiate the group A or 'typical' virus isolates from those belonging to the other rotavirus groups depends on a diagnostic change in the segment 7–9 region of the gel. In all the non-group A viruses studied so far, there is a reduction in this characteristic tight triplet of segments to a doublet, with in some cases (groups B and E) the displaced segment migrating more rapidly on polyacrylamide gels and in others (groups C and D), more slowly (Fig. 1). Unfortunately, the molecular changes involved in alterations of the migration rates of double-stranded RNA on polyacrylamide gels are not understood, and this is a potential source of confusion in drawing conclusions solely from genome profile studies. Early work on the temperature sensitive-mutants of reovirus (Ito & Joklik 1972) showed that a single point mutation is able to induce visible mobility shifts of genomic segments on polyacrylamide gels. Also, our own work using terminal fingerprinting to probe the reasons underlying segment mobility shifts in group A rotaviruses showed quite clearly that degrees of sequence relationship between corresponding RNA segments cannot be inferred from their respective migration rates on gels (Clarke & McCrae 1982). That is, two RNA segments of identical gel mobility can have sequence differences as large as or larger than those of segments that do not co-migrate. Consequently, conclusions about rotavirus grouping based solely on the results of genome profile analysis could result in confusion in the literature, particularly if the number of rotavirus groups continues to rise.

An example of how genome profile data can be misleading is shown in Fig. 2. This compares the genome profiles of the type members of several of the delineated rotavirus groups with that of the adult diarrhoea rotavirus (ADRV) isolates responsible for a large epidemic of gastroenteritis in young adults in China (Hung et al 1983). On the basis of this result, and in the initial absence of serological data on this virus, we tentatively concluded that the profile of ADRV most closely resembled that of the porcine group E virus and therefore ADRV was probably either related to group E, or represented the first isolate of a new group (F). In fact, additional nucleic acid and serological studies have clearly shown that ADRV isolates fall into the group B rotaviruses (Chen et al 1985). Therefore, workers studying non-group A rotaviruses should be encouraged not to speculate on the final grouping of an atypical virus isolate in the absence of data to corroborate genome profile analysis.

To conclude, genome profile analysis is a relatively simple technique that can be applied to quite large numbers of specimens even in unsophisticated laboratory environments. It is certainly reasonable at present to use it as a screen for the initial detection of non-group A viruses. However, it should not be used

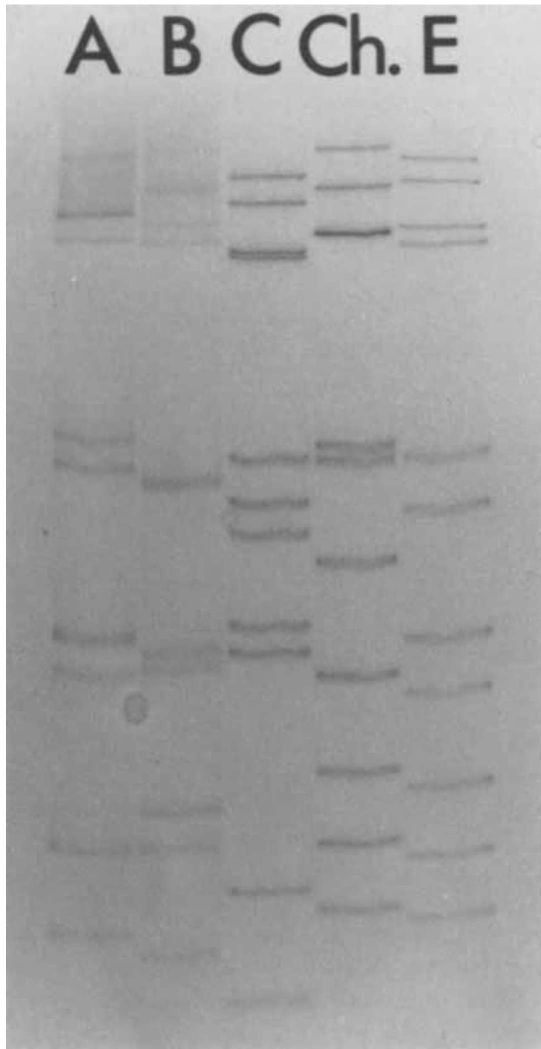

FIG. 2. Comparison of the genome profile of the Chinese adult diarrhoea rotavirus (ADRV) isolate (Ch.) with those of other rotavirus groups.

as the sole experimental criterion by which to assign an isolate to one of the rotavirus groups.

Terminal fingerprint analysis

This technique was designed initially to investigate the level of sequence diversity underlying variations in RNA segment mobility in genome profile

studies (Clarke & McCrae 1982). As a consequence of the results achieved it has become one of the main experimental criteria used in defining different rotavirus groups. Interpretation of the results obtained with any particular isolate is based on the observation, made first on group A viruses, that two levels of sequence conservation exist at the termini of rotavirus genomic RNA segments. At the extreme termini of all the RNA segments is an absolutely conserved sequence of eight nucleotides; internal to this is a region of 35–45 nucleotides which exhibits a segment-specific conservation of G-nucleotide position—that is, the fingerprint pattern of G-residue positions relative to the terminus is diagnostic for a particular RNA segment, irrespective of the species of origin of the isolate (McCrae & McCorquodale 1983, Clarke & McCrae 1983).

The appearance of the non-group A isolates, with their single major change in genome profile, coupled to the lack of group antigen reactivity, posed two epidemiological questions: did these new isolates constitute one or more completely separate gene pools which are unlikely to exchange information with each other, or did they differ radically from group A only in a single gene, namely that encoding the group antigen? Comparative terminal fingerprinting of two non-group A porcine isolates with a standard group A virus clearly demonstrated that these two isolates not only differed radically in terminal sequence from the group A isolate, but also differed from each other. These results, together with corroborating serological data, led us to propose a revised grouping system for the rotavirus genus in which virus isolates sharing both the group antigen (demonstrable by indirect immunofluorescence) and the terminal fingerprint pattern of their genomic RNAs were considered to be in the same virus group (Pedley et al 1983). We have subsequently extended this type of comparative study to include further non-group A viruses and at present the number of clearly defined virus groups stands at five, A–E (Pedley et al 1986).

Despite the successes achieved with terminal fingerprinting, at its inception it was regarded as a first-generation assay procedure and as such has a number of shortcomings. First, it is labour intensive and requires considerable technical expertise and experience in nucleic acid handling, and also access to a well-equipped laboratory with a ready supply of the requisite radioisotopes. All these factors strongly militate against the widespread dissemination of the technique, particularly to more primitive laboratories in the third world. A second and possibly more fundamental problem in the longer term concerns the universality of the basic observation on which the technique is founded. It was always conceivable that the conservation of near-terminal fingerprint patterns observed for the group A rotaviruses would not hold true for the other groups, thereby undermining the use of the technique to define virus groups. Our comparative fingerprinting of the first two isolates that fell into a group other than A (group C) was reassuring on this point, since it confirmed an apparent conservation of near-terminal pattern in group C viruses (Pedley et al 1983).

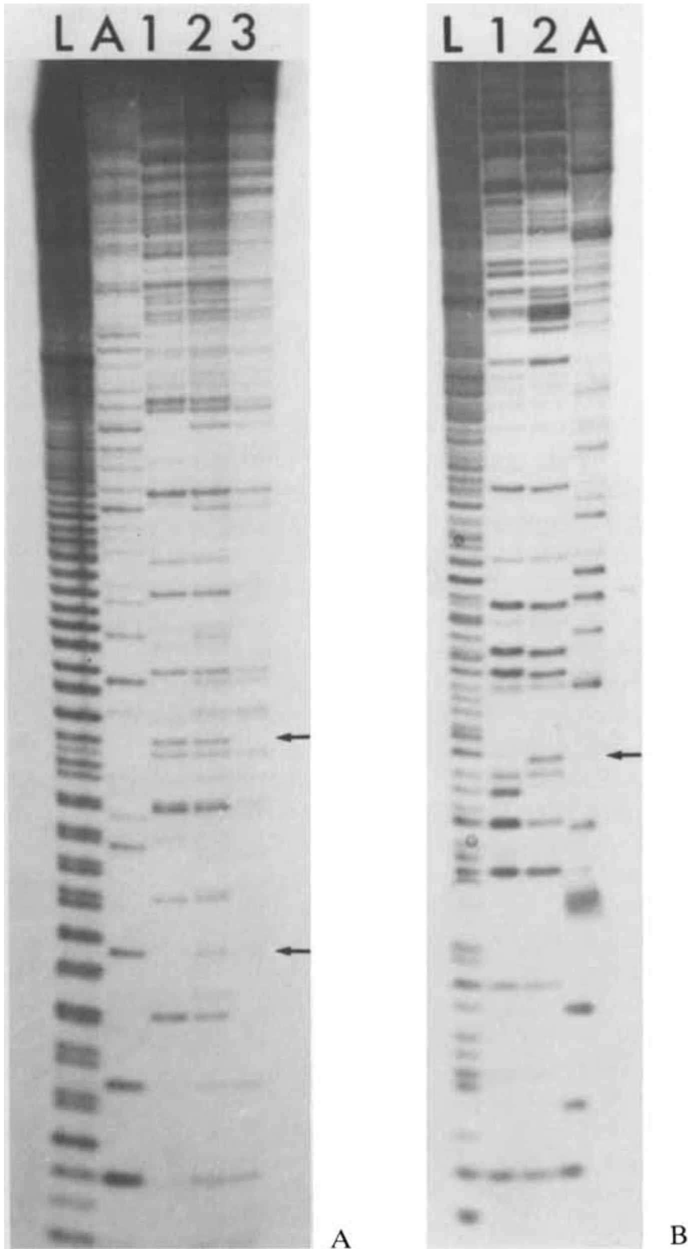

FIG. 3. Terminal fingerprint analysis of different virus isolates from the same rotavirus group.

(A) Comparative fingerprints of genome segment 8 of three independent isolates of ADRV (group B), made at distances of greater than 1000 kilometres from each other.

However, as time has progressed and with the increasing number of non-group A isolations, we have been able to do further intra-group comparative studies. These have shown (Fig. 3) that in groups B and E some minor variations of pattern within the segment specific conserved regions do occur, and these are indicated by arrows in Fig. 3. At present these variations have not been on a scale that prevents the unequivocal assignment of a fingerprint pattern to a particular RNA segment from a particular virus group; but we have had access only to very few virus isolates from each group, and as more isolates become available for analysis the value of fingerprinting for virus group determinations needs to be continuously monitored.

Nucleic acid hybridization-based assays

Hybridization-based assays are potentially the most useful of all the nucleic acid-based approaches to both the characterization and the epidemiological surveying of rotaviruses, and it is in this area that our work and that of others has become focused. Two basic types of assay procedure are being developed. First there is the 'dot-blot' type of assay, which is designed to allow the simultaneous screening of large numbers of virus isolates for the presence of viral sequences related to the particular hybridization probe being used. Using this approach on group A rotaviruses we have developed an assay protocol in which molecularly cloned cDNA copies of genomic segments are used as probes for the presence of particular genomic segments or groups of segments in a viral isolate (Pedley & McCrae 1984). A basically similar procedure, but not one using molecularly cloned probes, has been independently developed by Chanock and his co-workers (Flores et al 1983). The practical advantage of using molecularly cloned copies of viral genes as probes is that they are 'cleaner'; that is, they are much less likely to contain labelled nucleic acids that will cross-react with non-viral sequences in the extracted faecal specimen and thereby generate a false-positive result. Cloned cDNA probes are also more amenable to tagging by stable non-radioactive procedures and, of course, if these assays are to be widely disseminated and used in underdeveloped and remote geographical locations, non-radioactive probe/hybridization detection procedures are essential. Problems with the currently available rapid screening dot-blot assays of faecal extracts do remain, the majority being concerned with variability in the amounts of virus present in individual faecal specimens and variability in the signal/noise ratio on the dot blot, making discrimination between a weak positive and a

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Track L: partial alkaline hydrolysis ladder track to denote base position. *Track A*: terminal fingerprint of the corresponding genome segment from a group A isolate for comparison.

(B) Comparative fingerprints of genome segment 10 of two independent porcine group E isolates. Other track designations as in (A).

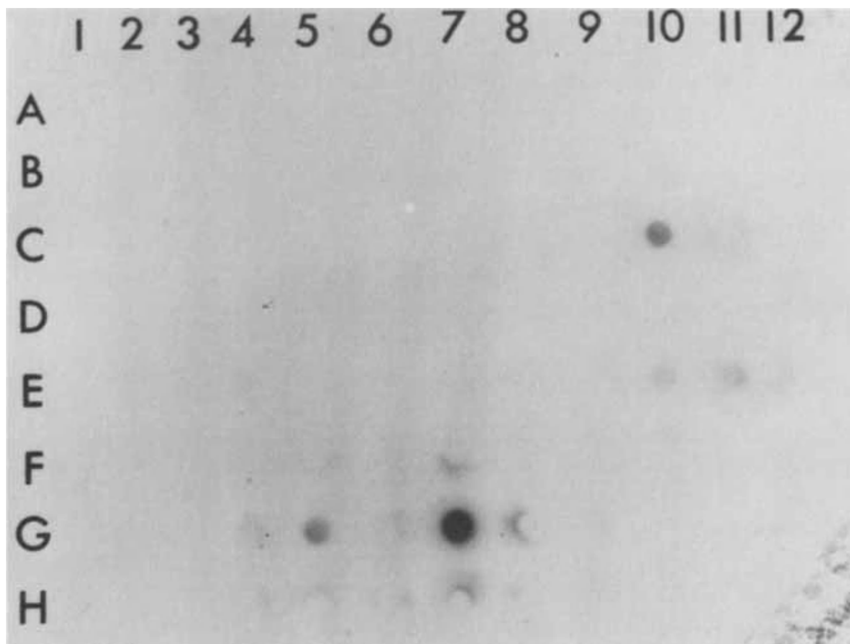

FIG. 4. Dot-hybridization screening of 96 different faecal specimens for the presence of group B rotaviruses. Samples were prepared, applied to nitrocellulose using a 96-well hybridot manifold (Bethesda Research Labs.) and screened as described by Pedley & McCrae (1984). The probe used was a mixture of cDNA clones from four segments of the Chinese adult diarrhoea rotavirus (ADRV) isolate.

strong negative difficult. This problem can be partially solved by pre-grouping specimens containing similar amounts of virus (S. Pedley & M.A. McCrae, unpublished observations) and by comparing signal strength with different probes. Despite these technical difficulties, some of which have only been partially solved, the 'dot-blot' approach offers the way forward for the rapid screening of large numbers of clinical specimens for the presence of non-group A viruses. An example of the type of result that can be achieved is shown in Fig. 4.

The second assay procedure uses Northern blot hybridization, in which the genome RNA segments are fractioned on a one-dimensional gel (genome profile analysis) followed by electro-blotting on to DPT-activated paper to which the RNA binds covalently, giving a facsimile copy of the original genome profile (Alwine et al 1979). This copy can then be hybridized with a variety of radioactively labelled probes to detect particular viral sequences. This technique has the advantage that because of the sharp bands produced on gels by double-stranded RNA, in contrast to the dot-blot assay it is practically feasible to use RNA probes generated by direct labelling of extracted faecal specimens

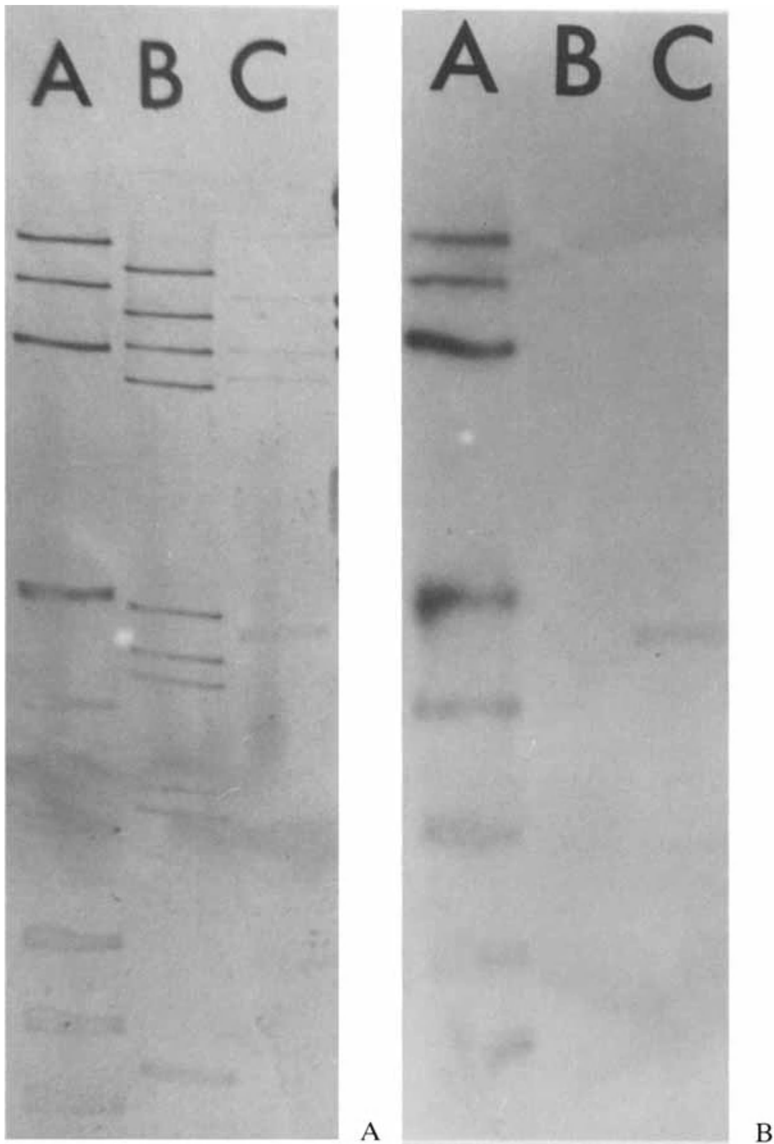

FIG. 5. Northern blot hybridization analysis of three non-group A virus isolates.
(A) Gel showing the genome profiles of the three virus isolates under study. *Track A*: genome profile of the Chinese ADRV isolate (group B). *Track B*: genome profile of the type isolate (C/Cowden) from group C. *Track C*: genome profile of the rat virus isolate of Vonderfecht et al (1984).
(B) Autoradiogram of gel shown in (A) after Northern transfer and hybridization with ^{32}P-labelled ADRV virus RNA.

without hybridization to other nucleic acid in the samples completely obscuring the result. This type of assay, which logistics dictate would be applied only to the more 'interesting' samples picked up in genome profile or dot-blot screening, permits a range of comparative studies to be done on the samples under test. Thus, using whole genome RNA probes, the sequence relationships of different isolates can be investigated, which provides an attractive alternative nucleic acid-based experimental criterion to terminal fingerprinting on which to base the group designation of particular isolates. An example of the type of result obtained using labelled genomic probes in this type of assay is shown in Fig. 5. From this it is clear that group C viruses do not cross-hybridize with any of the genomic segments of ADRV (group B), whereas the rat isolate made by Vonderfecht et al (1984) does show homology with some segments of the group B virus.

As cloned cDNA probes for the genomic segments of the different rotavirus groups become available, more quantitative measurements of the extent of sequence relationship between corresponding genomic segments from different isolates will also be possible.

Concluding remarks

The non-group A rotavirus field is still at a very early stage in its development with many questions posed, for only a few of which do we have even partial answers. Clearly, the main drive in the near future needs to focus on answering two major interconnected questions. First, how common and widespread are non-group A infections in humans and in domestic livestock? That is, what is their contribution in their own right to the overall problem of acute viral gastroenteritis in human and veterinary medicine? Secondly, how distinct are they from their group A counterparts? In other words, in the development of vaccines to combat group A viruses, what cognizance needs to be taken of the possibility of intergroup reassortants emerging under the selective pressure of a group A vaccine? Complete answers to these questions will obviously take time to achieve and will demand both serologically based and nucleic acid-based studies.

In the nucleic acid area, genome profile analysis is still the technique in widest use and will continue to be a major route by which non-group A isolates are first identified. However, the limitations of the technique are such that workers using it must be strongly encouraged either to do more definitive analyses themselves, or to collaborate with laboratories that can undertake such studies, before drawing conclusions on the virus group of a new isolate. This situation will be helped by the wider dissemination of recombinant DNA-generated reagents for use in dot-blot-based screening assays. There is also an acute need for the production of reagents for use in rapid serological assays of the ELISA type, particularly for doing large antibody screens of human and animal populations,

as a guide to focusing the virus isolation work. Recombinant DNA studies on the expression of viral proteins from cloned cDNA copies of non-group A virus genes are an attractive route to providing the large amounts of viral antigen needed for such procedures to be used on a wide scale.

The Northern blot hybridization type of assay, using either whole genome RNA probes or cloned cDNA probes as the nucleic acid-based criterion for establishing the virus grouping of a particular virus isolate, is an attractive alternative to terminal fingerprinting. This is particularly true if the number of new 'interesting' virus isolates continues to grow at its recent rate, since the hybridization techniques are considerably less labour intensive than fingerprinting. Hybridization technology does have its limitations, particularly when unfractionated RNA probes are used, as they will be in the first instance. Therefore in the immediate future it would still be prudent to subject any isolate thought to be outside the existing virus groups to terminal fingerprinting before drawing final conclusions as to its grouping.

At the less immediately applied level of achieving a more complete characterization of the type member of each virus group in its own right, recombinant DNA technology has a great deal to offer. With the exception of the group D isolate, none of these non-group A viruses has been adapted to growth in tissue culture. In the absence of tissue culture growth, definition of the proteins encoded by the different viral genes, elucidation of the coding assignments of the various viral genes, and so on, will all rely heavily on exploiting cDNA clones that can be generated from viral double-stranded RNA extracted directly from infected faeces. Work in this area has begun in our laboratory and partial cDNA clones for a number of the ADRV isolate genes are already available. Such cDNA clones can be of use both at the practical level in epidemiological studies and at the fundamental level of making a more detailed characterization of these viruses, and effort should therefore be focused on their isolation from the type members of each virus group.

To conclude, the importance of the non-group A rotaviruses to the overall medical and veterinary problem caused by acute viral gastroenteritis remains undefined, but the fact that members of one group (B) have already been shown to cause large epidemics of gastroenteritis in China (Hung et al 1984) means that they certainly merit further attention.

Acknowledgements

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DISCUSSION

Holmes: In your Northern blot hybridization studies of the Chinese rotavirus (ADRV), using your total genomic probe, how many segments do you think show sequence homology?

McCrae: In my view, the Chinese and the rat virus showed strong homology only in the 5–6 region. If we expose the gel for long enough we see some homology in the 1–4 region. One problem with interpreting this technique is that ideally one wants identical amounts of RNA in the two gel tracks being compared, and there was insufficient material for us to run a number of gels to achieve this balance.

Holmes: How do you control the amount of RNA, and also the stringency of hybridization?

McCrae: Controlling the stringency of hybridization is not difficult. The stringency set in that study was 70%. Controlling the amount of RNA is more difficult. One disadvantage of the dot-blot hybridization technique is the background noise. Analysing faecal material, which may have very variable numbers of virus particles, by hybridization techniques can give problems. We get round these to some degree by doing particle counts before extraction, which is a lot of work, or by using a method rather like an immunodiffusion assay. The extracted nucleic acid is put in a well and allowed to diffuse out. We stain with ethidium bromide, to get a halo of fluorescence. The intensity of fluorescence correlates rather well with the amount of double-stranded RNA in the sample. It is not an absolute measure but it allows a large number of samples to be put into roughly equivalent groups for hybridization.

Woode: A problem with the group A rotaviruses is that serotype specificity is not reflected directly in other biological properties of the virus, such as host specificity and virulence. Is there any way, using molecular biological techniques, in which one could correlate the host specificities of the group B human or pig viruses with factors other than their serotypes?

McCrae: Not in the short term. If one makes the analogy with poliovirus, the difference between vaccine virus and that from a vaccine-associated case of polio that is clearly virulent in humans is 11 nucleotide changes in an approximately 7500-base pair piece of nucleic acid. In the analysis of non-group A viruses we are still at the stage of setting up techniques for diagnosis and initial identification. So the new technology hasn't much to offer yet by way of predicting that an isolate will be virulent or not. Those differences are likely to be too subtle for present methods. We can take the cloned cDNAs and sequence them, or look for sequence differences, but both those things are a lot of work and require many isolates where the virulence is known. I can't think of a human or animal system, other than poliovirus, where it will be easy to do

such studies, because it would not be easy to get biologically well-characterized isolates.

Greenberg: You discussed hybridization of your group C genomic probe to both group C and group A RNA, and your group A blot looked positive. Was that due to the background, or if you did a true Northern blot, could you, at a low level of stringency, show cross-hybridization of your non-A probe to the group A genome? Is there sequence homology between group A and C rotaviruses?

McCrae: If hybridization stringency is set at 70%, it is a non-specific result, or backgound noise. That is the problem with that sort of hybridization assay. If the stringency is lowered to 50% you start to see cross-hybridization. The question is how different two genomic segments have to be before we consider them different. We use 70% stringency for this reason.

Greenberg: We have done similar studies with two group A rotaviruses, the avian and mammalian group A viruses. At a hybridization stringency of 60% there is no cross-hybridization in a Northern blot, for any genes. If we do a Western blot with hyperimmune sera for the same two specimens, there is total homology: that is, every structural protein of an avian virus appears to be antigenically related to every structural protein of the mammalian virus. At the same time, with relatively non-stringent conditions, their nucleic acid seems to be unrelated. So you can have a close antigenic relationship with very small amounts of nucleic acid homology.

Wadell: Is there any advantage in performing hybridization at different levels of stringency?

McCrae: Yes: if you vary the level of stringency, you are asking different questions. If the stringency level is set at 95% you are asking a different question from when you set it at 50%. But because the atypical rotaviruses will not grow in tissue culture, we have the problem of generating diagnostic reagents, so we are not trying to look for these subtle differences in genomes yet, but are aiming to set up technologies for simple diagnosis, to ascertain how important these new rotaviruses are in relation to disease. The experience in China suggests that they can be important in humans, but we also have anomalous situations: for example, the problem of why, given the prevalence of group B antibody in pigs in the UK, we don't see the group B rotavirus spreading into humans in this country. It is difficult to do those studies because we cannot generate the necessary reagents. We have no ready source of antigen, to allow us to do antibody surveying, which is the first technique to apply on a wide scale.

Wadell: When that work has been done, would you see an advantage in performing restriction enzyme analyses of cDNA clones, to get information on relationships without sequencing?

McCrae: The evidence from group A rotaviruses is that restriction enzyme profiles of cDNAs derived from particular RNA segments aren't very helpful.

For the glycoprotein-encoding segment of group A rotavirus RNA, for which many isolates have been sequenced, the restriction enzyme patterns of the RNA segments are very different from each other, yet the sequence homology between them is 80%. When people obtain full-length clones, it will be almost as quick, and as definitive, to sequence them. The longest segment is only 3kb, so it's a problem on a different scale from, say, adenovirus, with 36kb to be sequenced.

Flewett: Why would one expect that a virus which is virulent in pigs would also grow in humans? We would need to know much more about receptors than we do, to predict this. The foot-and-mouth disease virus grows in several animal species but the total number of human cases reported is only about 30. African swine fever has not got into humans, either: just as well!

McCrae: In China you have a rotavirus in pigs that serologically is related to the virus occurring in humans. I agree that they may have differences more subtle than we can detect at present, but on present evidence it is hard to see why it has spread to humans in China and not in the UK or in Europe.

Saif: Have you done Northern blot hybridization comparing the Chinese human group B rotavirus with the swine group B, either a UK isolate or a USA one?

McCrae: Yes. The UK isolate cross-hybridized for all segments with ADRV. We have not done the Ohio isolate, yet.

Flewett: Has anyone managed to infect pigs with the Chinese human virus?

Bridger: I attempted this, but the Chinese virus failed to multiply and there was no seroconversion in the pigs: but I cannot be certain the virus used was viable.

Snodgrass: We used two human rotavirus strains from gastroenteritis patients in Edinburgh with a group C genomic profile. Gnotobiotic piglets were inoculated orally with fresh faecal suspensions, and no diarrhoea, rotavirus excretion, or other evidence of infection was detected (Snodgrass et al 1984).

Bridger: We tried to infect pigs with a human group C rotavirus, but without success. It was from Brazil, so again there was the question of its viability.

Greenberg: When atypical viruses are put on monolayers and stained, do you get at least abortive infections with these non-cultivable viruses?

Saif: Up to 60% of our MA104 cell monolayers become infected in the first cycle of viral replication, but there was no productive infection, using our group C (swine) rotavirus. Dr Theil and I have done this with a group B (swine) rotavirus, and saw syncytia formation in MA104 cell monolayers (Theil & Saif 1985), but this was not found consistently with all or even the same group B rotavirus isolates.

Greenberg: Has this been investigated with the human atypical rotaviruses?

Bishop: We tried to grow the group C human virus, using techniques that have been successful with group A rotaviruses, but we were unsuccessful.

McNulty: We obtained abortive infections with avian rotaviruses which belonged to serogroups other than A and D. This was the basis for assigning them to different serogroups, but the number of infected cells was very low (McNulty et al 1984).

Bridger: In our studies with Dr McNulty's viruses it was difficult to find positive cells.

Saif: Two points seem to have been useful in getting even the abortive infection. We have used a cell-culture immunofluorescence test, and the first point is to treat the virus-inoculated MA104 cell monolayer with proteolytic enzymes, as was effective with group A rotaviruses. We have used pancreatin. Also, we can't get abortive infection unless we centrifuge the viral inoculum onto the cell monolayers (Bryden et al 1977).

Bishop: Have people restricted themselves to MA104 cells, or have a variety of cell lines been tried?

Saif: We tried primary swine kidney cells and primary bovine kidney cells. Dr Theil tried primary African green monkey kidney and Vero cells and could not establish an infection, using the conditions used successfully with group A rotaviruses.

Greenberg: Dr Bishop, with human group C, did you get an abortive infection, or no infection?

Bishop: We produced an abortive infection. Any success we may have had was later obscured by overgrowth of a group A rotavirus also present in small numbers in the inoculum.

Holmes: One of my students, H.S. Nagesh, has tried primary pig kidney cells and several other cell lines to get a pig atypical virus (group unknown) growing in culture, unsuccessfully. He has tried various proteolytic enzymes and also heparin treatment of the cells, rather than centrifugation, to make the viruses stick to them. None of these devices appeared to work, but we didn't have a good antiserum with which to look even for immunofluorescence.

Bridger: In Dr McNulty's successful avian virus cultures the immunofluorescence seems to be much fainter than we find with group A rotaviruses, and one has to examine cultures at higher magnification (400×) than with group A. I wonder what Dr Saif finds with her successful abortive culture of group C: is immunofluorescence less bright, and do you need higher magnification to see it?

Saif: The immunofluorescence is weaker than with group A. This may relate to how good the antiserum is.

McNulty: The titre of the antiserum is important. With some of the more recently discovered avian serogroups the titres of available antisera are low.

Bridger: Good group A immunofluorescence can be obtained with a convalescent-phase serum; I wonder, having worked with your viruses, whether we have been looking carefully enough, at high enough magnification, for faint fluorescence.

McNulty: We normally use a ×40 objective with a numerical aperture of 1.30. This is important in detecting faint fluorescence.

Bishop: Perhaps the lack of success in culturing novel rotaviruses is partly because the virus is often excreted in small amounts. To what extent does the tendency of these atypical viruses to 'fall apart' in faeces play a part? Is the fragility of novel rotaviruses real, or an artifact of staining?

Flewett: There is something odd about the Chinese (group B) virus, that you can take it apart very easily, simply by exposing it to phosphotungstate. We had great difficulty with the Chinese virus at first. Then Hiroshi Suzuki, an advocate of uranyl negative stains, showed lots of particles by uranyl acetate staining when he was working with us. What is the chemical difference between the Chinese virus and the typical rotaviruses, which remain intact in phosphotungstate? There must be a difference in the protein linkages holding the virus together.

Horzinek: Was the uranyl acetate buffered, or just an aqueous solution? If the latter, then the stain is very acid, which may account for the better demonstration of the Chinese virus.

Flewett: The uranyl acetate is certainly acid, about pH 3.84. If you take it up to 4.5 it all comes out of solution, so you can't buffer it to neutrality, but in the faeces the virus is almost neutral, so pH cannot be the whole explanation. (*Note added after the symposium*: Chinese virus is disrupted by phosphotungstate at pH levels down to pH4: H. Suzuki et al, to be published.)

Saif: We have two different strains of swine group B rotavirus: Ohio and N338. One (Ohio) seems to be very labile and to behave like the Chinese (group B) virus in that we usually see viral cores on electron microscopy, whereas with the other strain (N338) we see complete double-shelled particles similar to group A rotaviruses: so there may be strain differences in stability within the group B viruses.

Bishop: And do you feel that the appearance is unrelated to the staining methods?

Saif: Yes. We used the same stain, 3% phosphotungstic acid, pH7, with both group B rotaviruses.

Kapikian: This issue of which stain was used to diagnose rotavirus infections by electron microscopy is very critical, especially since much electron microscope (EM) work has relied on phosphotungstate. A review of two of the largest and longest cross-sectional studies of hospitalized paediatric patients with diarrhoeal illnesses in the USA (Brandt et al 1983) and in Japan (Konno et al 1983) reveals that practically all of the EM-positive rotavirus specimens tested were also positive by ELISA (Brandt and Konno, personal communications), thus indicating that the non-group A rotaviruses were probably not important as aetiological agents of severe paediatric gastroenteritis. It is of interest that in the USA study the negative stain used was phosphotungstate, whereas in Japan it was uranyl acetate. Those who are working actively on the

non-group A rotaviruses who suggest that these agents are of greater medical importance than some current studies indicate use two main arguments to support their point of view: (1) these agents are shed in small amounts in the stool and thus may be missed by conventional electron microscopic examinations. Is this also the case in animals infected with non-group A rotaviruses? And (2), as we have just heard, the lability of non-group A rotaviruses to phosphotungstate staining may have impeded the detection of these agents in previously described studies. I am not convinced yet that a child admitted to a hospital with a severe diarrhoeal illness caused by a non-group A rotavirus sheds so little virus in the stool that EM detection is not possible. We need an efficient serological test, to help answer the question about the importance of non-group A rotaviruses in severe paediatric diarrhoea. If such a test were available, paired acute and convalescent sera from children admitted to the hospital with diarrhoeal illness could be examined for a serological response to the non-group A rotaviruses. This could be done with existing paired sera from previous cross-sectional studies and would quickly elucidate the role of these agents.

Bishop: Dr Flewett, is it only phosphotungstate that produces this effect?

Flewett: Yes. You must avoid phosphotungstate. Uranyl formate is all right. We haven't tried silicotungstate or borotungstate. Ammonium molybdate may be all right: as far as I understand it, Dr Suzuki has done almost all the electron microscopy for Dr Konno's group in Sendai. He has been using uranyl acetate as a negative stain for a long time: had there been a lot of atypical rotaviruses around, he would have picked them up.

Bishop: How consistent is the finding that only small amounts of novel rotaviruses are excreted?

Bridger: With the porcine group B virus, NIRD-1, by electron microscopy, levels of virus were extremely low: but we were using phosphotungstate. However, when we send faecal samples to Malcolm McCrae for nucleic acid analysis, he is constantly complaining about the low levels of RNA, which may be a more direct answer! But the group C virus of Linda Saif grows marvellously and produces good levels of RNA. You have studied several different strains?

Saif: Yes. In our experience, group B is shed in lower titre than any of the group A or C rotaviruses. Group C is shed in somewhat higher titre than B, but still less than A.

Bishop: Are these field isolates, or from germ-free piglets?

Saif: This is true of both, in terms of detecting atypical rotaviruses from naturally infected pigs, and also after passage through gnotobiotic pigs.

McNulty: What is known about the epidemiology of atypical rotaviruses in pigs? Has Dr Saif done longitudinal surveys on pig farms, looking at litters sequentially, to see what rotaviruses are being excreted? There is a tendency to look for viruses in diseased animals or humans, but if these atypical rotaviruses

are not important in terms of producing disease, we should be looking at healthy individuals. We took this approach in chickens and we found four different serogroups. Has anything similar been done in mammalian species?

Saif: We have done limited surveys of atypical rotaviruses in swine, particularly weanling swine. As I mentioned, we find the highest incidence of group A, then group C; the least prevalent are the group B rotaviruses. We have done only a few non-diarrhoeic pigs and we need to examine more of these. We have followed some litters through, sampling twice per week, and in these the order of prevalence is also A, then C, and very few group B.

McNulty: What is the prevalence of antibodies to group B in pigs in Ohio?

Saif: Dr Theil and I published a survey of group A and B antibody prevalence in a limited number of adult swine. Prevalence was lowest for group B rotaviruses (Theil & Saif 1985). My student and I found similar figures to ones that Dr Bridger reported for the prevalence of group C antibodies in swine in the USA (Terrett et al 1985).

Bridger: Your figure for group B was 23% (Theil & Saif 1985) whereas we found 86% positive from pigs of all ages, so there may be differences between the UK and USA. The prevalence of group C antibody was similar in both countries.

McNulty: There was no apparent difference in the age of acquisition of antibody, which suggests that the epidemiology of the atypical rotaviruses is much the same as that of group A rotaviruses.

Bridger: There was a shift to older ages of pigs for group E. Dr Debouck et al (1983) followed the excretion of groups C and A in litters of swine and showed that group C was very common in Belgium in pigs over three weeks of age, but not particularly associated with disease. This is similar to what you found in chickens; that is, that group C was there, as part of the normal gut flora.

Greenberg: Is diagnosis by silver staining of RNA on polyacrylamide gels as sensitive as is needed? If faecal samples were screened using that technique, would it be an adequate epidemiological tool? This is now a rapid and simple technique.

McNulty: We used both electron microscopy and the silver-staining technique and found good agreement between the two sets of results.

Bishop: We need to decide what diagnostic test to recommend to a laboratory interested in the epidemiology of rotaviruses, but not necessarily in sophisticated research.

Holmes: We have found that workers in Bangladesh and in China have readily been able to set up the RNA extraction, electrophoretic technology needed for the electropherotyping technique. The sensitivity of silver-staining techniques compares well with that of electron microscopy. Some claim that it compares equally well with ELISA, but this may be because many people are doing ELISAs that are not as sensitive as the WHO assay. Even there, the loss of sensitivity is important only in the weakest samples, which are only a small

percentage, if you are looking at acute disease or epidemics. So the electrophoretic technique seems to be the way to go at the moment, and if Malcolm McCrae can produce enough mixtures of nucleic acid probes for the different serogroups, then for large numbers of samples, dot-blot hybridization will be the way of the future: but it will have to be done with non-radioactive probes, because the short half-life of ^{32}P-labelled probes creates a considerable problem in many places.

McCrae: I agree about the labelling of probes. Our experience is that dot blots are not very useful. What I tried to emphasize is that for detecting non-group A viruses, genome profile analysis is a valuable technique, but many people are trying to take the extra step to say which group of virus they are dealing with. This is not valid, in my view, but it is difficult to discover a methodology that allows people to do serogrouping, in their own laboratories. At present there is no technology where the reagents are available for grouping.

Flewett: The reagents could be developed, given an adequate supply of antigen. What is needed is a good set of group-specific monoclonal antibodies. Unfortunately, when monoclonals are made against rotaviruses, you usually get a group-specific monoclonal, simply because the group-specific 46000 molecular weight antigen, in the group A rotaviruses at any rate, is a strong and abundant antigen, so most B cell clones will be producing antibodies reacting with that antigen. If people would attempt to make group-specific monoclonals for the groups of atypical rotaviruses we might have a set of reagents that could be disseminated for general use, and people should be able to pick up these atypical viruses. The ELISA test can be made extremely sensitive, ten times more sensitive than the WHO test, very easily, by using tetramethylbenzidine as a substrate.

*McCrae:*The expression of cloned copies of the non-group A virus genes in heterologous systems is one way of making large amounts of viral antigen. The first test one wants is an antibody-screening test, which requires a ready supply of antigenic material. It doesn't need to be whole virus. We have achieved tentative expression of one of the cDNA clones from the Chinese virus in *E. coli*. Once the viral protein is expressed in this system, you can have a limitless supply of antigen.

Bishop: Provided it's the right antigen for the detection of group antibody. Do you know this?

McCrae: We don't know that, but that is also true of monoclonal antibodies: there's no reason to believe, as Tom Flewett pointed out, that any group B monoclonal antibody will be group specific. It may well not be.

Bishop: Is anybody at the stage of knowing which is the key gene that codes for group antigen of atypical rotaviruses?

McCrae: No real protein analyses have been done on the non-group A viruses. They are so 'dirty' when we get them, and you can't propagate them:

their purification and their analysis on gels would be very difficult.

Appleton: How do you treat faecal specimens for the dot-blot technique, for use as a diagnostic test for screening large numbers of specimens?

McCrae: Direct phenolic extraction of infected faeces can be done on a small scale (100 μl of infected faeces) quite easily. The extracted material often contains particulate matter which will clog the nitrocellulose filter. So we do a simple salt precipitation of the phenolic extract, making it 2M for lithium chloride, which precipitates almost all the protein and single-stranded nucleic acid. The double-stranded nucleic acid stays in solution, so you spin that down (the whole procedure can be done in a microfuge tube). The supernatant now filters well onto nitrocellulose. Unfortunately, the salt precipitation technique requires an overnight incubation for good precipitation, which is a disadvantage from the diagnostic point of view.

You can directly 'dot' the RNA sample onto nitrocellulose but you can't put much on without a lot of spread, and it is slower than using a commercial manifold.

Bridger: An ELISA test has been described for group C viruses which Debouck et al (1983) used for their epidemiological study. For the rat virus, an ELISA test has also been described (Vonderfecht et al 1985). The titres that we obtained to groups B and C in polyclonal sera are in the range of tens of thousands, so one should be able to set up ELISA tests for these viruses.

Flewett: This is essential, before one makes monoclonal antibodies. A test is needed to tell one if specific antibodies are being produced.

Hung: We have now found another novel rotavirus in China, in Hunan Province. Children as well as adults were affected. The epidemic was sporadic. There were about 60 registered cases. Patients manifested with symptoms of acute gastroenteritis, watery diarrhoea associated with abdominal cramps, and vomiting, but milder symptoms than those in ADRV. Morphologically the virus looks like a typical rotavirus, but the RNA profile is distinct from both group A and group B profiles. Immuno-electron microscopy showed aggregation of virus particles with convalescent sera. We got only very few faecal samples (three), one from a child, one from a 14-year-old adolescent and one from an adult. We did RNA hybridization with cDNA from ADRV. There was no cross-reaction to groups A and B, so this virus is distinct antigenically and genetically from both ordinary rotaviruses and our ADRV (Fig. 1).

Holmes: You should also check against a group C antiserum, because the RNA electrophoretic pattern looks very like those of previously identified group C rotaviruses.

Hung: (*comment added after the symposium*): We have now tested using a serum against group C rotavirus supplied by Dr Bridger, which cross-reacted with all three isolates on counter-electrophoresis.

Bridger: I don't disagree at all with Malcolm McCrae's general point, but perhaps with some groups one can get some idea of serological specificity by

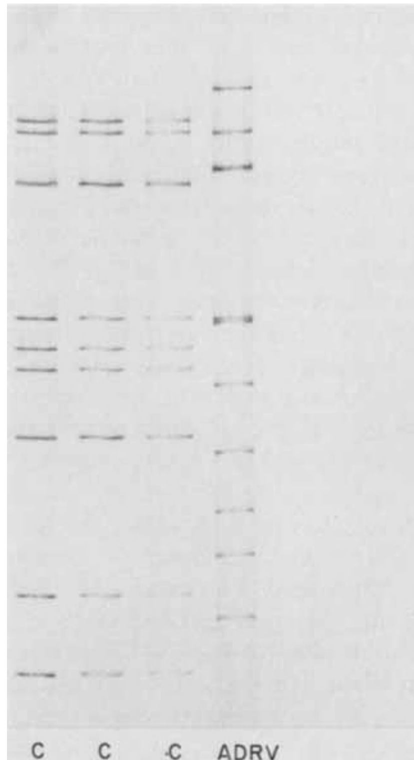

FIG.1 (*Hung*). RNA profiles of ADRV and of putative group C viruses from three diarrhoea patients (aged one, 14 and 35 years) from Hunan Province. (G.M. Chen et al, unpublished material.)

genome profile. We are saying that group A has the triplet in its RNA profile. Perhaps group C has a pattern with a more widely spaced triplet? We have looked at five different strains, all in the serological group C, and they all had a similar pattern. Perhaps there are some groups, such as group B, where it may be difficult to predict serogroup from their genome profile. Some of the five were from pigs and some from humans: they all had the sort of pattern that Dr Hung Tao was describing. Are we saying that, for group A, we can predict serogroup from RNA profile, but for the novel groups we cannot? If so, are we being inconsistent? Perhaps it is just some of the groups, such as B and E, where you have to be careful.

McCrae: There is no reason not to play hunches and one would be foolish not to do so. From Dr Hung Tao's RNA profiles of these recent isolates, like Ian Holmes I would guess that this is a group C virus, and one would want to check that possibility by hybridization or fingerprinting. The problem is that creeping

into the literature is this terminology that people are dealing with, say, a group C virus, when they may have something else. That is the caution I want to stress.

Bishop: Maybe it is now time for those with experience of the non-group A rotaviruses to provide guidelines about what to do next after identifying a possible novel rotavirus.

McCrae: For a start, is our suggested nomenclature acceptable? And what experimental criteria are going to be set to define the different groups? Agreed criteria seem to be desirable, particularly as we shall probably find additional groups. The rat rotavirus is being commonly referred to as a group B virus, yet on hybridization analysis we find only limited homology for some of the segments.

Bishop: Everyone appears to be happy with the nomenclature. Some general guidance on criteria for determining grouping seems desirable.

Flewett: The confusion over nomenclature has come from people who see new and interesting electrophoretic patterns of RNA and decide to put their virus into a new rotavirus group without further evidence on its serological nature. We must insist on good serological evidence before anybody puts a new virus into a new group. If, after that, something unusual is found on the RNA pattern, the grouping might have to be modified.

McCrae: I would be disturbed if we accepted serological cross-reaction, or lack of it, as the only criterion on which to name groups of atypical rotaviruses. Dr Bridger and I have preferred a dual methodology. It may not be easily transferable to a wide variety of laboratories, but the group antigen is almost certainly a single polypeptide in the virus, and one may well get reassortment.

Greenberg: If one does fluorescence and looks for cross-reactivity among group A rotaviruses, you are not only measuring antibody to VP6, you are detecting cross-reactive antibody to virtually every protein that the virus makes.

McCrae: One can get very weak fluorescence of some groups: so you have to be cautious. In principle I agree but in practice there are difficulties; to take the rat rotavirus from the USA as an example, this doesn't cross-react for many of its genes but does give a serological cross-reaction. What should we call it? This presents a problem where only a small number of isolates have been looked at. So there seems to be a need to decide what constitutes a new group, at least. (See Final General Discussion for suggested criteria, p 250–251.)

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Seroepidemiology and molecular epidemiology of the Chinese rotavirus

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Abstract. The Chinese rotavirus which causes epidemics of diarrhoea in adult humans was isolated in 1983. This virus, designated adult diarrhoea rotavirus (ADRV), resembles typical rotaviruses morphologically and has a genome made up of 11 discrete segments of double-stranded RNA. Because the Chinese rotavirus has a unique RNA pattern on polyacrylamide gel electrophoresis and is antigenically distinct from group A rotaviruses, it is tentatively included in group B. Infection with ADRV or ADRV-related viruses (as shown by serological study) is detected in human populations as widespread as mainland China, Hong Kong, Australia, the United States and Canada, and in some domestic animals. RNA co-electrophoresis has shown homology of isolates from 12 different outbreaks (with some minor variations at segments 10, 11, 3 and 5). cDNA probes and monoclonal antibodies have been prepared to improve the detection and further characterization of the virus.

1987 Novel diarrhoea viruses. Wiley, Chichester (Ciba Foundation Symposium 128) p 49–62

Rotaviruses are now the major cause of diarrhoea in infants and children. In adults, rotavirus infections are said to be rare and subclinical (Flewett & Woode 1978). In recent years, however, the People's Republic of China has experienced several nation-wide outbreaks of non-bacterial diarrhoea. In the stools of adult patients we discovered a novel rotavirus (Hung et al 1983) and designated it adult diarrhoea rotavirus (ADRV), thus distinguishing it from the rotavirus causing infantile diarrhoea. This virus resembles the previously characterized (typical) rotavirus morphologically (Fig. 1), but antigenically is distinct. It possesses a genome made up of 11 segments of double-stranded (ds) RNA. The RNA profile of the virus, however, is unusual (Hung et al 1984a, Wang et al 1985b (Fig. 2).

In collaboration with British colleagues, we used immuno-electron microscopy and indirect immunofluorescence to demonstrate a reciprocal serological

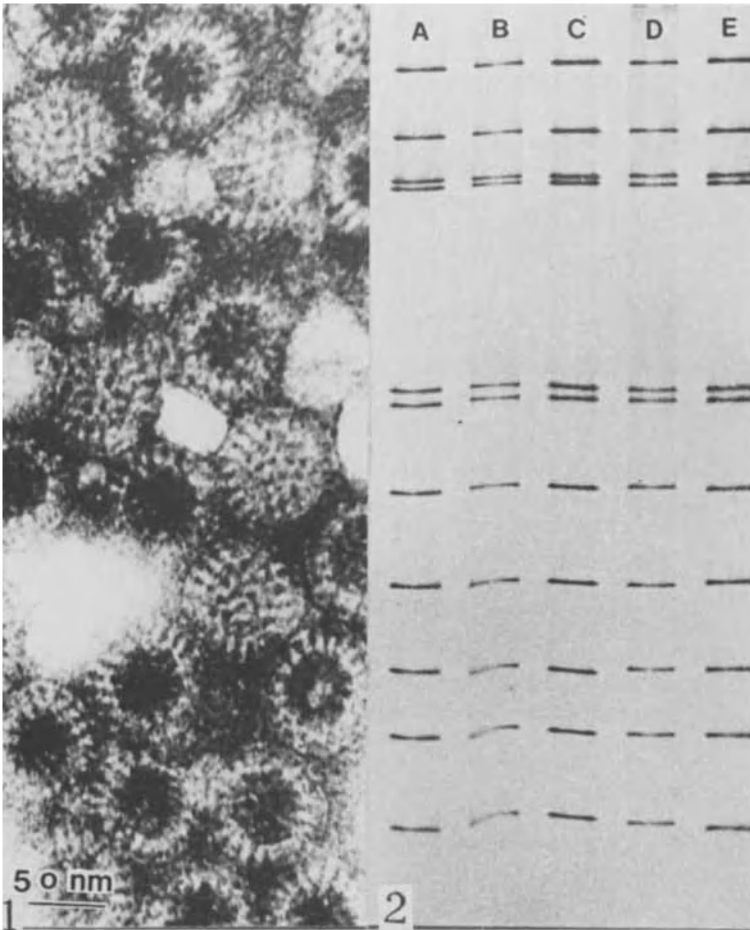

FIG. 1. Negative staining of adult diarrhoea rotavirus.

FIG. 2. RNA profiles of adult diarrhoea rotavirus isolated from five different areas of China: A, Chinchou; B, Lanchou; C, Fuhsin; D, Hunan; E, Guangsi.

cross-reaction between ADRV and the type member (B/NIRD–1) of group B rotaviruses. No cross-reactions were detected between ADRV and viruses belonging to groups A, C, D and E. As an independent corroboration of the serological results, RNA–RNA hybridization was used to show that ADRV and B/NIRD–1 are related in their nucleic acids (Chen et al 1985).

The epidemics of Chinese rotavirus diarrhoea subsided after the nation-wide outbreaks in 1982–1983, but local outbreaks have not stopped. To study the extent of the spread of ADRV infection we did a series of serological

investigations in healthy people, both in China and abroad, and also in domestic animals.

We used a single preparation of ADRV purified from the stools of five patients during the 1983 outbreak. Extracts of all the stools had the characteristic electrophoretic pattern of segmented viral ds RNA (Wang et al 1985a). The negatively stained appearance of the partly purified virus was typical of ADRV, and on immuno-electron microscopy the virus particles were aggregated by rabbit hyperimmune sera against ADRV, but not by hyperimmune sera produced against group A rotavirus. The virus did not react against rotavirus antibody in the standard WHO enzyme-linked immunosorbent assay (ELISA) kit (Hung et al 1984b, Wang et al 1985b).

To evaluate counter-immunoelectrophoresis (CIE) as a method for detecting ADRV antibodies, we tested the same virus preparation against a panel of control sera. The sera were kept at −20°C until use. 15 μl of the serum and purified ADRV were added to the cathodic and anodic wells, respectively, in a 0.8% agarose slab, measuring 10 × 0.25 cm, in barbital (0.1 M)–Tris (0.036 M) buffer (pH 8.6). Electrophoresis was done in the same buffer at 2 V/cm at room temperature for about 14 h. The agarose gel was washed for 10–15 h with two or three changes of phosphate-buffered saline. The reaction was scored immediately and again after drying and staining with Coomassie brilliant blue. A positive reaction was obtained with all six sera from patients convalescing from confirmed ADRV diarrhoea, and with two hyperimmune rabbit sera produced against the virus (Wang et al 1985a). Sera from the two rabbits were negative before inoculation with ADRV, as were sera from two patients with bacterial diarrhoea, two patients with rotavirus diarrhoea, and five pigs recovering from porcine rotavirus diarrhoea. Of sera from 53 human subjects collected 20 months after the diarrhoea episode caused by ADRV, only 25 gave a positive reaction. The method is thus specific for ADRV but may not be sensitive enough to detect low levels of antibody months after recovery from infection.

Using this method, we tested sera collected from healthy people from mainland China, Hong Kong, Australia, USA and Canada and found that infection with ADRV or ADRV-related viruses is widespread (Table 1). Antibodies were detected in 9.5–20% of healthy people from areas of China and other countries where the disease has not yet been reported. The seropositivity rate was higher in Jinzhou (the region experiencing ADRV diarrhoea in 1983) than in other areas and was similar in subjects who had not had symptoms of ADRV infection (41%) and those who had (53%).

Human cord blood sera collected in Buffalo, USA (supplied by Dr Marie Riepenhoff-Talty) were also tested for ADRV antibodies, but only one out of 80 was positive.

Our investigations on healthy people showed that the seropositivity to ADRV or to ADRV-related virus(es) was rather low; this was also true for those recently recovered from the infection. The low seropositivity rate in human

TABLE 1 ADRV antibodies in randomly selected healthy human subjects in various countries

Location	*Outbreaks of ADRV diarrhoea reported*	*CIE positives*
Jinzhou, China	Yes	14/34 (41%)
Beijing, China	No	7/51 (14%)
Shandong, China	No	23/114 (20%)
Hopei, China	No	6/50 (12%)
Hong Kong	No	6/34 (18%)
Australia	No	6/40 (15%)
USA	No	22/202 (9.5%)
Canada	No	5/40 (12.5%)

CIE, counter-immunoelectrophoresis.

TABLE 2 ADRV antibodies in domestic animals in China

Animal	*CIE positives*
House rats	28/60 (47%)
Pigs	73/202 (36%)
Laboratory rodents:	
Wistar rats	6/35 (17%)
Guinea-pigs	1/29
Swiss mice	2/100
Chickens; ducks	2/47; 1/42
Cattle; sheep; horses	0/46; 0/29; 0/5

CIE, counter-immunoelectrophoresis.

populations suggests that the virus could spread easily through a large population.

Among domestic and laboratory animals from different locations in China, ADRV antibody and/or antibodies cross-reacting with ADRV were most frequent in house rats and pigs (Table 2). Antibody was not detected in cows, sheep and horses. However, a positive reaction does not distinguish ADRV antibody from antibodies to antigenically related but not identical agents. Humans are probably an important reservoir for ADRV. Domestic animals may contribute to the natural reservoir, and thus the search for epidemic sources appears to be important. Efforts to detect ADRV from randomly collected diarrhoeic stools by means of a newly developed ELISA test have failed to find ADRV-positive patients so far. Both hyperimmune sera and monoclonal

FIG. 3. Variations in RNA segments of adult diarrhoea rotavirus isolates from different outbreaks, as revealed by co-electrophoresis.

antibodies to the virus have been prepared and an ELISA kit for the diagnosis of ADRV is now available (Wang et al 1986, Yei et al 1986).

Genome analysis designed to see whether variants of ADRV were present in the outbreaks revealed that isolates of ADRV from 12 different outbreaks shared an identical RNA pattern, but minor variations were found by co-electrophoresis at segments 10, 11, 3 and 5 (Fig. 3) in some isolates (Wang et al 1985b). For more specific and large-scale screening of field isolates, we use a 'dot-blot' technique developed by Pedley & McCrae (1984). We employ cloned cDNA copies of ADRV RNAs as probes to detect ADRV or ADRV-related viruses. To give the probe a longer shelf-life, which is especially important in developing countries, such as China or India, we labelled the nick-translated

cDNA with biotin, rather than radioactively. It has not yet proved possible to cultivate ADRV in cells or in laboratory animals, and stool specimens from patients with the acute disease still remain the only source of virus material for study. Searching for cells or animals susceptible to ADRV is now a prime consideration.

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DISCUSSION

Flewett: We also have made an ELISA test, with the idea of screening for antibodies to the Chinese (group B) rotavirus (ADRV). These are all group-specific antibodies, of course. We separated the virions and ensured that the ELISA test was reacting only with the fractions of 1.38 and 1.36 g/ml density. We are reasonably happy that the assay is specific. We set up a blocking test with sera against rotaviruses of various serogroups. We studied various species, animals and man (see Fig. 1). A group of vets was studied, and three out of 110 had antibody to ADRV. We took a 60% point as our cut-off, based on a sample of pigs which mostly had group B antibodies.

The problem is of seeing where to draw the line. If we draw it at 60%, antibodies seem to be rare in humans. Out of 100 blood donors in Birmingham,

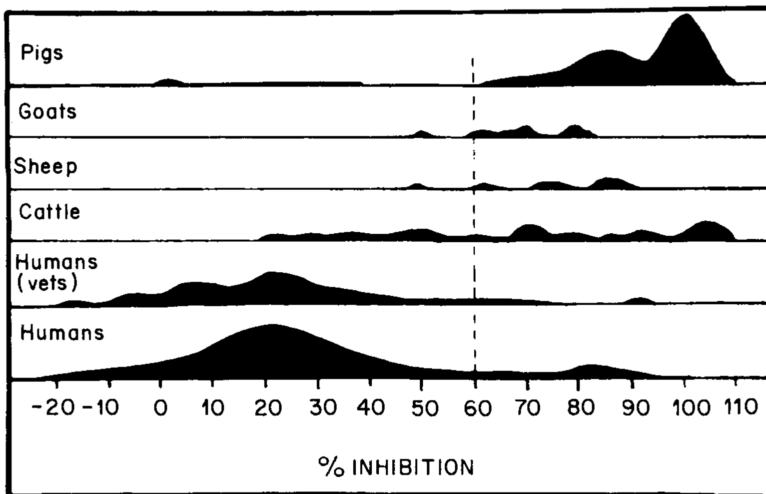

FIG. 1 (*Flewett*). Distribution of sera giving antibody inhibition in various percentages (group B rotavirus antigen).

only 10% had antibody. Goats and sheep have the antibody (91%); of the cattle studied, 71% had it. All the pigs tested were positive, with two exceptions. When there are so few people with antibodies, the question is whether this means that virus is circulating in the population, or that there are occasional importations of virus into a human population, but that virus doesn't then spread. I would guess this picture means the latter. If so, where do these importations come from? Do they come in from other countries? We know that some Chinese people visit the UK, but we have never seen the Chinese virus or anything like it in faecal material. We hardly see any atypical rotaviruses at all. Are these perhaps zoonoses? Nobody knows this, and it is something for the epidemiologists to sort out in the future.

Bishop: Perhaps we could explore the epidemiology a little more directly. You called it the 'adult' diarrhoea virus, Dr Hung. Were children affected also? If so, then how frequently? Was there any evidence of children and their parents being simultaneously infected, and was there spread from parents to children?

Hung: In all the epidemics that we studied, children were always involved, but less severely and less frequently than the adults. We didn't know whether the virus was transmitted from mothers to their children, but I am sure that it could be.

Flewett: I gained the impression, when I was in China, that the distribution of this diarrhoeal infection is almost entirely in the countryside and the villages, and not in the cities. Is that so?

Hung: No, that is not so. The epidemics have occurred everywhere, in the countryside and in cities, and at all times of year.

Kapikian: When you say that children were also infected with the virus, it is important to know the severity of their illnesses. I have the impression that adults develop a more severe disease than children, and that is a paradox which is difficult to explain.

Hung: A severe dehydration occurred in adults, rather than in children. I don't know how to explain this paradox, either.

Kapikian: In earlier studies in China, it was shown that the group A rotavirus infections occurred in the cooler seasons, in temperate regions (Pang et al 1980), but I understand that these atypical rotaviruses (ADRV) do not show that pattern?

Hung: No. There is no distinct seasonal pattern of ADRV epidemics, unlike group A rotavirus outbreaks.

Kapikian: So this virus has very distinct epidemiological features: temporally it is different in its occurrence and the age distribution is paradoxical. Usually one expects children to get the more severe disease, especially since they don't have antibodies to even a related agent. It is a difficult disease to understand, because the epidemiology is perplexing. What sort of illness developed in volunteers who received the atypical rotavirus under experimental conditions?

Hung: It was exactly the same as the natural disease, with very severe watery diarrhoea and dehydration, after an incubation period of about 2½ days.

Kapikian: Would any of these infections have been fatal, if oral rehydration therapy were not available? Was it of that severity?

Hung: No: it is a self-limiting disease, lasting 3–5 days, with spontaneous recovery.

Mathan: It is intriguing that in a country with a similar ecology to China, such as India, we have never seen the adult diarrhoea rotavirus, even though about seven epidemics of acute non-bacterial diarrhoea have been studied. Can you tell me what the seroepidemiological pattern of the group A rotavirus is in the Chinese population, and its prevalence?

Hung: Infantile diarrhoea (caused by group A rotavirus) was first identified in China in 1979, and there have been many clinical, serological and aetiological studies. In Henan province, a group of virologists headed by Dr Fu Bing-Nan have been screening diarrhoea cases, including bacterial diarrhoea. They found no evidence of ADRV in their cases, by electrophoresis and ELISA: they found bacterial diarrhoea, as well as the group A virus, but to my surprise they have never found ADRV. We suggested that they study their negative cases using our ELISA kits for ADRV, because I felt there should be some cases of ADRV there. We are also now investigating this, to see whether there is ADRV in any sporadic diarrhoeal patients, but we have no results yet. We have been studying the age distribution of antibody to ADRV, and we hope to have definitive results in the near future.

Blacklow: Does the epidemiology of ADRV suggest predominantly a common source for these diarrhoeal outbreaks with little secondary spread of

infection, or is secondary spread common in these outbreaks?

Hung: In the first epidemics in the North-east of China, in Jinzhou, the water supply was heavily contaminated by stool (as demonstrated by *E. coli* cultivation), but we also found that a small proportion (about 10% of patients) were infected through direct contact with affected individuals, not through contaminated water. So I think that the major form of the epidemic is water-borne, but that the virus can also spread by direct person-to-person contact.

Blacklow: The amount of secondary spread seems to be less than the amount of disease that occurs with the initial common source, then?

Hung: We don't know that, but we noticed that during the epidemics, beside the common water source, there were some individual contacts.

McCrae: What is the current incidence of the disease? You had some very large epidemics in China two or three years ago: has the virus more or less disappeared now? Are you no longer isolating ADRV in China?

Hung: There are still sporadic outbreaks of ADRV in China, but on a smaller scale than in 1983, several hundred people being affected rather than the one million cases in the nation-wide epidemics in 1982–1983. But we are still having outbreaks in different parts of the country. It is now more difficult for us to obtain samples in Beijing because the local virologists want to study the local outbreaks themselves.

Bishop: Is there any possibility that contamination of foodstuffs with human night soil could have precipitated some of these epidemics, or do you feel that water-borne contamination was the major source?

Hung: I don't know, but I think food could be an important additional source, especially in the countryside, where individual sanitation and public hygiene are still poor. Contamination of foodstuffs seems to present severe problems.

Chiba: Have you ever tested any sera collected before the major outbreaks, say 10 years ago, in China?

Hung: No. This is a good suggestion. We might be able to study sera collected from hepatitis patients in large hospitals, to get a retrospective estimate of the disease.

Bridger: We have done some retrospective serology on the porcine group B and C viruses. We looked at two serum pools from pigs in the UK, collected in 1974 and 1978, and antibody to groups B and C was present in those (Pedley et al 1983). Has anyone else any information on serum pools, of any species, collected before the 1980s?

Hung: I would certainly like to have sera, preserved for years, to see whether there is antibody to ADRV in the older sera.

Kurtz: Is there any evidence of reinfection with ADRV? If a patient has had the illness once, will he or she get it again, or is there long-lasting immunity?

Hung: I am not sure, but I recall that people infected with ADRV could be reinfected with the virus after a period of time, and showed the same symptoms

as in the first infection. We have been tracing antibody in people recovered from ADRV infection and watching them carefully for reinfection.

Kurtz: That is rather different from the picture with group A rotaviruses.

Hung: Yes, it has been difficult to get exact and detailed information on it, because volunteers for reinfection were not available.

Kapikian: I am intrigued by this point that the rechallenge of volunteers led to an illness as severe as the first illness: how many volunteers were used, and was the challenge inoculum the same size as the first inoculum? Was it an overwhelming dose?

Hung: I cannot say much about this volunteer study, which has not been published yet. The dose level of ADRV was low, a few drops of faecal filtrate, containing plenty of viral particles. We did the study twice and succeeded in obtaining infection on the second occasion, using pooled faeces from ADRV patients.

Kapikian: What about second natural infections, then? When you have had outbreaks in certain cities and certain areas of China, has there been a 'second wave' of cases?

Hung: Yes: in some areas, outbreaks have recurred over several years. The disease had been reported previously, before we found the virus. It had been referred to as non-bacterial diarrhoea, and in these areas doctors have told me that they observed recurrence of the diarrhoea in the same individuals.

Hall: Is anything known about the intestinal lesions in the persons affected in these outbreaks? Has there been an opportunity for microscopic examination of biopsies?

Hung: No. Only in very rare circumstances do people die of severe dehydration, usually older and weaker people. We couldn't get biopsy samples from these patients.

Kapikian: The major unanswered question about the atypical rotaviruses seems to be their role in disease, world wide. What should we do now to answer this question? Dr Hung is now able to test for ADRV in China with an ELISA kit, and there is a monoclonal antibody to this virus. What test should be recommended for detecting the ADRV? How does electron microscopy compare to the ELISA? Perhaps some pharmaceutical company that is already marketing a kit for detecting group A rotaviruses would be interested in preparing a kit for the non-group A rotavirus. Are the non-group A viruses important enough in terms of disease outside China for such a test to be produced and marketed? Or is it a limited problem, and we can thus wait for large outbreaks to occur before we tackle this issue? The conclusion I would draw at the moment is that the atypical rotaviruses outside China are not an important cause of severe gastroenteritis in infants and young children. Thus, if a vaccine were available for this non-group A rotavirus, it would not be recommended universally for the paediatric age group. Is my perception correct, or do we need a diagnostic test at this time?

Cubitt: A few centres should be looking at the epidemiology of atypical rotaviruses. At the moment in the UK, more and more laboratories are relying on ELISA and latex tests for the diagnosis of rotavirus infections. As a result fewer samples are being sent to laboratories that are running gels or more sophisticated tests, such as those performed in Dr McCrae's laboratory at Warwick University.

McCrae: I agree with Al Kapikian and I suspect that he is right, that the present evidence is that these atypical viruses are probably not particularly important, at least in causing human disease. However, most of the tests that have been done, with the exception of genome profile analyses, have been possible only on a small scale. We were struck, when we used to analyse animal samples, that about 10% had mixed genome profiles. We put that down to two group A rotaviruses, but perhaps that was wrong. Given the difficulty of the systems being non-cultivable and so on, perhaps we should encourage the funding of one or two centres to work on these systems, to develop diagnostic reagents of a type that can be distributed widely. At present the assays are not suitable for that, even the serological assays.

Flewett: We and others have been finding antibodies to group B viruses in comparatively small numbers in our local populations in the UK, but it doesn't follow that these are antibodies to the Chinese virus, although we used it as our antigen. It may be something like the pig virus, which probably doesn't infect people: certainly the vets that we examined don't seem to have antibodies to group B antigen and they must be handling infected pigs. There have been enormous outbreaks of group B rotavirus diarrhoea in China. Has it moved to adjacent countries like Viet Nam, or Siberia? Is there evidence of it in Tibet? There used to be big movements of sheep flocks across the Himalayan passes between Tibet and Northern India but we haven't heard of group B rotavirus in that part of India. How is it that it hasn't come out of China? It is odd that this 'Chinese' virus has caused vast outbreaks in China and not anywhere else. There are now many Japanese tourists visiting China and this virus has been looked for in Japan, but has not appeared there, or in America: and China now has many American tourists. It does not seem to be an exportable virus, but why not?

Bishop: I wonder if it is a case of the rest of the world having given something to China that it hadn't experienced before, although the suggestion that it is a zoonosis is probably a more likely explanation. There is evidence that group B rotaviruses exist in Australia, America and elsewhere. It is possible that once China opened her borders to Western visitors, some viruses crept through in the reverse direction and that you are seeing in China a new virus introduced into a population that was particularly susceptible.

Woode: What is the general view of the role of zoonoses in the whole rotavirus field? Is there any evidence that they are important in any group, including group A, and how are we to tackle group B rotaviruses?

Bridger: Cross-species infectivity has been demonstrated with some novel rotaviruses (see my paper, p 5–15) but whether the animal viruses can infect man has not been shown.

Woode: We have also experimentally cross-infected group A rotaviruses between mammalian species, but the infections are often non-clinical.

Saif: When we used group B rotaviruses to cross-infect between gnotobiotic pigs and calves, in both directions, we got some mild diarrhoea in pigs infected with calf group B virus.

Woode: Effort has been put into determining whether any of the natural human rotavirus strains (A or B) are actually animal strains, but is there any firm evidence for this?

Flewett: We have looked at many rotaviruses from around the world and run their electrophoretic patterns, subgrouping and sometimes also serotyping them. If an animal rotavirus is infecting children it ought to show itself as a 'long' RNA electrophoretic pattern and be a member of subgroup 1, and we have only seen that twice.

Kapikian: Dr O. Nakagomi and colleagues in Japan (personal communication) have detected a human rotavirus which has a long RNA pattern but belongs to subgroup 1. To my knowledge, this is the first demonstration of a subgroup 1 human rotavirus with a long RNA pattern.

Flewett: They are not common, certainly.

Greenberg: My feeling is that the best definition of the host range of a virus, rather than the species in which it was isolated, is its ability to passage in a specific host. Work by Paul Offit and myself shows that virtually any mammalian group A rotavirus can cause diarrhoea in a mouse, but only murine rotaviruses are capable of passing from one mouse to another. The limited evidence in humans at present is that only group A rotaviruses from humans will passage in humans. We have the perfect example with the animal vaccine strains, where the evidence is that bovine and simian rotaviruses are capable of infecting humans and causing immune responses, and may be capable of causing a mild illness. However, these vaccine strains seem incapable of passaging from the person inoculated to other susceptible humans, or do so much less efficiently than a homologous human virus does.

Woode: How then would you say that a virus is a human virus? We can't use serotyping. What about hybridization studies?

Greenberg: No, because the genetic basis of host range is not known.

McCrae: Why do you regard this as a problem? These rotaviruses certainly reassort in tissue culture, which suggests that they could be reassorting in nature. The fact that a bovine virus doesn't go very well in humans probably reflects the fact that it is adapted in terms of its RNA segments to grow well in the bovine system, and may not have acquired whatever RNA segment is needed to give it the host range to propagate well in human cells. That doesn't mean to say that viruses from other species can be disregarded when we are

working out a strategy for producing effective vaccines for a particular species.

Woode: There is an important epidemiological requirement to know whether animals act as a major reservoir of human rotavirus infection and vice versa, and WHO has recommended research on this for that reason. In terms of the Chinese virus, it seems worth developing criteria to decide whether or not ADRV is really an animal rotavirus with poor adaptability to humans (and hence it hasn't spread anywhere except in China).

Holmes: Currently rotaviruses are classed as 'human' or 'animal' simply on the basis of whether they were isolated from humans or other animals. A subgroup 1 rotavirus with a long RNA pattern is most likely to be of animal origin, but then there is the porcine Gottfried virus, a serotype 4, subgroup 2 virus: you could not say on serological grounds whether it was a human or pig virus. There is no clear dividing line. Malcolm McCrae is right. Even though the influenza experts have never been able to be in the right farmyard when influenza virus reassorted, and a demonstration of this therefore seems improbable, not many people doubt that the gene pool of influenza A is spread between various animals and birds and that new pandemic strains affecting humans have arisen by reassortment.

Woode: There is evidence that swine influenza virus (H1N1) and the Fort Dix human isolate (A/NJ/8/76) are closely related and that the virus can spread directly from swine to humans without reassortment (Hinshaw et al 1978). One would like similar information on rotaviruses like the Chinese ADRV: but how would one do it—by fingerprinting?

Holmes: It will depend ultimately on gene sequencing. The influenza workers were certain about the basis of antigenic drift and shift only after sequencing a large number of strains. With rotaviruses it is now technically feasible but there is much work to be done, if the sequencing of many isolates is to be attempted.

McCrae: You don't know which segment, even, has anything to do with host range. There is evidence that segment 4 may be involved to some degree in this. With many molecular biologists working on this segment and sequencing it from viruses of different species of origin, we may find a region of that segment that can be said to be a pig-specific region: then it is fairly easy to make a probe to that and start asking if we find that sequence in human viruses. This is all a long way off.

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Enteric adenoviruses

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Abstract. The 41 serotypes of human adenoviruses are classified into six subgenera (A–F) with different tropisms. Enteric infections are caused in children by serotypes Ad40 and Ad41 of subgenus F. Serotypes Ad40 and Ad41 transform embryonic cells but cannot induce tumours in newborn hamsters. They differ from all other (established) human adenoviruses by being unable to replicate in conventional cell cultures. Ad40 and Ad41 grow in 293 cells (human embryonic kidney cells immortalized by transfection with the E1A, E1B regions of Ad5). In spite of the difficulty of isolating Ad40 and Ad41 they can be directly identified in stools by enzyme-linked immunosorbent assay (ELISA) and solid-phase immuno-electron microscopy. The amount of viral DNA in stool preparations is sufficient for identification by DNA restriction or dot-blot analysis.

Adenoviruses have been associated with 7–17% of cases of diarrhoea in children. Ad40 and Ad41 cause diarrhoea throughout the year. Clinical features are watery stools, vomiting and moderately elevated temperature; respiratory symptoms are infrequent. The diarrhoea is protracted (mean 8.6 and 12.2 days for Ad40 and Ad41 respectively). Children with rotavirus diarrhoea vomited more frequently and had a higher temperature and diarrhoea of shorter duration. The impact of enteric adenoviruses in the aetiology of diarrhoea world-wide is not known but is accessible to investigation.

1987 Novel diarrhoea viruses. Wiley, Chichester (Ciba Foundation Symposium 128) p 63–91

Human adenoviruses were first detected by Rowe et al (1953). So far, 41 different serotypes have been identified. The enteric adenoviruses differ from other diarrhoea viruses by being members of a family with a wide variety of tropisms. The established adenoviruses have a predilection for lymphoid tissue, hence their name. They can persistently infect these organs, and they are also characterized by their propensity for causing outbreaks of respiratory disease or keratoconjunctivitis. These established human adenoviruses, members of subgenera A to E, replicate well in tissue culture.

In 1975, Flewett et al reported an outbreak of infectious diarrhoea in children. Their stools contained large numbers of adenovirus particles that could not be

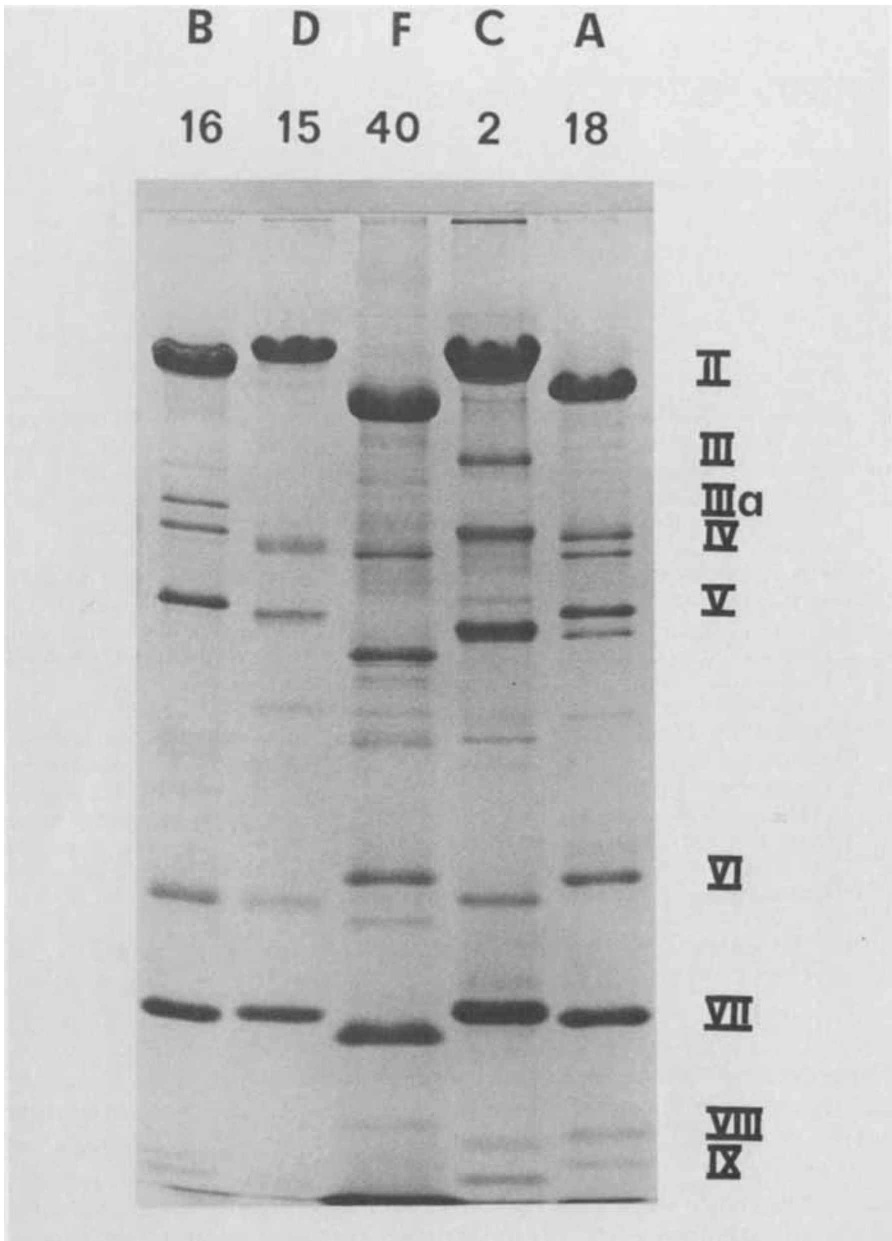

FIG. 1. SDS-polyacrylamide gel electrophoresis of virions of human adenoviruses Ad16 (subgenus B), Ad15 (subgenus D), Ad40 (subgenus F), Ad2 (subgenus C) and Ad18 (subgenus A). (Reprinted from Wadell et al 1986 by permission of the Royal Swedish Academy of Sciences.)

propagated in tissue culture. This hampered their identification and classification by accepted serological methods such as neutralization or haemagglutination inhibition techniques, and acted as a challenge to the development of new methods for identifying and characterizing the enteric adenoviruses. These methods are necessary if we are to elucidate the epidemiology and clinical relevance of the fastidious enteric adenoviruses in the aetiology of diarrhoea in children.

Morphology and classification

The adenovirus particle is a non-enveloped icosahedron with a diameter of 80 nm. The virion is composed of at least 10 different structural polypeptides and a linear 33–45 kilobase pair DNA molecule. The virus capsid is formed of 252 capsomers. Of these, 240 capsomers (the hexons) are symmetrically arranged so that each hexon is surrounded by six capsomers. The 12 corners of the virion each contain a capsomer from which an antenna-like projection (fibre) extends.

Human adenoviruses are now classified into six subgenera, A to F (see Table 1). Adenoviruses have traditionally been classified by their haemagglutinating or oncogenic properties, and more recently by the size of the structural polypeptides of the virion and the DNA homology of the viral genome. The enteric adenoviruses (that is, serotypes Ad40 and Ad41) have been shown to display a hardly discernible agglutination of rat erythrocytes, similar to that of members of subgenus A (de Jong et al 1983).

The established adenoviruses serve as a model for oncogenicity in newborn hamsters. Adenoviruses of subgenus A induce tumours in most animals within two months. Adenoviruses of subgenera B and E induce tumours in a few animals after 4–18 months. Neither Ad40 nor Ad41 induces tumours in newborn hamsters (Wadell et al 1980, Wadell 1984). Adenoviruses of subgenera C, D and F transform rat cells *in vivo* (van Loon et al 1985b).

Analysis of the apparent molecular mass of virion polypeptides by sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis provides insight into the relationships between human adenoviruses. The internal structural polypeptides V, VI, VII and VIII are evolutionarily conserved and do not vary in size, between serotypes that are members of the same subgenus. Distinctly different sizes were observed when members of different subgenera were compared. The polypeptide pattern could consequently be used to classify the 39 adenovirus serotypes then known into five subgenera. Analysis of the enteric adenoviruses (Ad40 and Ad41) showed that they could be classified in a sixth subgenus, designated F, on the basis of the size of polypeptides V, VI, VII and VIII (Wadell et al 1980, Fig 1).

Precise information on the relation between human adenoviruses could be obtained by analysing differences in their nucleotide sequences. The DNA homology of adenoviruses has been studied by filter and liquid hybridization

TABLE 1 Properties of human adenovirus serotypes of subgenera A to F (modified from Wadell 1984)

		DNA				*Apparent molecular mass of the major internal polypeptides (kDa)*						
		Homology (%)			*No. of SmaI[a] frag-ments*							
Sub-genus	*Serotype*	*Intra-generic*	*Inter-generic*	*G+C (%)*		*V*	*VI*	*VII*	*Haema-glutina-tion pattern[b]*	*Length of fibres (nm)*	*Oncogenicity in newborn hamsters*	*Tropism/ symptoms*
A	12,18,31	48–69	8–20	48	4–5	51.0–51.5 46.5–48.5[c]	25.5–26.0	18	IV	28–31	High (tumours in most animals in 4 months)	Cryptic enteric infection
B:1 B:2	3, 7, 16, 21 14[d] 11, 34 35	89–94	9–20	51	8–10	53.5–54.5	24	18	I	9–11	Weak (tumours in a few animals in 14–18 months)	Respiratory disease Persistent infections of the kidney

C	1,2,5,6	99–100	10–16	58	10–12	48.5	24	18.5	III	23–31	nil	Respiratory disease persists in lymphoid tissue
D	8,9,10,13, 15,17,19, 20,22–30, 32,33,36, 37,38,39[e]	94–99	4–17	58	14–18	50.0–50.5[f]	23.2	18.2	II	12–13	nil	Kerato-conjunctivitis
E	4		4–23	58	16–19	48	24.5	18	III	17	nil	Conjunctivitis Respiratory disease
F	40,41	62–69	15–22	52	9–12	46.0–48.5	25.5	17.5	IV	28–33	nil	Infantile diarrhoea

[a] The restricted DNA fragments were analysed on 0.8–1.2% agarose slab gels. DNA fragments smaller than 400 bp were not resolved.
[b] I, complete agglutination of monkey erythrocytes; II, complete agglutination of rat erythrocytes; III, partial agglutination of rat erythrocytes (fewer receptors); IV, agglutination of rat erythrocytes discernible only after addition of heterotypic antisera.
[c] Polypeptide V of Ad31 was a single band of 48 kDa.
[d] Members of subgenus B are divided into two clusters of DNA homology based on pronounced differences in DNA restriction sites.
[e] Only DNA restriction and polypeptide analysis have been performed with Ad32–39.
[f] Polypeptides V and VI of Ad8 showed apparent molecular masses of 45 and 22 kDa, respectively.
Polypeptide V of Ad30 showed an apparent molecular mass of 48.5 kDa.

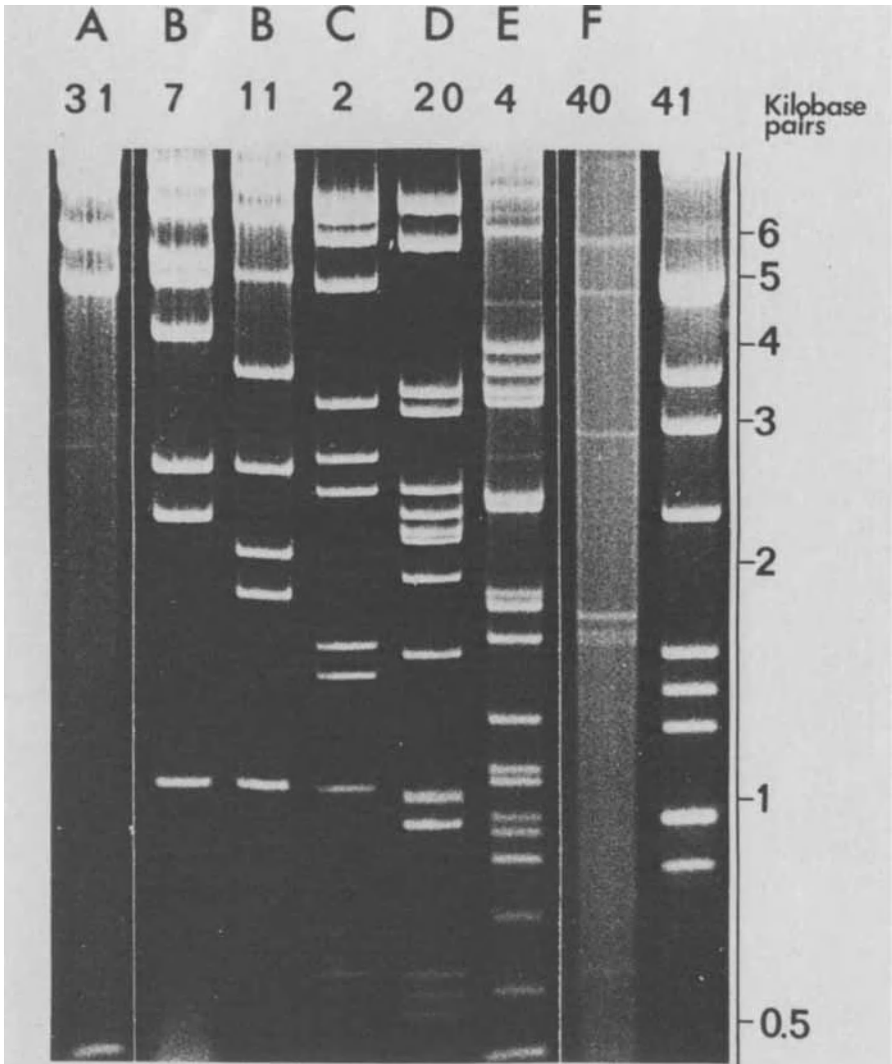

FIG. 2. *Sma*I DNA restriction patterns obtained after digesting DNA from adenovirus serotypes from subgenera A to F. (Reprinted from Wadell 1984 by permission of Springer-Verlag.)

techniques (see Table 1). The degree of homology between members of different subgenera is less than 23%. This means that recombination between adenoviruses of different subgenera can be expected to be very infrequent, and subgenera can therefore be expected to act as barriers to recombination. Human adenoviruses are also characterized by distinct differences in the GC content of the DNA: subgenus A, 47–49%; subgenera B and F, 51–52%, and subgenera C,

D and E, 57–59% (van Loon et al 1985a). This variation in nucleotide composition was exploited by analysing all adenovirus prototypes by the restriction endonuclease *Sma*I, which cleaves the DNA at 5′ CCCGGG (Wadell et al 1980, Fig. 2). This approach revealed two principles: (a) the members of each subgenus displayed a characteristically limited range of numbers of *Sma*I restriction fragments: (b) pairwise comparison of DNA restriction fragments from two members of the same subgenus showed that in general more than 50% of the fragments co-migrated. This rule is not valid for sub-genera A, E and F.

The amount of genetic variability between members of the six different subgenera varies substantially, as indicated below:

Subgenus A. Ad12, Ad18 and Ad31 are distinctly different from each other in both polypeptide pattern and genome composition (Table 1). However, the tip of the fibre and the hexon carry epitopes common to the three serotypes, since a pronounced cross-reactivity is demonstrated by both haemagglutination inhibition and neutralization assays. All three members cause cryptic enteric infections and, in particular, Ad31 has frequently been isolated from children with diarrhoea; but the causal relation remains to be established.

Subgenus B. DNA restriction analysis of the eight members of subgenus B revealed two distinct clusters of DNA homology. The first, B:1, consists of Ad3, Ad7 and Ad21, which all cause outbreaks of respiratory disease. They can also cause a more generalized infection and diarrhoea may occur. These serotypes account for 33% of all adenovirus isolates typed and reported to WHO. The second cluster (by DNA homology), namely B:2, consists of Ad11, Ad34 and Ad35, which are closely related to each other and all cause persistent infections of the urinary tract.

Subgenus C. Ad1, Ad2, Ad5 and Ad6 display a DNA homology of 98% in pairwise comparison. They represent 59% of all adenovirus isolates reported to WHO. They can persist for years in lymphoid tissue and be intermittently shed into the stools.

Subgenus D. The 23 serotypes of this subgenus are closely related and characterized by a predilection for infecting the eye.

Subgenus E. Ad4 is the only human adenovirus serotype classified into subgenus E. It has been associated both with respiratory disease and with epidemic follicular conjunctivitis. The Ad4 and Ad4a genome types are strikingly different at the genome level, since only 45% of the DNA restriction fragments co-migrate. No other adenovirus serotype studied displays such pronounced genetic differences within the one serotype.

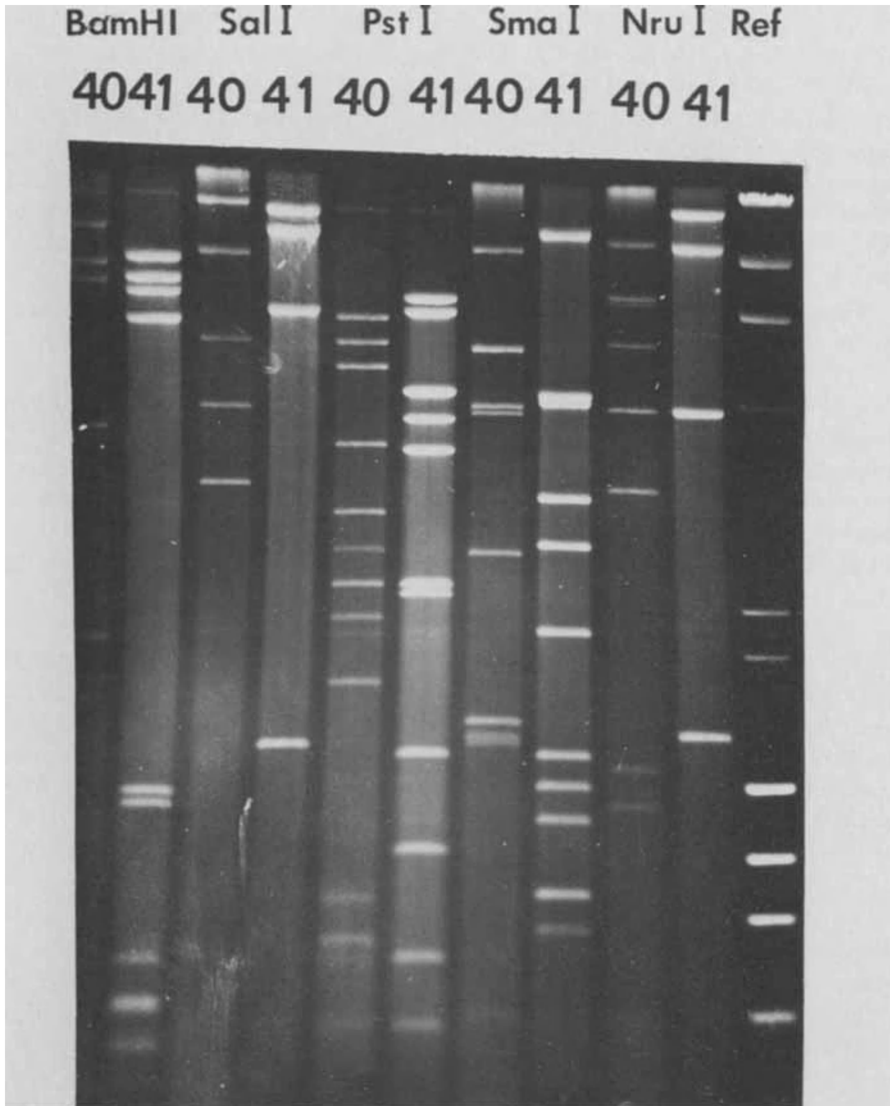

FIG. 3. DNA restriction patterns of the enteric adenoviruses Ad40 and Ad41 (subgenus F) obtained with restriction endonucleases *Bam*HI, *Sal*I, *Pst*I, *Sma*I and *Nru*I. Lambda DNA and ϕX174 digested with *Hin*dIII and *Hin*cII, respectively, were used as size references. (Reprinted from Wadell et al 1986 by permission of the Royal Swedish Academy of Sciences.)

Subgenus F. Subgenus F contains serotypes Ad40 and Ad41. The DNA homology between them, determined by liquid hybridization, was 62–69% (van Loon et al 1985a). Determination of the co-migrating DNA restriction frag-

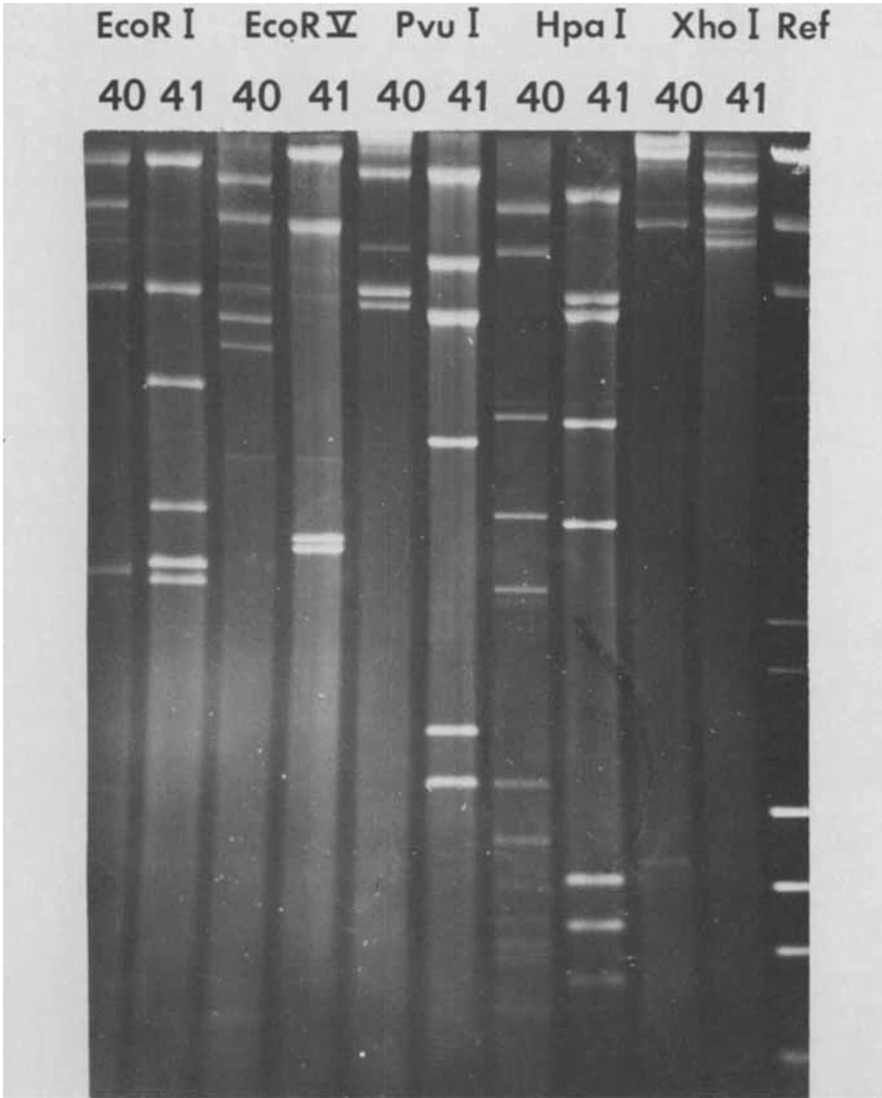

FIG. 4. DNA restriction patterns of Ad40 and Ad41 (subgenus F) obtained after digestion with *Eco*RI, *Eco*RV, *Pvu*I, *Hpa*I and *Xho*I. The size reference is described under Fig. 3. (Reprinted from Wadell et al 1986 by permission of the Royal Swedish Academy of Sciences.)

ments obtained after digestion with *Bam*HI, *Bgl*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Hpa*I, *Nru*I, *Pst*I, *Pvu*I, *Sal*I, *Sma*I and *Xho*I revealed that only 18 of the 177 DNA restriction fragments co-migrated (Uhnoo et al 1983, Wadell 1984, Figs. 3 & 4). This means that subgenera A and F are both highly heterogeneous.

However, members of both subgenera are characterized by a pronounced subgenus-specific cross-reactivity between the epitopes on both the fibre and the hexon, as measured in haemagglutination inhibition and neutralization assays (de Jong et al 1983). A corresponding cross-reactivity can occasionally be seen between members of different subgenera. Shared epitopes on Ad4 (subgenus E) and Ad16 (subgenus B:1) hexons are responsible for a pronounced two-way cross-reactivity in neutralization assays. Ad40 virions (subgenus F) express epitopes common to Ad4 virions (subgenus E), detected by solid-phase immuno-electron microscopy (Svensson et al 1983).

The serotype and genome type concept

The definition of a serotype relies on the distinct antigenic determinants that are capable of inducing neutralizing antibodies. Restriction endonuclease digestion of DNA from various strains of the same serotype revealed a pronounced genetic variability, expressed as distinct viral entities designated 'genome types' (Wadell et al 1980). In subgenera B, C and D the genetic variability within a serotype and between serotypes of the same subgenus can be of a similar order.

Genetic variability of enteric adenoviruses

In an analysis of 26 Ad40 strains from South Africa, Canada and Europe by seven DNA restriction endonucleases, Kidd et al (1984) demonstrated six different genome types. The dominating DNA restriction pattern was the same as the characteristic Hovi-x strain (Wadell et al 1980). This was detected among 73% of the strains representing all three continents.

The analysis of 15 strains of Ad41 from Europe, Canada and South Africa (Kidd 1984) revealed that all displayed a *Sma*I restriction pattern compatible with the original DNA restriction pattern of Ad41 (Uhnoo et al 1983). The Canadian and European strains could not be distinguished by means of 10 restriction endonucleases, whereas four different genome types could be demonstrated among the South African strains.

Mapping of the Ad40 and Ad41 genomes

Takiff et al (1984) have presented DNA restriction site maps of *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, *Sma*I and *Xho*I of an Ad40 strain that cannot be the Dutch Dugan strain as stated, and a strain of Ad41 designated 1105 from the USA. Allard et al (1985) have mapped the DNA restriction sites of *Bam*HI, *Eco*RI, *Hpa*I, *Nru*I, *Pvu*I and *Sal*I of a Swedish Ad41D389 strain. The *Eco*RI maps of the two Ad41 strains were identical. The *Bam*HI maps suggest that the strains used are two slightly different genome types. Both patterns are common among Swedish Ad41 strains. However, the DNA restriction site maps of *Bam*HI

presented by Takiff et al (1984) and Allard et al (1985) are not compatible. Van Loon et al (1985a) have established the *Bcl*I, *Bst*EII, *Cla*I, *Eco*RI, *Pvu*I and *Sal*I restriction site maps of the Ad40 strain Dugan, and the *Cla*I, *Eco*RI, *Sal*I and *Xho*I maps of the Ad41 strain Tak. Kidd et al (1985) have cloned Ad40 strain N5911 and Ad41 strain M4550. Restriction fragments obtained by *Bam*HI, *Pst*I and *Bgl*I were cloned by Allard et al, Kidd et al and Takiff et al, respectively. The *Bam*HI clone J, mapped at the extreme left end of the Ad41 genome, displayed a broad cross-reactivity, hybridizing with representative adenovirus serotypes of subgenera A, B, C, D, E and F. The Ad41 *Bam*HI clone H, located at map units 1.5–3.4, hybridized only with DNA from Ad40 and Ad41, giving a 100-fold stronger signal to Ad41.

Kidd chose larger *Pst*I clones, making up 5.3% and 18.7% of the Ad40 and Ad41 genomes, respectively. The N26 clone appeared to be specific for Ad40, whereas the large M9 clone reacted strongly with both Ad40 and Ad41. The *Bgl*D clone, comprising 16% of the Ad41 genome, could be used to differentiate between enteric and non-enteric adenoviruses. However, the differential sensitivity for detection of Ad40 versus Ad41 was not evaluated (Takiff et al 1985).

In conclusion, the *Bam*HI clone J of Ad41 may be used to detect adenoviruses in general, whereas the *Pst*I N26 clone of Ad40 is valuable for detection of Ad40, and the *Bam*HI clone H of Ad41 can be used to detect Ad41.

Growth properties of enteric adenoviruses

The enteric adenoviruses Ad40 and Ad41 of subgenus F differ from all other adenoviruses by being unable to grow in human embryo kidney cells or in most heteroploid cell lines. De Jong et al (1983) made a careful study of the growth characteristics of several enteric adenovirus strains in Chang, tCMK (tertiary monkey kidney), Graham 293, HDF, HeLa-B, HeLa-H and Hep-2 cells and concluded that growth depended on virus strain, cell type, subline and batch, and further unknown conditions. This complicates the interpretation of results obtained in different laboratories. In general, 293 cells and tCMK cells were the most reliably permissive cell lines for enteric adenoviruses (Takiff et al 1981). Strains were best differentiated serologically into either Ad40 or Ad41 by serum neutralization titrations on tCMK cells (de Jong et al 1983). The host cell range dependence appears to be a function of the early regions, E1A and E1B, since enteric adenoviruses Ad40 and Ad41 can grow in 293 cells. These cells are human embryonic kidney cells that have been immortalized by transfection by the E1A and E1B regions of Ad5, which apparently can complement the Ad40 and Ad41 genome and enable growth to occur in these cells. The 293 cells are only semipermissive to enteric adenoviruses, since one-step growth curves revealed that the yield of infectious Ad1 virions was 150- to 500-fold higher than the yield of infectious Ad40 and Ad41 virions. However, the number of Ad1

virus particles produced was only 10-fold higher than the number of enteric adenovirus particles. The reason for this difference is not known (Brown 1985).

Pathogenesis

Enteric adenoviruses replicate in the gut to 10^{11} virions per gram of stool. Adenovirus particles have been demonstrated in the duodenal mucosa analysed after the autopsy of a child who died from infection with adenovirus 41 (Whitelaw et al 1977, Johansson et al 1985). The observation by Uhnoo et al (1984) that 70% of paired sera from children with enteric adenovirus-associated diarrhoea displayed a type-specific seroconversion measured by haemagglutination inhibition supports the notion that enteric adenoviruses are causative pathogens in diarrhoea. The respiratory tract is naturally involved in infection with the established adenoviruses, but only a limited number of respiratory symptoms were noted in children with diarrhoea caused by enteric adenoviruses (Uhnoo et al 1984), and none at all according to Chiba et al (1983). We, together with O. Meurman in Turku, have analysed 24 nasopharyngeal specimens (NPS) and stool pairs. Adenovirus group-specific antigen was detected in all stools and in 17 of the NPS samples; enteric adenovirus type-specific ELISA was positive in three stools only and in none of the NPS samples.

Relation to coeliac disease

Kagnoff et al (1984) screened 1498 proteins for their homology to gliadin and noted that the Ad12 E1B protein contained a region of 12 amino acid residues that included eight identities and a hydrophilic identical pentapeptide. The native antibodies from rats carrying an Ad12-transformed baby rat kidney cell tumour reacted both with gliadin and with a synthetic heptapeptide of gliadin from the region of homology. The significance of this molecular mimicry has to be evaluated by determining the conservation of this peptide in the early proteins of other adenoviruses infecting the gut, and by estimating the relative frequency of infection by the adenoviruses in question in the population of healthy and sick HLA-B8, DR3, DR7 and DC3 individuals.

Clinical characteristics

The clinical features of childhood gastroenteritis associated with enteric adenovirus (and other pathogens) are shown in Tables 2–5. However, established adenoviruses belonging to subgenera A to E can definitely also cause diarrhoea; vomiting is less frequent. All symptomatic patients also have fever, usually to a high degree, and as a rule have respiratory symptoms. The mean duration of diarrhoea due to established adenoviruses was 6.2 days (Table 2).

After an incubation period of seven to eight days, children infected with

TABLE 2 Clinical characteristics of 55 children with gastroenteritis due to adenovirus

		No. (%) with indicated clinical finding:								
					Fever					
Type of infection	*Total no. of patients*	*Diarrhoea*	*Mean duration (days)*	*Vomiting*	*37.5–38.9 °C*	*≥39 °C*	*Total no. with fever*	*Abdominal pain*	*5% dehydration or more*	*Respiratory symptoms*
Ad40	14	14 (100)	8.6	11 (79)	7 (50)	1 (7)	8 (57)	1 (7)	2 (14)	3 (21)
Ad41	19	18 (95)	12.2	15 (79)	7 (37)	1 (6)	8 (42)	7 (37)	3 (16)	4 (21)
Established adenovirus	14	14 (100)	6.2	7 (50)	5 (36)	9 (64)	14 (100)	5 (36)	2 (14)	11 (79)
Untyped adenovirus	8	8 (100)	4.5	6 (75)	2 (25)	3 (38)	5 (63)	4 (50)	1 (13)	2 (25)

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TABLE 3 Clinical features in 393 children with acute gastroenteritis in relation to enteropathogens detected in the stools

	Groups of patients and number (%) with each finding				
	1 *Rotavirus* ($n = 168$)	2 *Enteric adenovirus* ($n = 32$)	3 *Bacteria* ($n = 42$)	*4* *Bacteria and virus* ($n = 16$)	5 *No pathogens* ($n = 135$)
Diarrhoea	164 (98)	31 (97)	42 (100)	16 (100)	132 (98)
Diarrhoea >10 times daily	36 (21)	7 (22)	15 (36)	3 (19)	27 (20)
Vomiting	146 (87)	25 (78)	18 (43)***	15 (94)	72 (53)***
Vomiting >5 times daily	62 (37)	3 (9)**	3 (7)***	5 (31)	19 (14)***
Fever	141 (84)	14 (44)***	29 (69)*	15 (94)	83 (61)***
Fever >39 °C	71 (42)	1 (3)***	18 (43)	7 (44)	45 (33)
Abdominal pain	31 (18)	8 (25)	21 (50)***	4 (25)	40 (30)*
Blood present in stools	2 (1)	1 (3)	17 (41)***	1 (6)	14 (10)***
Mucus present in stools	28 (17)	6 (19)	11 (26)	1 (6)	34 (10)***
Respiratory symptoms	56 (33)	6 (19)	16 (38)	8 (50)	57 (42)
Hospitalization	65 (39)	9 (28)	16 (38)	8 (50)	33 (24)*

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ denote significant differences between the rotavirus group and each of the other groups (chi-squared test).
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TABLE 4 Clinical course of gastroenteritis in children infected with different enteropathogens

	Groups of children and mean (SEM) duration (days) of each symptom				
	1 *Rotavirus* ($n = 168$)	2 *Enteric adenovirus* ($n = 32$)	3 *Bacteria* ($n = 42$)	4 *Bacteria and virus* ($n = 16$)	5 *No pathogens* ($n = 135$)
Symptoms before hospital contact	2.9 (0.16)	5.3 (0.75)***	5.4 (0.59)***	3.0 (0.50)	3.9 (0.25)***
Diarrhoea	5.9 (0.28)	10.8 (1.71)***	14.1 (2.18)***	8.4 (1.70)**	8.0 (0.57)***
Vomiting	2.5 (0.10)	3.2 (0.80)	2.1 (0.34)*	2.1 (0.24)	2.1 (0.16)
Fever	2.2 (0.12)	2.4 (0.35)	3.3 (0.39)**	2.5 (0.40)	2.5 (0.16)
Hospital stay	2.4 (0.19)	3.6 (1.18)	3.6 (1.20)	2.6 (0.56)	2.8 (0.48)

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ denote significant differences between the rotavirus group and each of the other groups (Mann-Whitney U-test).
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TABLE 5 Clinical and biochemical findings in children with acute gastroenteritis investigated in hospital[a]

	Groups									
	1 Rotavirus		*2 Enteric adenovirus*		*3 Bacteria*		*4 Bacteria and virus*		*5 No pathogens*	
	n	%	*n*	%	*n*	%	*n*	%	*n*	%
Dehydration	78/142	55	11/30	37	16/38	42	9/16	56	29/114	25***
Dehydration > 5%	19/142	13	3/30	10	4/38	10	1/16	6	5/114	4*
General condition: moderately > severely ill	42/142	30	3/30	10*	11/38	29	4/16	25	18/114	16**
Intravenous fluid therapy	23/65	35	2/9	22	6/16	38	3/8	38	8/33	24
ESR > 20 mm/h	18/113	16	4/20	20	14/30	47***	3/10	30	17/76	22
Leucocyte particle count > 12×10^9/l	7/116	6	7/21	33***	7/31	23**	1/11	9	15/84	18**

[a] Excluding patients who were not medically examined because their parents consulted the doctor by telephone only.
$^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ denote significant differences between the rotavirus group and each of the other groups (chi-squared test).
ESR, erythrocyte sedimentation rate.
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enteric adenoviruses Ad40 and Ad41 had a clinically moderate disease, with diarrhoea and vomiting, where elevated temperature or respiratory symptoms were the exception. Their parents consequently decided to seek medical attention after a longer duration (5.3 days) of enteric adenovirus diarrhoea than did parents of children with rotavirus gastroenteritis (2.9 days, Table 4). The symptoms of patients infected with Ad40 and with Ad41 were on the whole similar. However, children infected with Ad41 more frequently had abdominal pain (Table 2), and had a more protracted diarrhoea (mean duration of 12.2 days) than those infected with Ad40 (mean duration 8.6 days). The mean ages of the children were 15 months for Ad40 and 28 months for Ad41 infection.

A comparison of the clinical features of 393 children with acute gastroenteritis during 1981 in Uppsala (Uhnoo et al 1984, 1986) (Tables 3–5) demonstrated that enteric adenoviruses caused a more protracted, milder infection with a reduced frequency of vomiting and more moderate elevation of the temperature than did rotaviruses. When bacteria were considered to be the causative agent of the diarrhoea, the children frequently had abdominal pain and blood in the stools, whereas vomiting was significantly less frequent than in children infected with rotaviruses. At follow-up of the 32 children infected with enteric adenoviruses, three children had difficulties in digesting lactose-containing products, 5–7 months after the diarrhoea, and one child had not tolerated gluten-containing food for nine months.

Epidemiology

Information on the distribution of enteric adenoviruses has been gained from seroepidemiological surveys, the study of outbreaks, and prospective studies. Antibodies against enteric adenovirus were found in significant titres in pools of sera from Holland and West Germany (de Jong et al 1983). Kidd et al (1983) surveyed neutralizing antibodies using an Ad41 strain reactive with antibodies induced by both Ad40 and Ad41. More than a third of the sera collected from children in England, New Zealand, Hong Kong and Gambia contained enteric adenovirus-specific antibodies.

Enteric adenoviruses have been associated with outbreaks of diarrhoea in a children's ward (Flewett et al 1975) and an RAF base (Richmond et al 1979). We later identified the latter adenoviruses as Ad40. Chiba et al (1983) reported on an outbreak of Ad40-associated diarrhoea in an orphanage in Sapporo.

Brandt et al (1985) studied the association between adenoviruses and diarrhoea in children in Washington, D.C. from 1974 to 1980. Adenoviruses were found during each calendar month in 8.6% of 900 children with diarrhoea, using differential growth in 293 cells as a criterion for enteric adenoviruses. Whenever possible, typing was also confirmed by DNA restriction analysis. Similar studies have been made in Toronto (Middleton et al 1977), Glasgow (Madeley et al 1977), Turku (Vesikari et al 1981), Baltimore (Yolken et al 1982),

Buffalo (Riepenhoff-Talty et al 1983), Manchester (Ellis et al 1984) and Uppsala (Uhnoo et al 1984). In most of these studies adenoviruses were detected throughout the year at a frequency of 7–17%, a frequency second only to that of rotaviruses. It is however vital to distinguish between enteric adenoviruses and established adenoviruses, which may be shed for years in stools. By all available enteric adenovirus-specific assays, 7.9% of the children in the Uppsala study had enteric adenovirus-associated diarrhoea (Uhnoo et al 1984).

Few studies have been published from developing countries. In a two-year study, Leite et al (1985) reported from Brazil that 2% of 746 children under five years of age, with diarrhoea, shed enteric adenoviruses. A seven-month study of 616 black children in South Africa by Kidd et al (1986) revealed that 13.8% shed rotaviruses and 6.5% shed either Ad40 or Ad41. The highest peak of enteric adenoviruses was noted in midsummer, in contrast to rotaviruses, which peaked during the autumn.

Diagnosis

Electron microscopy is the method of choice for evaluating unknown causative agents of viral diarrhoea. Enteric adenoviruses were first observed by this technique in 1975. They can then be identified by immuno-electron microscopy, or by the more versatile solid-phase immuno-electron microscopy (SPIEM) applied by Svensson et al (1983). Although epitopes of Ad40 and Ad41 cross-react, these viruses can be typed by SPIEM using serial dilution of hyperimmune type-specific sera.

We first used counterimmune electro-osmophoresis to evaluate the feasibility of using absorbed Ad40-specific sera in differentiating between Ad40 and established adenoviruses (Jacobsson et al 1979).

ELISA or radioimmunoassay is well suited to the direct detection of enteric adenoviruses. It is critical to absorb the anti-virion sera exhaustively with adenoviruses of other subgenera. Two ELISA procedures for typing Ad40 or Ad41 by this approach have been described (Johansson et al 1980, 1985). Neutralizing monoclonal antibodies specific for Ad40 and Ad41 have been developed by J.C. de Jong (personal communication). Singh-Naz & Naz (1986) have isolated type-specific monoclonal antibodies that apparently react with a polypeptide corresponding in size to polypeptide VII.

The SDS–polyacrylamide gel electrophoresis that has been useful in characterizing enteric adenoviruses can hardly be used for routine purposes. However, DNA restriction analysis can be done directly on viral DNA extracted from stools (Kidd et al 1985). The restriction patterns obtained can be compared to those in Fig 5. The *Sma*I patterns of adenoviruses of subgenera D or E are composed of 16 or more fragments and are easy to distinguish from those of enteric adenoviruses. A catalogue of restriction patterns of all 41 adenovirus prototypes has been published by Adrian et al (1986).

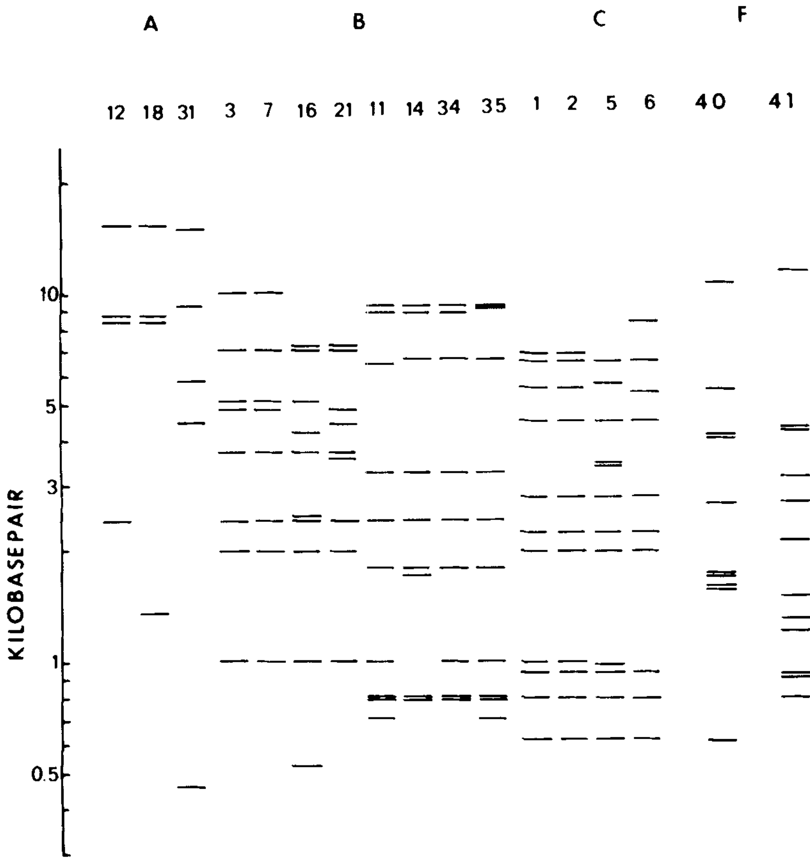

FIG. 5. Schematic presentation of the *Sma*I DNA restriction patterns of all the human adenovirus prototypes of subgenera A, B, C and F.

Enteric adenoviruses may be cultivated in tertiary monkey kidney cells (tCMK) or in 293 cells. The latter are more widely distributed and therefore more commonly used. In interpreting the results obtained it is vital to be aware that the growth of enteric adenoviruses is only semipermissive and that the 'blind' passages may allow overgrowth by the almost ubiquitous adenoviruses of subgenus C. Isolation in tissue culture can be combined with an assay based on fluorescent infected cells, using enteric adenovirus-specific sera. Haemagglutination inhibition cannot distinguish between Ad40 and Ad41. They also cross-react in serum neutralization assays, requiring careful studies, preferably in tCMK cells (de Jong et al 1983).

In conclusion, screening for enteric adenoviruses may be initiated by a group-specific ELISA assay, followed by type-specific ELISA based on

absorbed reagents or monoclonal antibodies. Alternatively, viruses may be screened in a laboratory equipped with an electron microscope and confirmation obtained by the use of immuno-electron microscopy. The third possibility is a more widespread application of specific DNA probes. The fourth method, differential growth in 293 cells and heteroploid cell lines, requires confirmation by DNA restriction or any of the above procedures. A fifth possibility would be DNA restriction analysis on DNA from stool specimens, using the silver staining technique.

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DISCUSSION

Hung: Are there any animals susceptible to type 40 or 41 adenovirus?

Wadell: This is what we want to discover. The problem with the adenoviruses in general is that they are usually strongly host restricted. We haven't found an animal that is susceptible, but then we haven't looked very much, either. The enteric adenoviruses are not oncogenic in newborn hamsters, unlike some of the established adenoviruses, but Ad40 and Ad41 do transform rat embryo cells. It should not be impossible to find an animal model for the enteric adenoviruses, since human Ad5 has been demonstrated to cause hepatitis in mice and pneumonia in cotton-tail rats.

Hall: Adenoviruses are reported to cause haemorrhagic enteritis in turkeys (Arbuckle et al 1979), and the Belgians have reported adenovirus enteritis in weaned piglets (Coussement et al 1981). I don't know how the turkey and pig viruses relate to those discussed by Professor Wadell. Adenovirus in pigs causes a mild enteritis in weaned animals, which is quite common and causes poor growth; persistence of infection is also recorded.

Flewett: There is a rare enteric adenovirus infection in Arabian foals, which are subject to a form of immunodeficiency due to a recessive mutation (McChesney et al 1973, McGuire et al 1974). When they develop an adenovirus infection on top, there is a severe, usually fatal enteritis. Another animal example is an enteritis in mice caused by an adenovirus (Takeuchi & Hashimoto 1976).

Wadell: There is no evidence to suggest that these animal adenoviruses should be analogous to the human fastidious enteric adenoviruses.

Saif: We detected an adenovirus associated with diarrhoea in a three-week-old nursing pig and passaged the virus in a few gnotobiotic pigs. It was detected in low titre from the gnotobiotic pigs; typical particles are shown in Fig. 1.

McNulty: One of our pathologists, Dr Joan Smyth, has recognized peracute and acute outbreaks of diarrhoea in nine-month-old cattle at pasture which were associated with adenovirus infection. Virus inclusion bodies were present in the endothelium of small blood vessels in the small intestine and colon (Smyth et al 1986).

Wadell: Is it an arteritis, then?

McNulty: Strictly speaking no, but virus was detected in arterioles, venules and capillaries.

Snodgrass: It is unlikely that enteric adenoviruses are a common cause of diarrhoea in young farm animals. We frequently use faecal electron microscopy

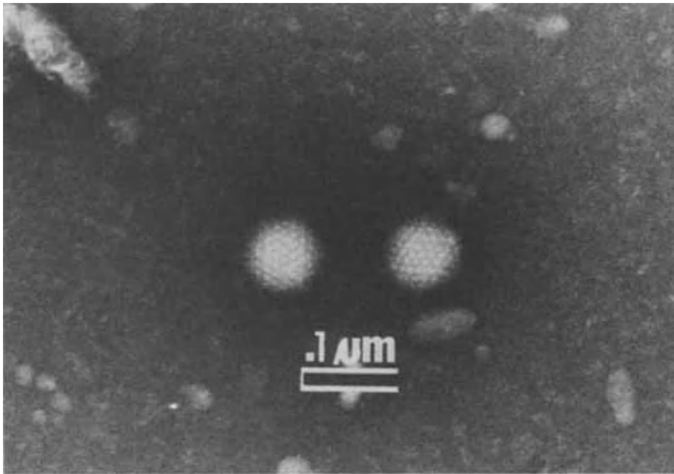

FIG. 1 (*Saif*). Electron micrograph of adenovirus particles from the intestinal contents of an infected gnotobiotic pig with diarrhoea.

as a diagnostic tool and have never observed adenoviruses in faeces from calves, lambs or piglets.

McNulty: It is unusual to find adenoviruses in pig faeces, I agree.

Saif: Yes, but they are sometimes in very low titre when found. I have seen them in older weaned pigs with diarrhoea, but only in low numbers.

Bridger: Is that true of the human specimens as well?

Wadell: They are there, and the amount would be up to 10^{11} virus particles per gram of faeces. The infectious titre may be low, because these viruses don't grow in most heteroploid cell lines.

Bridger: We should have seen them by electron microscopy, and we have not.

Hung: We have sometimes found adenovirus particles in the stools in infantile rotavirus diarrhoea (group A) in China. This suggests a possible mixed infection. Also, last year in China (1985) we had an outbreak of acute conjunctivitis, and we identified Ad7; some children had diarrhoea as well. Do you know whether serotype 7 causes diarrhoea?

Wadell: If you have a severe generalized infection with adenovirus in a young child, you can have both conjunctivitis and diarrhoea. These symptoms are often reported for Ad3, but also occur with Ad7. In Ingrid Uhnoo's study, serotypes 3,7,18, and also nine strains of Ad2 and 5, were isolated from children with diarrhoea; at least half of the children seroconverted against adenoviruses (Uhnoo et al 1984).

Flewett: We have seen enteric adenoviruses and rotaviruses in the same

child: it is not uncommon to see the two together, in quite large numbers.

What we have heard on the epidemiology of enteric adenovirus is very interesting and I hope people will follow it up over several seasons. We normally don't see very many enteric adenoviruses, in our region, in the UK, but I do remember one winter in which for a brief period of about three months we had more of these in diarrhoeic children than we had rotaviruses: the rotaviruses overtook them after the end of March.

Also, when one finds large numbers of adenoviruses in the stools and isolates an adenovirus of, say, serotype 2, one must not assume that all these virions are adenovirus 2 virions. One needs to try them on HEp II cells and then on 293 cells and stain them by immunofluorescence, and see how much is neutralized by the different serotype-specific sera. I am sure that both kinds of adenovirus are coming out together, perhaps the Ad2 ones from the throat migrating down through the intestinal tract into the faeces, and the Ad40 or Ad41 viruses probably coming from the enterocytes.

Caul: We detect enteric adenoviruses by electron microscopy in about 10% of our faecal samples from symptomatic children. We have no evidence of adenovirus infection in adults. Is the specificity in your experience restricted to children, Professor Wadell, and are there sites of infection other than the gastrointestinal tract involved with the Ad40 and A41 serotypes?

Wadell: As regards symptoms from other parts of the body, Ingrid Uhnoo saw respiratory symptoms in 20% of the children (Uhnoo et al 1984). With Dr O. Mäki in Finland we studied nasopharyngeal–stool pairs and did not find enteric adenovirus in the respiratory specimens, only in the stools.

In relation to Dr Flewett's comments on the epidemiology of enteric adenoviruses, Dr J.C. de Jong has typed enteric adenoviruses at RIVM, Bilthoven since 1981. He found 72% of Ad40 and 28% of Ad41 for 1981. During 1984 and 1985 the reverse ratio, 20% and 80%, respectively, of Ad40 and Ad41 was noted. Consequently, in a one-year survey in a given population, we can get misleading information on the relative occurrence of Ad40 or Ad41. This information may be taken as a sign of cross-protection between Ad40 and Ad41, but a direct study of cross-protection has not been done, to my knowledge.

Flewett: These enteric adenoviruses do infect adults. In our first study we had the infection in children and in a nurse. We had a good demonstration of the epidemiology and incubation period recently when a colleague evidently brought it home from the well-baby clinic and her small son came down with an incubation period estimated at about eight days. His grandmother, who had come from Scotland to look after him, went down with diarrhoea with the same incubation period, and adenoviruses were isolated from her too. So adenovirus can infect grandmothers!

Caul: Can we assume, though, that clinical expression in adults is rare? We have studied a fairly large enteric adenovirus outbreak in a military camp. The

mothers and fathers did not get the infection, but it was widespread in their children.

Flewett: This grandmother certainly had diarrhoea and so did the young nurse in our original study, but of course children are affected more frequently.

Kurtz: I have the impression that enteric adenovirus isolation or electron microscopic findings, and the correlation with symptomatic disease, is extremely difficult to resolve. There are so many subgroups and serotypes, some of which are excreted normally, and others which may be causing disease. Is there an easy way in which a diagnostic laboratory could try to sort out this question?

Wadell: If a virus has caused the diarrhoea, there should be plenty of virus particles in the stool. The asymptomatic shedding of adenoviruses over extended periods is usually of low titre. ELISA methods are useful for identification but require absorbed reagents or monoclonal antibodies (Johansson et al 1980, 1985). One can use specific probes, but we are also fond of the restriction enzymes. Restriction analysis can be performed directly on DNA preparations extracted from stools. A catalogue of restriction patterns of all 41 adenovirus prototypes is now available (Adrian et al 1986).

Bishop: What would you accept as an accurate diagnosis of types 40 and 41? I tend to do it by exclusion. If I see adenoviruses in large quantities in stool, and can't adapt them to cell culture, then I assume that they are probably 'enteric' adenoviruses. What requirements ought to be satisfied for the diagnosis of an enteric adenovirus infection?

Wadell: I don't think your criteria are sufficient, because there is a gradient in permissiveness in established cell lines. Serotypes 12,18 and 31 grow poorly, which makes them hard to distinguish from 40 or 41 with your criteria.

Kapikian: This question of how to associate a diarrhoeal episode aetiologically with adenovirus infection is fraught with difficulty, since even adenoviruses associated with respiratory illnesses are characteristically shed efficiently in the faeces and of course in the respiratory tract also. In addition, such respiratory adenoviruses can be shed in the faeces for long periods of time—such as several weeks or even months. Various studies, such as the longitudinal studies at Junior Village in Washington, DC (Bell et al 1961), the community-based Virus Watch Program in New York City (Fox et al 1969) and cross-sectional studies at Children's Hospital in Washington, DC (Brandt et al 1972), have wrestled with the problem of how to associate an illness with a temporally occurring adenovirus infection. I would like to highlight the hallmark studies of Bell, Huebner et al at Junior Village, a welfare institution for homeless but otherwise normal children. In this study, adenovirus types 1, 2 and 5 were frequently isolated but the mere recovery of an adenovirus during an illness was not considered to be enough to establish an aetiological relationship. Rather, this was studied further by comparing the illness attack rate of the test children during the week of adenovirus recovery with the illness attack rate observed in

two control groups: (1) the illness attack rate of these same children (who were adenovirus positive) two weeks before and two weeks following the calendar week of virus isolation (horizontal controls); and (2) the illness attack rate for all other children in residence during the calendar week(s) when the adenovirus infection was occurring in the test group (cross-sectional controls). In this way, for example, adenovirus types 1, 3 and 5 were associated with clinically undifferentiated definite illness, whereas adenovirus type 2 was not (Bell et al 1961).

It is of interest that the type 4 and type 7 adenovirus vaccines used in military recruits are live enteric-coated preparations which are administered orally. They infect the small intestine, are shed in the faeces, induce serum neutralizing antibody and are effective in preventing acute respiratory disease caused by these two serotypes (Edmondson et al 1966, Top et al 1971a,b). So, in a sense, these are 'enteric' adenoviruses, since they are multiplying in the small intestine and are shed in the faeces, since the upper respiratory tract has been by-passed by the enteric coating. My plea is that we do not associate any virus with an enteric illness just because the virus is found in the faeces in association temporally with an illness.

With regard to the detection of adenovirus 40 and 41, Brandt et al at Children's Hospital in Washington, DC have reported that as a general guideline, the quantity of adenovirus particles visualized by direct electron microscopy in a stool specimen from a patient with gastroenteritis could provide strong presumptive evidence for the presence or absence of enteric adenovirus types 40 and 41 (Brandt et al 1984). They showed that the mean adenovirus concentration in a stool specimen which contained enteric adenovirus types 40 or 41 was eight times greater than that observed in specimens containing a non-enteric adenovirus. This rapid procedure could prove useful in the clinical setting for a presumptive diagnosis.

Kurtz: Just to look at diagnosis from another angle, do the children who have type 40 or 41 infections develop antibody rises to the infecting serotype?

Wadell: Yes; Ingrid Uhnoo had serum pairs; 12 of 18 children showed a type-specific seroconversion (Uhnoo et al 1984).

Greenberg: There are two newer methods for detecting adenoviruses; one is the polyacrylamide gel electrophoresis silver-stain method, looking for a large band of nucleic acid at the top of the gel and then using specific restriction enzymes to confirm that the nucleic acid comes from an enteric adenovirus. The other method involves monoclonal antibodies specific for types 40 and 41. What would you recommend as the method to use in a laboratory that occasionally wants to identify adenoviruses?

Wadell: I would cleave that band of nucleic acid; it doesn't involve much work, and it gives all the information, after electrophoreses on agarose gels. Dr Singh-Naz has monoclonals reacting with a polypeptide of the same size as polypeptide VII (Singh-Naz & Naz 1986) that are reported to distinguish Ad40 and Ad41, and Dr Blacklow has monoclonals that also distinguish the enteric adenoviruses.

Blacklow: My colleague, John Herrmann, and I have developed type-specific monoclonal antibodies reactive solely with adenovirus types 40 or 41. The monoclonals react by radioimmunoprecipitation against a type-specific component of the hexon of the virus. The monoclonal against adenovirus 40 neutralizes this virus, and does not react by ELISA against any of the other adenovirus serotypes. Similarly, the monoclonal antibody against adenovirus 41 neutralizes this virus, and does not react by ELISA against any of the other adenovirus serotypes. We have used these two monoclonal antibodies in ELISA tests for direct detection of human enteric adenoviruses in stool specimens. We tested 24 stool specimens known to contain adenovirus type 40 by DNA pattern and 35 stool specimens known to contain adenovirus type 41 by DNA pattern. Twenty-three of 24 adenovirus 40 stools were detected by the monoclonal ELISA, as were 34 of 35 adenovirus 41 stools. The adenovirus 40 monoclonal ELISA did not react with adenovirus 41-containing stools, and the adenovirus 41 monoclonal ELISA did not react with adenovirus 40-containing stools. Neither monoclonal ELISA reacted with 12 stools containing non-enteric adenoviruses, nor with normal stools or with faecal specimens containing rotavirus or Norwalk virus (J.E. Herrmann & N.R. Blacklow, unpublished work).

Saif: Is anything known about the exact site of replication in the intestine of the human enteric adenoviruses, why they tend to persist, and also the cells in which the human oral vaccine strain replicates in the intestine?

Wadell: The information is very scanty. There was a child who died from infection with an enteric adenovirus in England (Whitelaw et al 1977). We did the restriction analysis and identified this specimen as Ad41. You could see inclusions in the duodenal cells of this child.

The Ad4, Ad7 and Ad21 vaccines represent a different issue. They have been evaluated in adults. We know that respiratory adenoviruses like Ad7 and Ad3 cause severe, sometimes fatal disease in children under the age of two. The seldom cause such a severe disease at older ages. The military recruits sleep together in one room and are exposed to very high infectious doses and can be very ill; that is why the vaccine has been reserved for them. You cannot directly compare the growth of Ad4 or Ad7 vaccine in adults with the replication of enteric adenoviruses in small children. I know nothing about the cell types where the vaccine virus grows.

Hall: The Belgian work on pigs suggests that the target cell is the mature enterocyte (Ducatelle et al 1982). This work also suggests that infection, but not lesions, persists for up to 40 or 50 days in these cells. You can see large intranuclear inclusions for 40–50 days.

Bishop: Adenovirus particles have been seen in the nucleus of mature epithelial cells obtained by duodenal biopsy of a Melbourne child with acute diarrhoea, thought to be caused by graft-versus-host disease (C.W. Chow, personal communication).

Flewett: There was another fatal adenovirus infection, in Melbourne in fact,

in a child. I was shown the electron micrographs from this child and there was no doubt that the enterocyte nuclei were full of adenoviruses.

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Astroviruses: human and animal

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Abstract. The name astrovirus was used by Madeley and Cosgrove in 1975 to describe a small round virus (approximately 28 nm diameter) with star-like appearance on electron microscopy. It was first seen in faeces from a few children with gastroenteritis. An aetiological role in gastroenteritis has since been confirmed. The virus causes a mild illness after an incubation period of 3–4 days. Antibody studies indicate that infection is widespread and, in Britain, mainly occurs in the 2–5 year age group. Outbreaks occur in, for example, institutions and paediatric wards. The virus usually spreads by the faecal–oral route but food- or water-borne outbreaks have occurred.

Strains of astrovirus have been isolated from many animals including calf, lamb, pig, cat, dog, duck and turkey. The lamb strain can cause gastroenteritis but the bovine strain did not cause diarrhoea in gnotobiotic calves. Infected turkeys have scours, and infection in ducklings causes haemorrhagic hepatitis with a mortality up to 25%. Five human serotypes have been described, all antigenically distinct from the bovine and ovine strains. The human astrovirus does not replicate in conventional tissue cultures but undergoes a non-productive cycle in human embryo kidney cells, and productive replication in the presence of trypsin. It is a positive-strand RNA virus, which is acid stable (pH 3), survives at 60°C for five but not 10 minutes and, like the enteroviruses, resists inactivation by alcohols. It has a density of 1.35–1.37 g/ml in caesium chloride.

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Astroviruses were so called in 1975 (Madeley & Cosgrove) because of their characteristic 5–6-pointed star-like form. Earlier in 1975, Appleton & Higgins described an outbreak of diarrhoea in some infants in a maternity unit which they associated with a virus of this appearance seen in the affected infants' faeces. These reports were of human infection, but in retrospect a distinct type of hepatitis in ducks due to an astrovirus had been described by Mansi et al in 1964. Subsequently, astroviruses have been detected in a wide variety of animals including sheep (Snodgrass & Gray 1977), calf (Woode & Bridger 1978), pig (Bridger 1980), dog (Williams 1980), cat (Hoshino et al 1981), deer (Tzipori et al 1981), turkey (McNulty et al 1980), duck (Gough et al 1984) and mouse (Kjeldsberg & Hem 1985). In some of these species infection is associated with illness; in others the association is, at best, doubtful. Where studies have been

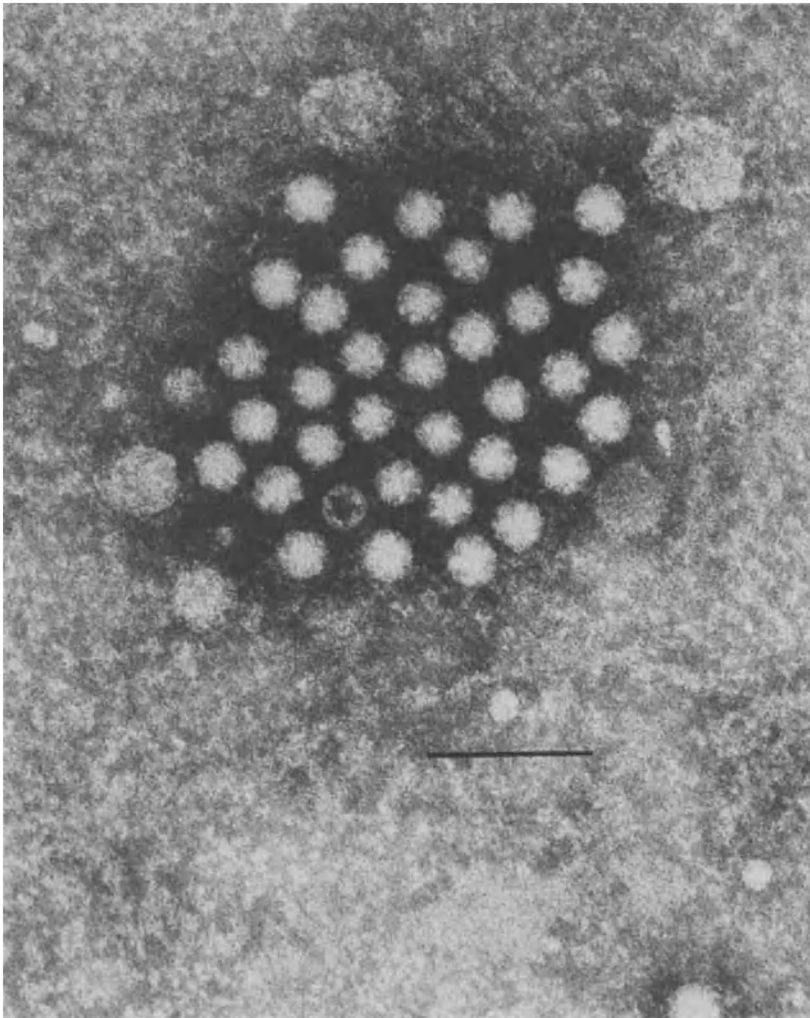

FIG. 1. Negatively stained (methylamine tungstate, pH 6.6) astrovirus particles from a human faecal sample. Bar, 100 nm.

done, no antigenic relationship has been found between viruses from the different species.

The virus

Astroviruses have a smooth or slightly crenated round outline with a solid star-shaped core (Fig. 1). Bridging structures (Snodgrass & Gray 1977)—

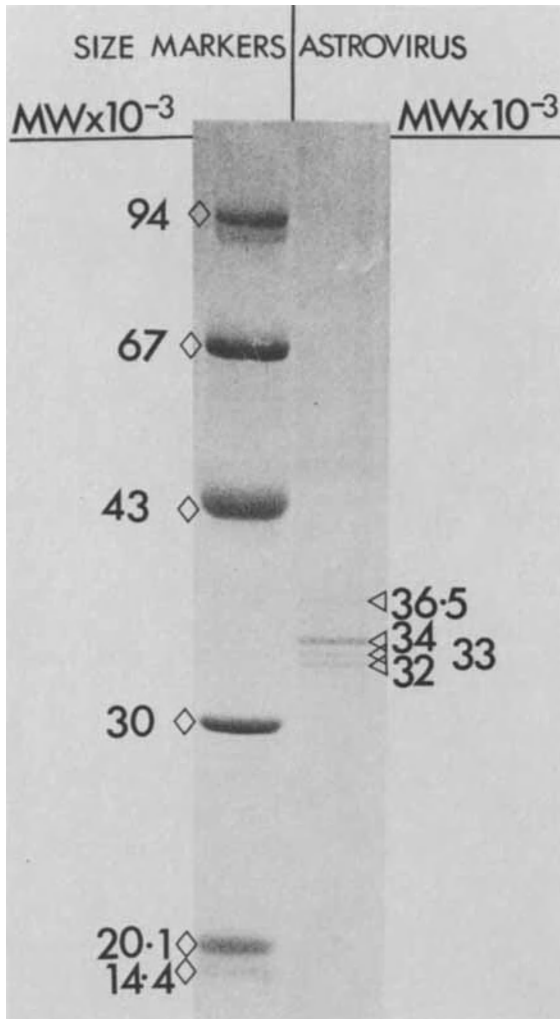

FIG. 2. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis of polypeptides from tissue culture-grown human astrovirus (serotype 4). (A. Easton, Warwick University.)

external projections associated with the points of the star—are occasionally seen. Not all particles show the characteristic appearance and in its absence identification may be impossible. Aggregation of the virus with antibody also interferes with its appearance. The diameter of the virus is 28–30 nm. Its density in CsCl is 1.35–1.37 g/ml. The ovine strain (Herring et al 1981) has a single-stranded RNA and only two major capsid polypeptides of similar relative

molecular mass (M_r), 33 000. The human virus likewise has a positive-strand RNA genome, of approximately 7500 nucleotides. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) of the human virus shows it to have four polypeptides of M_r 36 500, 34 000, 33 000 and 32 000 (Fig. 2). The largest of these is very faint and, by analogy with the enteroviral virus polypeptide O (VPO), may represent a precursor to one of the others. As the gel only resolved polypeptides down to M_r 14 000, a smaller polypeptide equivalent to the VP4 (M_r 5500) of enteroviruses could not have been detected (A. Easton, personal communication 1986). This pattern suggests that astroviruses are members of the picornavirus family. Physical characteristics of the human astrovirus include stability to acid (pH 3) and 10 minutes shaking in chloroform, and survival at 60°C for five but not 10 minutes.

The virus does not replicate in conventional tissue culture systems but human strains will undergo a non-productive cycle of replication in primary human embryo kidney (HEK) cells. There is no cytopathic effect but virus may be detected in the cytoplasm by fluorescent antibody techniques in 24–48 h infected cells. When 10 µg/ml crystalline trypsin is included in a serum-free medium, virus is released from infected HEK cells and a productive infection established (Lee & Kurtz 1981). Tissue culture-grown virus is morphologically and antigenically indistinguishable from faecally derived virus and after passage in HEK cells some strains can be adapted to a continuous line of rhesus monkey kidney cells (LLCMK2). In infected cells crystalline arrays of virus are seen in the cytoplasm adjacent to vacuoles (Kurtz et al 1979).

Infections in man

Astroviruses have a world-wide distribution. Infection may occur throughout the year but the peak incidence is in winter/spring in temperate zones. Overt illness is commonest in 1–3-year-old children; an antibody prevalence survey in Oxfordshire showed a rise from 4% in 6–12-month-olds to 64% in 3–4-year-olds and 87% in the 5–10 year age group. Symptomatic infection was found in 62 of 79 (80%) babies infected with astroviruses (Madeley 1979) while 11 (12%) had no diarrhoea and in six (8%) an association was doubtful. The frequency of re-infection and the likelihood of it being symptomatic are not known, but in the presence of detectable serum antibody, infection of volunteers did not result in diarrhoea (Kurtz et al 1979).

There are at least five different serotypes of human astrovirus (Kurtz & Lee 1984), demonstrable by immunofluorescent tests and immunosorbent electron microscopy (ISEM, Roberts & Harrison 1979) using rabbit antisera to various tissue culture-grown strains of the virus. Fig. 3 shows the virus counts of the heterologous reactions as a percentage of the homologous reactions for the five serotypes, using ISEM in which the grids were pre-coated with antisera to increase the adsorption of the homologous virus. Community-acquired strains

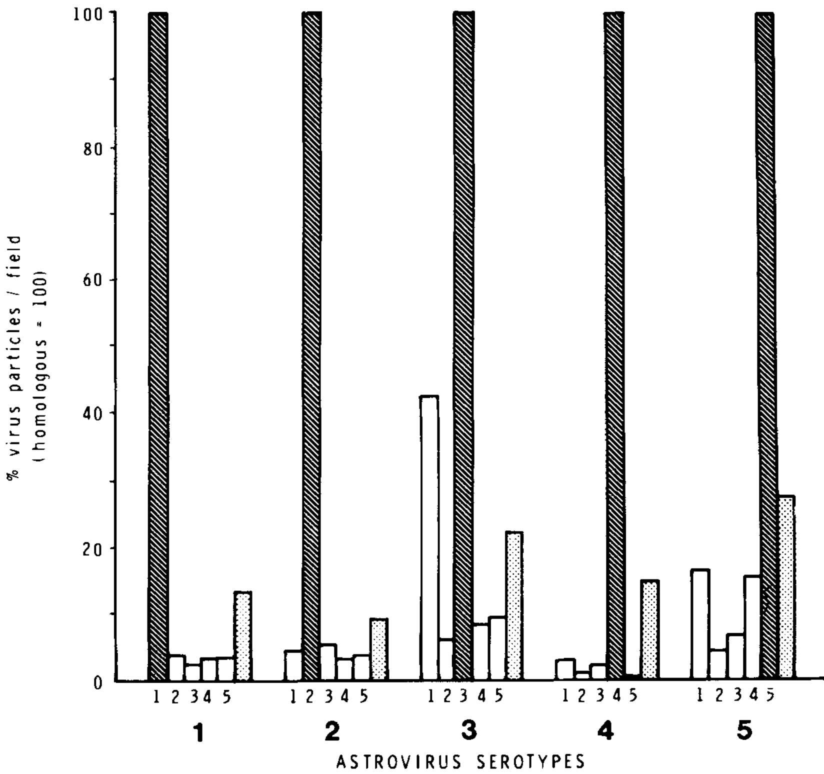

FIG. 3. Immunosorbent electron microscopy of astrovirus strains using anti-astrovirus sera. Cross-hatched bars indicate homologous reaction. Stippled bars indicate adsorption to uncoated grid. (Reproduced from Kurtz & Lee 1984 with permission of *The Lancet*.)

of astrovirus collected since 1975, mainly from the Oxford region, show the following distribution of serotypes: 72% type 1, 8% type 2, 8% type 3, 6% type 4 and 6% type 5.

Virus is transmitted by the faecal–oral route, either directly or via fomites, food or water. Person-to-person spread commonly occurs in the family, nursery and paediatric ward where infection may be endemic. Outbreaks have been associated with eating oysters (E.O. Caul, personal communication 1981) and drinking water from a contaminated culvert (W.D. Cubitt, personal communication 1986).

The clinical features of symptomatic infection include not only diarrhoea but also systemic signs and symptoms (Fig. 4). After an incubation period of 3–4 days, fever (≥37 °C), headache, malaise, nausea and occasionally vomiting may occur. Astroviruses become detectable in the faeces at this time, to be followed within a day by diarrhoea which is typically unformed or watery. Diarrhoea (2–6

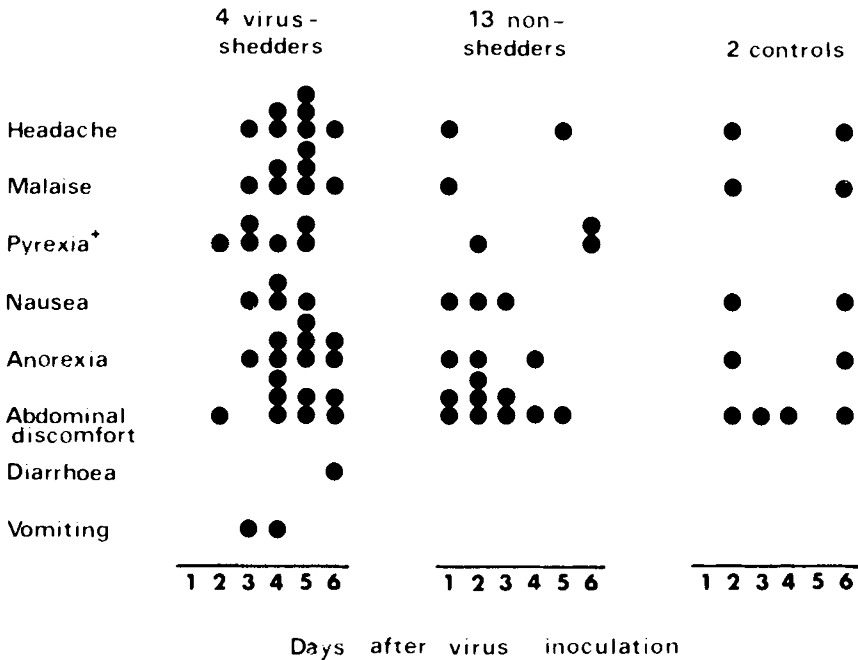

FIG. 4. Clinical responses of 17 volunteers inoculated with astrovirus diluted in orange squash in whom virus shedding was or was not detected, and in two controls who were given diluent alone. Each point represents the recording of a symptom or sign in one volunteer on each day after virus inoculation. Pyrexia$^+$ = a temperature $\geq$ 37.2 °C. (Reproduced from Kurtz et al 1979 with permission Alan R. Liss, Inc.)

motions/day) usually lasts only 2–3 days but may continue for 7–14 days and is accompanied by virus excretion. More persistent infection may occur in immunocompromised patients. Thus, one child with combined immunodeficiency who was given a bone marrow transplant became infected with astrovirus several weeks later. This infection, which was associated with diarrhoea and virus shedding, persisted for more than four months, until the child's death (E. Davies, personal communication 1986).

Duodenal biopsy during astrovirus infection has shown that the virus infects epithelial cells of the lower part of the villi (Phillips et al 1982).

Direct electron microscopy (EM) is the most useful method for detecting astroviruses in the routine laboratory. In the acute illness up to 10^{10} virus particles/ml faeces are present, although viable counts are lower (10^8/ml). This exceeds the lower limit of sensitivity of EM (10^6/ml). An advantage of EM is that other potential pathogens may be found in a specimen. Nazer et al (1982) reported that of 28 children infected with astrovirus 16 were co-infected with another enteric pathogen, 11 with rotavirus, four with bacteria (*Salmonella* spp.

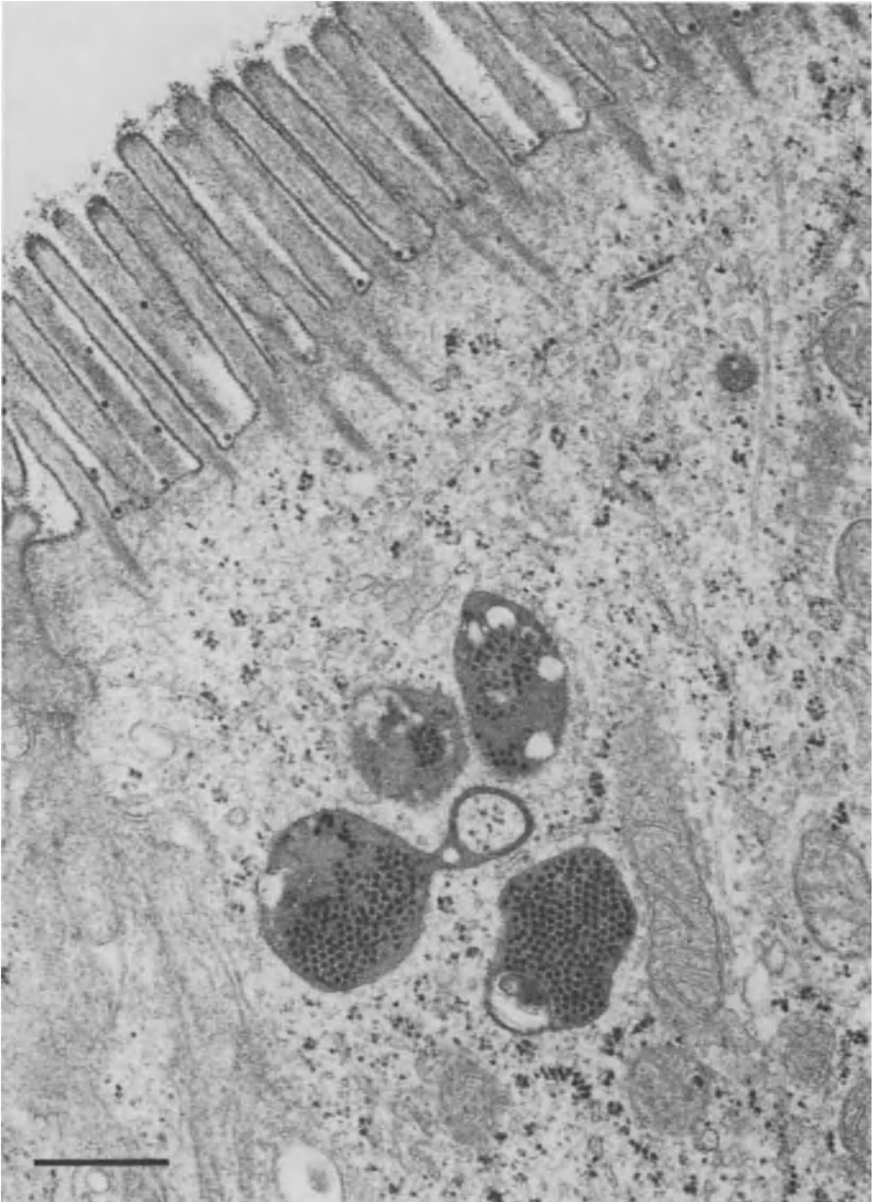

FIG. 5. Astrovirus particles inside autophagic vacuoles and free in the cytoplasm of a mid gut apical enterocyte, 38 h after infection of a gnotobiotic lamb. Bar, 400 nm. (E.W. Gray, Moredun Research Institute.)

or enteropathogenic *Escherichia coli*) and one with both rotavirus and bacteria. If more than one pathogen is found it is often not possible to establish the aetiology of the diarrhoea.

Infections in animals

The first species other than man in which astrovirus infection was observed was the sheep. Snodgrass & Gray (1977) reported an outbreak of diarrhoea in 4–6 week-old Suffolk lambs on a farm. Astroviruses were seen in the faeces of eight of 17 lambs examined. The virus was passaged twice through gnotobiotic lambs in which virus was excreted. It was associated with a yellowish diarrhoea developing on the 4th day after ingestion and lasting for two days in one lamb that was not sacrificed early. Virus excretion persisted from the 3rd to the 9th day in this lamb. After experimental infection (Snodgrass et al 1979), lesions appeared scattered throughout the small intestine by 23 h and were maximal in the mid gut and ileum at 38 h, after which gradual healing led to resolution by the 5th day. Virus replicated only in mature columnar epithelial cells situated in the apical two-thirds of the villi. These cells developed vacuoles and were replaced by immature cuboidal cells from the crypts, resulting in partial villus atrophy. Virus was seen by EM in the cytoplasm of these columnar cells 14–38 h after infection (Fig. 5). It was present as crystalline arrays, in apical pits and tubules in the microvillus border (which were suggested as the site of entry of the virus) and in autophagic vacuoles (Gray et al 1980).

In 1978 a bovine astrovirus was recognized by Woode & Bridger. A mixed infection with small round viruses, one of which was morphologically an astrovirus, caused diarrhoea in a calf. Experimental infection of gnotobiotic calves with this astrovirus produced no illness and the virus was considered non-pathogenic in this species. More recently (Woode et al 1984), single and dual infections (quite common in nature) with either bovine rotavirus or Breda virus 2 in gnotobiotic calves have confirmed the absence of illness with astrovirus infection alone, although the faeces became yellow and soft at the time of virus excretion (2–9 days after infection). Scours developed in the animals with dual infections or with Breda 2 or rotavirus infections alone. Astrovirus infection was confined to specialized M epithelial cells of the domes overlying Peyer's patches in the jejunum and ileum of the calf. As in the sheep, infected cells showed degenerative changes and were sloughed and replaced by cuboidal cells. The limited number of susceptible cells in the bovine gut compared to that of the sheep might well explain the lack of illness in the former. Infection however, is common, as shown in a serological survey in Iowa where 30% of calves had astrovirus antibodies. By cross-neutralization tests using antisera raised in gnotobiotic calves to two USA isolates and one British astrovirus isolate, three serotypes have been identified. More serotypes are suggested by the fact that

when 16 field isolates were tested, five could not be classified by the above system (Woode et al 1985).

Astroviruses have been reported in several other mammals. A feline strain has been reported in a four-month-old kitten with diarrhoea. The illness persisted for two weeks and required hospitalization (Hoshino et al 1981). Astrovirus was seen in five of 56 artificially reared red deer fawns who developed diarrhoea. The deer produced antibody to their virus (Tzipori et al 1981). In the dog, astrovirus was seen in diarrhoeal stools of beagle pups (Williams 1980). Its significance was difficult to ascertain because in each case the faeces contained a variety of viruses. The five pups were infected with astrovirus and typical coronaviruses, both of which disappeared when the diarrhoea stopped. In addition, one pup had a parvovirus, and two had atypical coronaviruses in their faeces which persisted after the pups recovered. An astrovirus has also been detected in three-week-old piglets who developed diarrhoea 3–4 days after weaning (Bridger 1980). Again the faeces contained a number of viruses (astro-, calici- and rotavirus-like, and enterovirus), making attribution of significance impossible.

The same problem of significance occurs in the turkey. Astroviruses have been seen in association with rotaviruses in two outbreaks of scours which caused an increase in mortality in the flock (McNulty et al 1980). Astrovirus has also been detected in the faeces of young chickens with scours but the virus does not appear to cause disease in chicks kept under laboratory conditions (R.E. Gough, personal communication 1986).

The most serious disease caused by astrovirus in the veterinary world is in the duck. Heavy losses, with a mortality rate up to 25% in 3–6-week-old birds, were noticed in fattening ducklings kept on open fields in Norfolk in 1983. The birds died acutely of hepatitis (Gough et al 1984), the livers showing haemorrhages, widespread necrosis of hepatocyte cytoplasm and usually bile duct hyperplasia. Experimental transmission of a virus, morphologically similar to astrovirus, from infected liver and faeces caused a haemorrhagic hepatitis 2–4 days after inoculation in five of 20 2–3-day-old ducklings. Mature ducks were unaffected by the infection. A similar disease caused by an agent serologically distinct from classical duck virus hepatitis virus had been described in 1964 by Mansi et al and was named duck hepatitis (DH) type II virus (Asplin 1965). Cross-protection experiments using sera and vaccines to the classic DH type I and DH type II viruses have shown that the astrovirus is closely related to the DH type II virus. The virus replicates with difficulty in chick embryos but one strain has been adapted to growth in them. Although no wild-life reservoir has been detected it is possible that wild birds may transmit the disease, especially as outbreaks have initially involved ducks kept on open fields. Control by vaccination with the attenuated chick embryo-adapted strain, or by reducing contact with wild birds by housing the ducks, has been successful; nevertheless, the disease continues to be a serious problem in East Anglia.

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DISCUSSION

Caul: In your seroepidemiological work in the Oxford region you found that about 75% of adults had antibody to astrovirus. In the laboratory, we detect astrovirus in perhaps 5–10% of symptomatic neonates or children. There are several possible explanations for this discrepancy. One is that subclinical infection with astrovirus is common. Alternatively, the symptoms are so mild that medical intervention is not warranted. There is support for this view from the Japanese study (Konno et al 1982), where only one of 46 ill children in a kindergarten required treatment. Another possible explanation is misidentification, because the characteristic surface star structure is lost when antibody is added to astrovirus preparations. This brings to mind the Marin County agent in the USA, which caused an outbreak of gastroenteritis in a geriatric population. It was described as Norwalk-like, but it resembles an astrovirus in the published immuno-electron micrographs. Immuno-electron microscopy is a useful tool for establishing an aetiological role, but should not be recommended where precise identification is needed.

Kapikian: The Marin County agent is a 27 nm virus detected by Oshiro et al (1981) in stool specimens from a gastroenteritis outbreak in elderly patients in a convalescent home in 1978, in Marin County, California. We also examined this agent by electron microscopy, and I agree with Dr Caul that it had the appearance of an astrovirus in certain preparations without antibody. However, only a few particles had the characteristic star-like appearance, as we had to examine many particles to observe the infrequent one which had that appearance, reminiscent of the astroviruses. Further studies have been carried out by Drs Midthun, Walsh, Greenberg et al, which Dr Greenberg can describe.

Greenberg: This was work done primarily by Karen Midthun. The Marin County agent seems to be an astrovirus, because its morphological characteristics are similar to those of astroviruses, and also because the purified virus, when iodinated and run on a polyacrylamide gel, has one or two protein bands in the M_r range of 30000. Thus it is not a calicivirus-like particle, because it has smaller molecular weight protein(s) than caliciviruses.

Marin County virus was given to volunteers, with results strikingly similar to those of Dr Kurtz's astrovirus volunteer study (Fig. 4, p 97) where, of 17 volunteers, only one had serious diarrhoeal disease. We gave Marin County virus to 18 volunteers; only one developed a clear-cut diarrhoeal disease, on the fifth and sixth days after inoculation. This contrasts with studies of Norwalk virus, where almost invariably the majority of inoculated volunteers become ill.

Finally, the Marin County epidemic occurred in an old-age home; aside from this outbreak, are there reports of other astrovirus outbreaks in adults, including the elderly? Mostly astrovirus infections seem to be occurring in young children.

Cubitt: We have recently studied an outbreak due to astrovirus type 1 in an

old people's home. Over a period of 22 days, 34 (80%) of the residents and 13 (44%) of the members of staff were affected. The illness was characterized by vomiting, diarrhoea and abdominal pains with a duration of 48 hours. Serological studies showed that patients developed significant antibody responses to astrovirus type 1 (titres rising from 20 to ≥160) (J. Gray, T. Wreghitt & W.D. Cubitt, unpublished work).

Kurtz: Astrovirus infections are mainly in children, either in paediatric wards or as sporadic cases; rarely they affect adults. There have been a few food-associated outbreaks of astrovirus diarrhoea in the UK in adults; the first one to be associated with the eating of oysters was described by Owen Caul (personal communication; see p 96).

Caul: Another small round virus from the USA, the Snow Mountain virus, also presents problems of identification as a result of applying the technique of immuno-electron microscopy. Do you know whether that is a Norwalk type of virus, or an astrovirus?

Kapikian: It is a Norwalk-like virus morphologically and has been shown by Madore et al to contain one major structural protein of M_r 62 000 (Madore et al 1986).

Caul: The astrovirus outbreak associated with oyster eating (unpublished observations) was in a naval base after an officers' dinner. About 24 hours after the consumption of the oysters, many of the officers went down with an apparently classical Norwalk virus infection. Disappointingly, the only virus found by electron microscopy was a small round featureless virus (which we call 'parvovirus-like') whose pathogenicity is unproven. Four days later, after recovery from the primary illness, the patients went down again with diarrhoea; this time they excreted large numbers of astroviruses. My feeling is that the first episode was probably due to a Norwalk-like virus which we were unable to detect and that the second episode was due to astroviruses, in a population who were probably susceptible to both viruses.

Bishop: Are you suggesting that they got a dual infection from the oysters?

Caul: Yes. Oysters are known to concentrate Norwalk-like viruses, so I expect that they concentrate other small round viruses as well.

Bishop: And the two episodes were due to the differing incubation periods initially of the two viruses?

Caul: That would be my interpretation.

Appleton: I would agree that this dual outbreak reflects the different incubation periods of two viruses. Shellfish frequently transmit several different viruses at once and mixed infections within one outbreak are quite common. In recent months we have examined specimens from several gastroenteritis outbreaks associated with molluscs and detected at least two different viruses in the majority of these outbreaks; and it is of course not unknown for persons to develop gastroenteritis 24–48 hours after eating shellfish and then hepatitis A three weeks later.

Mixed infections are probably responsible for the wide range of incubation

periods recorded within many other food-borne outbreaks of viral gastroenteritis.

Kurtz: Mixed viral infections are probably far more common than we realize. This makes determining the cause of an outbreak, or a single case, of gastroenteritis very difficult. Mixtures of rotaviruses, adenoviruses, astroviruses and other small round viruses occur in both humans and animals. In fact, in the latter, mixed infections are almost the rule, rather than the exception.

Kapikian: I am interested in the view of the veterinary experts here on the suggested classification of astroviruses as picornaviruses. For an ovine astrovirus, Herring et al (1981) described the presence of two polypeptide species in about equimolar amounts with molecular weights of about 33000. In addition, Konno et al (1982) have described the density of astroviruses from a kindergarten outbreak of acute gastroenteritis in Japan to be 1.39–1.40 g/cm^3 in caesium chloride. Thus, on the basis of morphology, polypeptides and density considerations, I thought that astroviruses were considered to be a separate family of viruses. Is there general agreement that they are picornaviruses? I have never seen a picornavirus with this 'star' appearance of astroviruses.

Kurtz: The molecular weight profile of the proteins in our culture-grown astroviruses looked more like picornaviruses than anything else; that is as far as we have gone.

Horzinek: Is anything known about the genome?

Kurtz: Only that it is positive-stranded RNA.

Horzinek: How do you know? Is it infectious? Has anybody put extracted RNA into cells and found progeny virus?

Kurtz: That has not yet been done.

McCrae: What is the evidence then that the RNA is positive-stranded?

Greenberg: It is polyadenylated, so it is likely to be positive-stranded RNA.

Woode: All known bovine astrovirus isolates share a common immunofluorescent (IF) antigen, but can be subdivided into serotypes by neutralization. We routinely screen for bovine astrovirus in faeces by IF of 24 h infected cell cultures. This is the most sensitive method, as there are few particles observed by electron microscopy but there is an infectivity titre of 10^3 to 10^4. This approach might be useful for the isolation of human astroviruses.

Horzinek: Is there an antigenic relatedness between any of the human serotypes and the duck hepatitis virus?

Kurtz: I don't know if that has been looked at. There is no antigenic cross-reaction between any of the animal and human astroviruses where it has been investigated. We examined ovine/human and bovine/human reactions, but did not find any crossing. Dr Snodgrass has looked at the ovine/bovine reaction.

Snodgrass: Yes. But even with convalescent serum, which you found to have a broader specificity, the lamb, calf and human astroviruses studied by us did not show any cross-immunofluorescence (Snodgrass et al 1979).

Kurtz: The chicken and duck astroviruses are also unrelated.

Greenberg: I have been interested in whether the small round viruses are important causes of mild diarrhoea in children. In young children, Norwalk virus causes a mild diarrhoea that does not take children to hospital and is frequently not seen by a physician. Over time, however, repeated episodes of mild illness may be an important cause of malnutrition. I wonder whether anybody has information on astroviruses, or any of the other small enteric viral pathogens, on the role of such viruses in children in developing countries. Do they cause mild diarrhoea in these countries?

Mathan: When we studied nearly a thousand cases of acute gastroenteritis in children in southern India, presenting at outpatient departments, astroviruses accounted for only 1.7% of cases. All children in whom astrovirus was found had a mild illness.

Bishop: Have you studied many children with malnutrition who may be excreting these small viruses chronically?

Mathan: No: I have no data on such children.

Cubitt: We recently looked at 200 faecal specimens from the Medical Research Council Unit in The Gambia, and found that only three patients with symptoms of diarrhoea were excreting astrovirus.

Kurtz: In immunodeficiency syndromes, some children have excreted astroviruses for several months, with liquid, rather unpleasant motions during that time. In normal children, virus excretion usually continues for only 4–5 days, occasionally for 10 days.

Caul: I am not aware of any report in immunologically normal children that chronic excretion of astrovirus, detectable at the electron microscope level, occurs. It certainly occurs in immunosuppressed children. We had a recent case of a leukaemic child who died from pneumonia. After autopsy we saw more astroviruses by electron microscopy in the small intestinal contents than we had ever seen previously. The child did not have diarrhoea, and there was no villous atrophy.

Bishop: How common are particles with the star-like appearance? Is the star something that you see only when the staining happens to be right, or is it a consistent finding in all astrovirus preparations?

Caul: We find phosphotungstic acid to be the best stain. In our experience, uranyl acetate or ammonium molybdate are not as good. With PTA, 5–10% of particles show the surface star reliably. Whether this is a 'real' star, I don't know!

Woode: In experimental infections, where you know that the virus is there, you always see some recognizably star-centred particles.

Holmes: Everybody who has looked at astroviruses, including Dr Madeley who first named them, has found that only a proportion of the particles show the stars. The few astroviruses that we saw were like that. It is probably a question of how the particles are oriented on the electron microscope grid.

Woode: The frequency of stars is quite high with bovine astrovirus, of one in 8–10 particles or better.

McNulty: In my experience with astrovirus, the stars may not be evident on the electron microscope screen, but in a photographic print it is easier to see the star-shaped profiles.

Flewett: Dr Madeley made that same point.

Wadell: I am wondering about the ubiquitousness of astroviruses. It occurs to me that they could play some part in less symptomatic diarrhoea. Are there any serological studies in populations who eat less shellfish?

Kurtz: We have looked at Zulus in South Africa and also at sera from North America and Australia. Antibody acquisition rates were similar to the rate in Britain.

Holmes: Roger Schnagl's studies of Aboriginals in central Australia showed that astroviruses were present in about 2% of Aboriginal samples, whether or not the patients had diarrhoea. That area is a long way from any shellfish! (Schnagl et et al 1979.)

Cubitt: We have tested the same batches of gammaglobulin as were used by Dr Bridger in her rotavirus studies. We found that in every country where we could get pooled immune globulin, namely Japan, South Africa, Belgium, France, Switzerland, Canada and the USA, there were very high levels of antibody to astrovirus type 1. The highest titre was in the American gamma globulin (Hyland Laboratories, Batch 2703 M00 AA).

Bishop: Would it be fair, then, to say that we believe astrovirus to be a common infection in animals and humans, and seldom a cause of severe diarrhoea, except perhaps in poultry?

Appleton: Although in general the symptoms are mild, in the first outbreak that we looked at (Appleton & Higgins 1975), in newborn babies in a maternity unit, the original symptoms of gastroenteritis were relatively mild but those babies went on with malabsorption problems for many weeks. At this period bottle-feeding was fashionable, and I think every affected baby was bottle-fed. It was not possible to get many of them back onto full-strength feeds for several weeks. So there may be longer-term effects.

Bishop: Perhaps there is a case, then, for a proper study of astrovirus infection in children in developing countries.

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Small round viruses: classification and role in food-borne infections

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Abstract. Since the first observation of Norwalk virus in the electron microscope in 1972, many different small virus particles in the size range 20–40 nm have been described world-wide in association with outbreaks of gastroenteritis. Progress in characterizing these agents has been hampered by the relatively small numbers of particles present in clinical material and the lack of success in culturing them. Although the relationship between some of these viruses remains confusing, a number of distinct groups has emerged, based on morphological features and limited physical data. Immuno-electron microscopy has proved valuable in detecting viruses but the addition of antibody can mask surface morphological features. Examination of viruses in negatively stained preparations without added antibody has revealed distinct morphological differences and viruses previously thought to be simply antigenic variants within the Norwalk group of viruses clearly belong to other groups. Preliminary evidence suggests that one human virus unrelated to Norwalk has a single-stranded DNA genome and is a parvovirus. Some groups have been implicated in outbreaks of food-borne gastroenteritis, particularly after the consumption of shellfish, and their role in other food-borne and water-borne outbreaks is being increasingly recognized.

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The use of electron microscopy since the early 1970s for the examination of faecal specimens from persons with non-bacterial gastroenteritis has revealed a host of previously unknown viruses, most of which cannot be propagated in conventional tissue culture systems. Viruses with a strikingly characteristic appearance, such as rotavirus and adenovirus, generally present few problems of identification. However, there have been many observations of smaller viruses ranging in size from 20 nm to 40 nm. These viruses lack such distinctive morphological features and may occur only in small numbers, which makes them difficult to detect.

The first small round virus described was the Norwalk agent. In 1972, virus particles, 27 nm in diameter, were detected by immuno-electron miscroscopy in

volunteers fed filtered faecal suspensions from an outbreak of winter vomiting disease which had originated in a primary school and spread to the local community at Norwalk in the United States (Kapikian et al 1972). Morphologically similar viruses, known as the Montgomery County and Hawaii agents, were subsequently found by the same investigators in two family outbreaks of gastroenteritis (Thornhill et al 1977). By immuno-electron microscopy and cross-challenge studies in volunteers the Montgomery County agent appeared to be serologically related to Norwalk, whereas the Hawaii agent was distinct. Investigation of other school outbreaks of non-bacterial gastroenteritis revealed further small round viruses, such as the Wollan agent (Paver et al 1973) and the Ditchling agent (Appleton et al 1977) in the UK and the Parramatta agent (Christopher et al 1978) in Australia. Immuno-electron microscopy is a technique often applied to the detection of viruses, and from published micrographs it was initially thought that the viruses involved in these and many other similar outbreaks were simply antigenic variants within one virus group. However, careful observation of viral particles without a coating of antibody, which may mask the surface appearance, has clearly shown that these viruses belong to more than one group.

Viruses detected in gastroenteritis outbreaks are often named after the places where the outbreaks occurred (e.g. Norwalk, Ditchling), or the name reflects the event with which the virus was associated. For example, the cockle agent (Appleton & Pereira 1977) was implicated in a large outbreak of gastroenteritis linked to the consumption of cockles. Other small round viruses with the descriptive names 'astrovirus' and 'calicivirus' were initially observed in specimens from sporadic cases of gastroenteritis in babies, and later were also associated with outbreaks of gastroenteritis or winter vomiting disease, both in babies and in older age groups. The distinct morphological features and differentiation of these two viruses have been reviewed by Madeley (1979).

Failure to culture any of these agents satisfactorily has greatly limited the amount of material available for biochemical study and hence has delayed definitive classification. An interim classification scheme, based largely on morphology with some physical data, was recently proposed (Caul & Appleton 1982) and has been adopted by the UK Public Health Laboratory Service for reporting purposes.

Classification

Small round viruses occurring in faeces fall broadly into two main morphological groups:

(1) Featureless viruses with a smooth entire outer edge and no obvious surface structure.

(2) Viruses with a clearly visible surface structure and/or ragged edge.

TABLE 1 Classification scheme for small round faecal viruses

	Type	Physical features	Examples
Featureless viruses: Smooth entire edge and no discernible surface structure	Enterovirus	RNA BD 1.34 g/cm^3 Size range 20–30 nm	Polio Hepatitis A
	Parvovirus	DNA BD 1.38–1.46 g/cm^3 Size range 18–26 nm	Feline/mink/canine Bovine
	Candidate parvovirus	DNA? BD 1.38–1.40 g/cm^3 Size range 22–26 nm	Wollan, Ditchling, Parramatta, cockle

	Type	Morphology	Physical features	Examples
Structured viruses: Surface structure and/or ragged outline	Astrovirus	5–6-pointed surface star	RNA BD 1.36–1.38 g/cm^3 Size range 28–30 nm	Lamb Human
	Calicivirus	Surface hollows, ragged outline 'Star of David' configuration	RNA BD 1.36–1.39 g/cm^3 Size range 30–38 nm	Human (UK1–4 and Japanese strains) Newbury (bovine) Pig
	Small round structured virus (SRSV)	Amorphous surface, ragged outline	BD 1.36–1.41 g/cm^3 Size range 30–35 nm	Norwalk, Montgomery County, Hawaii, Taunton, Amulree, Otofuke, Sapporo, Snow Mountain

BD, buoyant density in caesium chloride.

Features of the various members of these two groups are summarized in Table 1 and the appearance of representative members of each type of virus is shown in Figs. 1 and 2.

Featureless viruses

Enteroviruses. Although enteroviruses may be readily isolated from faeces and may sometimes be seen in faecal specimens by electron microscopy, there have been few reports of their involvement in outbreaks of gastroenteritis. Enteroviruses are more usually associated with illnesses whose target organs are other than the gastrointestinal tract, such as poliomyelitis and aseptic meningitis.

Parvoviruses are DNA viruses and are slightly smaller than enteroviruses, but there is considerable overlap in size and the two groups are more readily distinguished by their different buoyant densities in caesium chloride. Some animal parvoviruses have been well characterized biochemically and some have been clearly linked to gastroenteritis, including bovine parvovirus, feline parvovirus and the antigenically related mink and canine strains. An asymptomatic carrier state occurs with most of these viruses.

Candidate human parvoviruses. There are several agents from outbreaks of gastroenteritis in man which resemble parvoviruses in their morphology, size and similar high buoyant density, but where final classification awaits the identification of the nucleic acid. In our laboratory, nucleic acid extracted from the cockle agent has been labelled *in vitro* and preliminary evidence suggests that the genome is single-stranded DNA. On the basis of this observation, nucleic acid extracted from gradient-purified virus was cloned into plasmid pBR 322. Two recombinant plasmids containing inserts of 0.9 and 1.1 kb have been obtained (J. Clewley, unpublished work). These cross-hybridize with the human serum parvovirus, B19, but sequencing studies so far indicate that the serum and faecal viruses are different. B19 is the first, and so far only, human virus that has been officially classified as a parvovirus, excluding adeno-associated virus (Siegl et al 1985). The B19 virus was initially detected in the serum of an asymptomatic blood donor and is now known to be the aetiological agent of the childhood illness, erythema infectiosum or Fifth disease. Early comparisons by immuno-electron microscopy indicated that the faecal viruses and B19 are serologically distinct (Paver & Clarke 1976) and these observations have been confirmed using the more sensitive technique of radioimmunoassay with a monoclonal antibody (B. Cohen & H. Appleton, unpublished work). So far, only one serotype of B19 is known, but there appear to be a number of different antigenic strains of the faecal viruses (Table 2). Although the serum antibody response to faecal parvoviruses is not as striking as with some other enteric viruses, antigenic differences can be demonstrated by immuno-electron microscopy. It is also possible to identify IgM antibody on particles mixed with convalescent-phase serum.

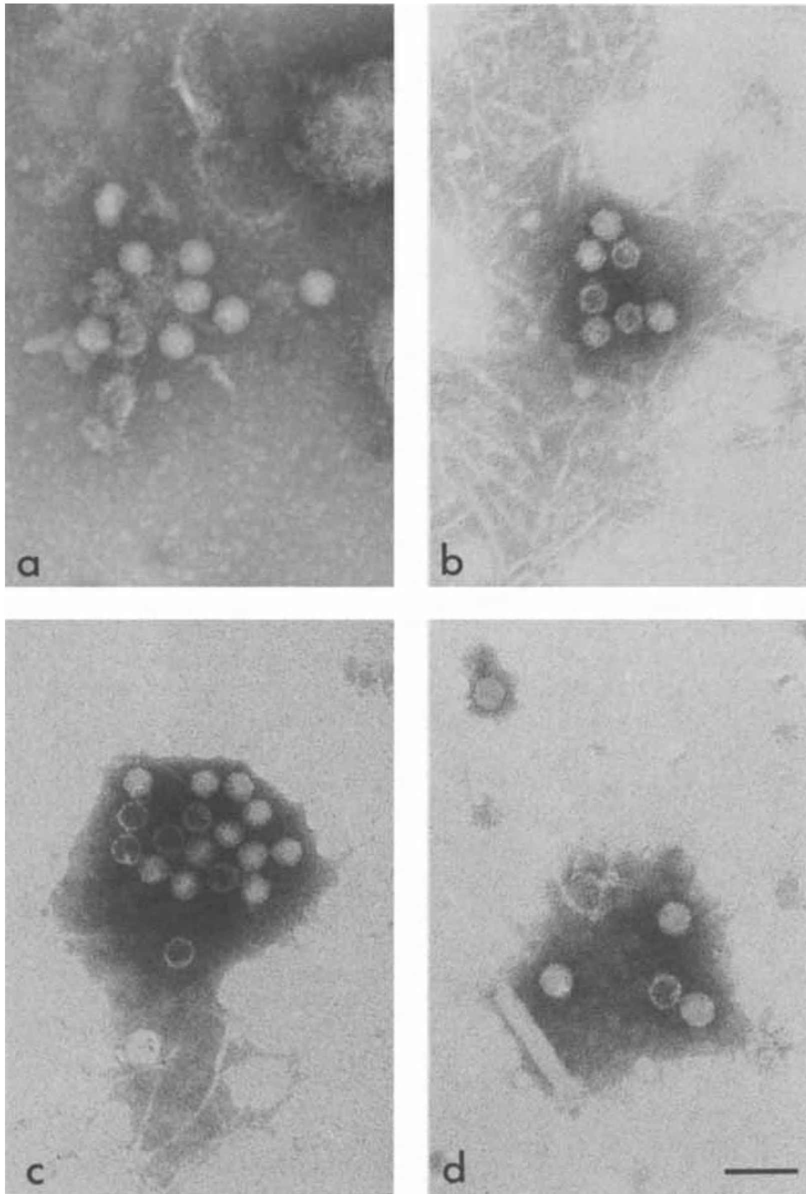

FIG. 1. Featureless small round viruses. (a) Enterovirus: poliovirus type 1. (b) Canine parvovirus. (c) Ditchling agent: virus from a primary school outbreak of winter vomiting disease. (d) Cockle agent: virus from a gastronomic outbreak associated with the consumption of cockles. In (b), (c) and (d) the viruses were purified on caesium chloride gradients. Some particles show the hexagonal outline characteristic of parvoviruses. Bar, 50 nm.

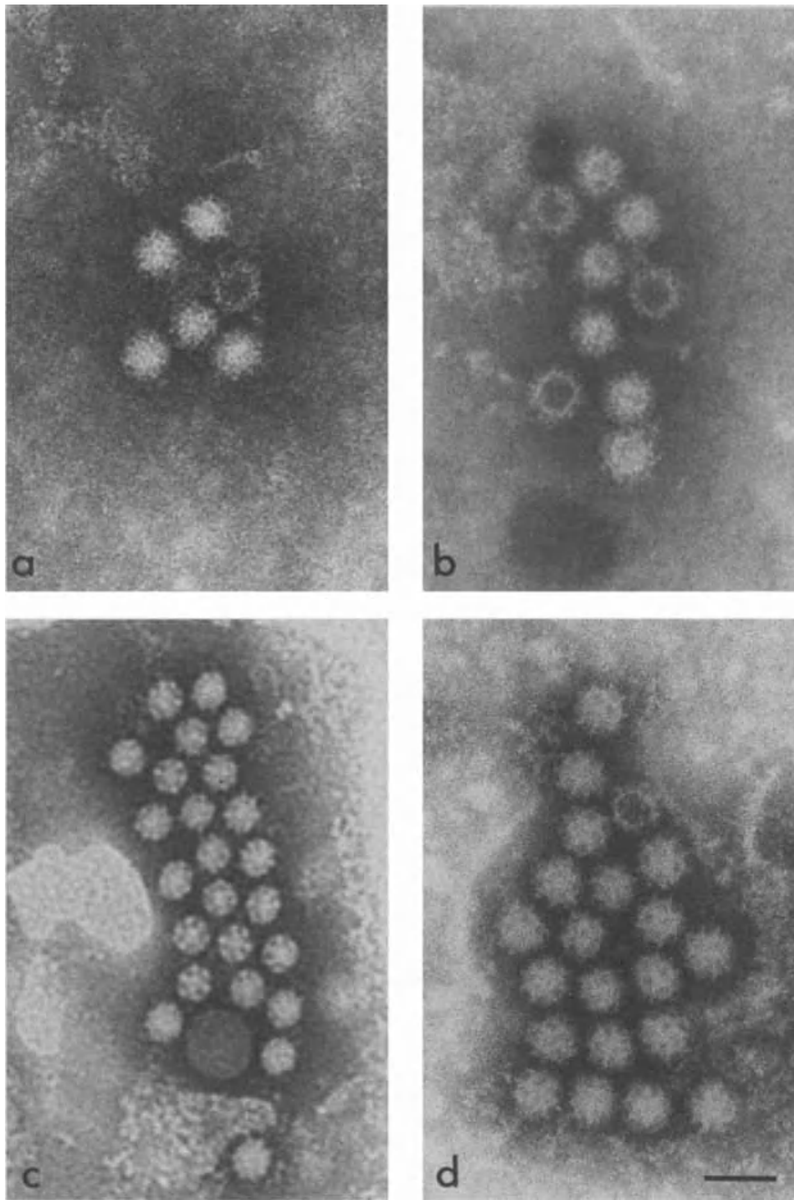

FIG. 2. Structured small round viruses. (a) Norwalk virus. (Micrograph by courtesy of Dr E.O. Caul.) (b) Amulree agent: a small round structured virus from a hospital outbreak of gastroenteritis, purified on a potassium tartrate/glycerol gradient. (c) Astrovirus. (d) Calicivirus. Bar, 50 nm.

TABLE 2 Serological relationship between small round viruses from outbreaks of gastroenteritis

	Agent	Relationship	Community affected	Country	Reference
Featureless viruses: Candidate human parvoviruses (Fig. 1)	Wollan	Related	School	UK	Paver et al 1973
	Ditchling		School	UK	Appleton et al 1977
	Cockle	Distinct	Widespread, food-borne outbreak	UK	Appleton & Pereira 1977
	Parramatta	Distinct	School	Australia	Christopher et al 1978
Small round structured viruses (SRSV) (Fig. 2)	Norwalk	Related	School	USA	Thornhill et al 1977
	Montgomery County		Family	USA	
	Hawaii	Distinct	Family	USA	
	Otofuke	Related to each other but distinct from Norwalk and Hawaii	Institute for mentally retarded	Japan	Taniguchi et al 1979
	Sapporo[a]		Children's home	Japan	Kogasaka et al 1981
	Taunton	Not compared to other agents	Hospital	UK	Caul et al 1979
	Amulree	Distinct from Norwalk and Hawaii	Hospital	UK	H. Appleton & J.V. Gostling, unpublished 1982
	Snow Mountain	Distinct from Norwalk and Hawaii	Resort camp	USA	Dolin et al 1982

[a] Sapporo agent is also unrelated to caliciviruses observed in other outbreaks in the same children's home.

Structured viruses

Norwalk virus has an amorphous surface structure and a ragged outline, and is morphologically quite distinct from the featureless viruses with their smooth surface and entire outer edge (Fig. 2). On the basis of its buoyant density it was originally suggested to be a parvovirus, but on morphological and biochemical grounds this has now been discounted. Greenberg and his colleagues (1981) have detected one major structural polypeptide with a molecular mass of 59 kDa, which puts Norwalk closer to the calicivirus family. Although Norwalk and other similar agents never reveal the classical appearance of calicivirus, it should be noted that some tissue culture-grown strains of feline calicivirus never demonstrate this morphology either, and the appearance of these strains and Norwalk is very similar. A one-way serological cross between human calicivirus and Norwalk has been demonstrated by radioimmunoassay and immuno-electron microscopy (W.D. Cubitt, personal communication, and see Blacklow et al, this volume). Nevertheless, the morphological differences between the two groups are reproducible.

Other Norwalk-like agents. Several morphologically similar agents have been reported world-wide, from both outbreaks and sporadic cases of gastroenteritis. There is little biochemical evidence available, and identification has been based totally on electron microscopic appearance. From volunteer studies in the USA and immuno-electron microscopy studies in many centres, different serotypes have been demonstrated (Table 2).

Calciviruses are so called because of the characteristic cup-like depressions visible on the surface of some particles, the name being derived from the Latin word *calix*, meaning cup. The viruses contain single-stranded RNA and have one major structural polypeptide of molecular mass 60–70 kDa. Human strains have been identified in several outbreaks by their characteristic appearance, and a 62 kDa structural polypeptide has been described in association with a virus obtained from a patient who developed gastroenteritis during an outbreak in an orphanage in Japan (Terashima et al 1983). The results of labelling experiments using [^{3}H]uridine in the presence of actinomycin D provide further evidence that these viruses contain RNA (Cubitt & Barrett 1984).

A morphologically similar virus known as the Newbury agent has been described in calves (Bridger et al 1984) and a virus from pigs, unrelated to vesicular exanthema virus of swine, has also been reported (Bridger 1980, Saif et al 1980). Both the calf and pig viruses occurred in association with diarrhoea.

Astroviruses have been recognized as a novel group of viruses because of their distinctive appearance. The name was proposed because a five- or six-pointed star-like configuration could be seen on the surface of some particles (Madeley & Cosgrove 1975). The particles are consistent in size at 28–30 nm and often occur as aggregates and sometimes as crystalline arrays. Studies on the lamb astrovirus showed that the particles contain an RNA genome with a short poly(A) tract,

resembling the genomes of both picornavirus and calicivirus. However, the possession of two major structural polypeptides places astrovirus in a separate group, intermediate between picornavirus and calicivirus (Herring et al 1981).

Food-borne gastroenteritis

The discovery of the new viral agents of gastroenteritis logically led to the investigation of outbreaks of food poisoning from which no bacterial pathogens could be recovered, and in 1977 for the first time a small round virus was detected in patients involved in a large series of outbreaks of gastroenteritis associated with the consumption of cockles (Appleton & Pereira 1977). It is this agent (cockle agent) that has subsequently been partially characterized as a parvovirus, as already described. Viruses have now been convincingly demonstrated to be the aetiological agents of several food-poisoning outbreaks linked with molluscan shellfish (Murphy et al 1979, Appleton et al 1981, Gill et al 1983, Sockett et al 1985), and, although the epidemiological evidence has been more difficult to establish, other foods have certainly been involved in the transmisison of viral gastroenteritis (Griffin et al 1982, Pether & Caul 1983, Riordan et al 1984).

Outbreaks of gastroenteritis associated with the consumption of food are usually investigated as incidents of bacterial food poisoning. In 1981 we reported that in almost a quarter of outbreaks of possible food-borne gastroenteritis in the UK, investigated by the Public Health Laboratory Service, bacterial food-poisoning organisms were not isolated (Appleton et al 1981). Many of these outbreaks were characterized by a very high attack rate, the incubation period was longer than the usual range for bacterial food poisoning, and the symptoms usually included vomiting as well as diarrhoea. Secondary cases were sometimes detected in close family contacts. These features suggest that an outbreak may be viral in origin.

Outbreaks of food-borne gastroenteritis are now being increasingly investigated for the presence of viruses. In 13 outbreaks in England and Wales in 1984–1985 where either small round structured viruses or parvoviruses, or both, were found, seven outbreaks were associated with shellfish, four involved cold buffet food, and in two the food was not stated (S. R. Palmer, personal communication). The high proportion of outbreaks associated with shellfish may possibly be an artifact, since previous reported successes in detecting virus in shellfish-associated outbreaks may mean that these outbreaks are investigated more thoroughly.

Bivalve molluscs such as oysters, mussels and cockles feed by filtering organic matter from the water passing over their gills, and in sewage-polluted estuarine waters they accumulate potentially pathogenic microorganisms. Statutory cleansing and heat treatment procedures appear to be adequate for the removal of bacterial contaminants, as since 1965 only two of 98 reported outbreaks in

TABLE 3 Outbreaks of illness associated with molluscs: England and Wales 1965–1983

Nature of outbreak	*Outbreaks 1965–1983*	*Outbreaks 1984–1986 (provisional figures only)*
Bacterial food poisoning	—	2
Hepatitis A	10	1
Viral gastroenteritis	22[a]	35[b,c]
Paralytic shellfish poisoning	1	—
Red whelk poisoning	1	—
Unknown	26	—
Total:	60	38

[a] Small round viruses detected in patients.
[b] These outbreaks fulfil the strictly defined criteria required by the PHLS Communicable Disease Surveillance Centre for inclusion as viral gastroenteritis. Material for laboratory confirmation was only available from some incidents. In the 1984–1986 period there were 31 further incidents of viral gastroenteritis in which seafood (mainly molluscs) was the suspected source of infection.
[c] In six incidents mixed seafood (including molluscs and crustacea) was incriminated.
Adapted from Sockett et al 1985 with additional unpublished information provided by the PHLS Communicable Disease Surveillance Centre.

England and Wales were due to bacterial food poisoning (Table 3). However, these procedures are clearly unsatisfactory for the removal and inactivation of viruses. Developments in laboratory techniques for identifying virus infections have indicated that, in the last 10 years, at least 10 outbreaks of hepatitis A and 22 outbreaks of viral gastroenteritis were associated with molluscs. In the period 1965–1983 a further 26 outbreaks of food poisoning were of unknown cause, but the symptoms recorded were highly suggestive of viral gastroenteritis. Despite increasing awareness of the problem of viral infections originating in shellfish, many more outbreaks of gastroenteritis have occurred in the UK over the winter of 1985–1986. Most outbreaks have been traced to cockles from one particular coastal area, and a few have been attributed to oysters and mussels. Material available from a small number of incidents was examined in the Virus Reference Laboratory (Table 4). In common with other reported food-borne outbreaks of viral gastroenteritis, small round viruses were detected. These included parvovirus, astrovirus, calicivirus and small round structured viruses. Rotavirus has not so far been detected in food-borne outbreaks, although water-borne outbreaks have been reported. Mixed infections are not uncommon and may be reflected in the variable incubation periods that may be reported within one outbreak. The occurrence of astrovirus is perhaps surprising, since this virus is more usually associated with very young children, but in a previous incident, connected with oysters, parvovirus was found in persons ill after 36 hours and astrovirus was detected when further symptoms developed at four days (E. O. Caul, unpublished observations).

Other food-borne outbreaks of gastroenteritis in which viruses have been

TABLE 4 Shellfish-associated outbreaks of viral gastroenteritis in the UK, investigated by the UK Virus Reference Laboratory, November 1985 to February 1986

Social function	*Shellfish incriminated*	*Virus detected*
Restaurant meal	Oysters	SRSV + parvovirus
Series of banquets	Cockles/mussels	Calicivirus + parvovirus
Private party	Cockles	Astrovirus + parvovirus
Restaurant meals (two groups)	Seafood cocktail (oysters, mussels)	SRSV + parvovirus
Family meal	Cockles	SRSV + parvovirus
College party	Oysters	Parvovirus

SRSV, small round structured virus.

detected have always been associated with cold foods. It is assumed that the food was contaminated by infected food handlers, although the possibility of water-borne spread, or cross-contamination from shellfish in the same kitchen, could not always be excluded. The high attack rate that is characteristic of these outbreaks must lead one to speculate that there may be some element of respiratory spread as well as faecal–oral transmission. Once again, only small round viruses—either small round structured viruses or parvoviruses—have been implicated.

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DISCUSSION

Caul: We have looked at some 132 community, closed-community or family outbreaks of non-bacterial gastroenteritis over the past 10 or so years. We find small round structured viruses (SRSV), morphologically indistinguishable from Norwalk virus, in about 32% of these outbreaks (unpublished observations). We also see a high proportion of dual infections with a small round featureless parvovirus-like particle and Norwalk-like virus in the same specimen. We have no doubt of which virus is causing the gastroenteritis, because of Dr Kapikian's work. That leaves the question of what the other small round (featureless) parvovirus-like virus is doing. We know that these small round featureless viruses are commonly excreted asymptomatically, from our non-bacterial gastroenteritis and volunteer studies; and we know from animal studies that parvoviruses replicate in crypt cells. Perhaps Norwalk virus infects mucosal epithelia, where it has been shown to give rise to a crypt cell hyperplasia, which in turn would boost the output of pre-existing parvovirus-like particles. This is one possible explanation of the commonly noted dual infections.

Bishop: Are you suggesting that this parvovirus is a persistent infection in the gut, and that its growth is enhanced when cells such as crypt cells are undergoing rapid multiplication in order to repair damage due to another agent?

Caul: Yes. There is good evidence that parvovirus-like particles are persistently excreted in a small proportion of normal people.

Bishop: This is analogous to the situation observed with parvovirus infection associated with rapid replication of red cell precursors (Cossart 1984).

Kapikian: I would be cautious about this parvovirus-like agent. The finding of a stool particle which is associated temporally with an illness, of course, as we know, does not establish an aetiological relationship. Also, we find such '22 nm particles' in stools occasionally in specimens that contain known, established, aetiological agents of disease, such as the 27 nm Norwalk virus or 27 nm hepatitis A virus (Purcell et al 1975, Kapikian et al 1980). Before associating a virus observed in a stool specimen with a temporally associated disease even in a preliminary way, one must demonstrate a serological response by immuno-electron microscopy (IEM) to that particle with paired pre- or acute and convalescent sera. We find that there is no significant change in the amount of 'antibody' observed on these 22 nm particles, in IEM studies with pre-illness or acute-phase and covalescent-phase serum specimens. To further complicate matters, the '22 nm particles' consistently appear in aggregates with a light 'antibody-type' coating or without any coating. In addition, the prevalence of such particles in control specimens should be considered before associating them with an illness. The Henle–Koch postulates and the later modifications by Huebner for associating an agent with an illness should be fulfilled in essential elements before an aetiological association is made (Huebner 1957, Evans 1976, Kapikian 1981).

Caul: I was careful, when speaking of dual infections, to stress that the SRSV, which seems morphologically indistinguishable from Norwalk, is the cause of the outbreak. We also see the parvovirus-like particles, which I don't think cause gastroenteritis, in association with Norwalk-like viruses. We don't show any seroconversions to the parvovirus-like agent, so I would agree with you; but we need more information on the parvovirus-like viruses and the role, if any, that they have in enteric disease.

Appleton: Serological differences have been demonstrated by immuno-electron microscopy between the Ditchling/Wollan agents, the cockle agent and the Parramatta agent. These are all featureless parvovirus-like agents. If indeed such viruses are part of the normal gut flora, and production is boosted by infection with another type of agent, it seems odd that these featureless viruses occur in a very high proportion of patients in some outbreaks, and may not even be detected at all in other outbreaks. Why is excretion not stimulated in all outbreaks with similar symptoms?

We have observed a cyclic pattern with the occurrence of faecal parvoviruses. They were very common around 1976–1978 and then greatly declined in the UK, but have been seen again frequently over the past winter (1985–1986). When parvoviruses are being detected frequently in association with gastroenteritis they can be detected in perhaps up to 20% of asymptomatic people, but in periods when they do not appear to be circulating the detection rate in asymptomatic persons falls well below 5%. A six-year cyclic pattern is known to occur with Fifth disease, which has recently been associated with the human serum parvovirus B19.

Flewett: In our early studies on gastroenteritis we certainly found parvovirus-like particles, in children with gastroenteritis, but in approximately the same number and frequency in children who did not have gastroenteritis. We concluded that these were an incidental finding and part of the normal faecal flora. Whether they lived in the crypts, or were adenovirus-associated viruses that have come down from adenovirus infections of the enterocytes, we could not tell, in the absence of sera with which to test them. We need specific sera to identify these particles, and until we can get better identification we can't draw any conclusions.

Woode: As there is evidence that serum antibody doesn't protect the gut in most virus infections, can you get repeat gut virus infections with no modification of the serum antibody titre? Is the criterion that there must be an alteration in serum titre for there to be an active infection, a general rule for enteric viruses?

Kapikian: With Norwalk virus, pre-existing serum antibody is not necessarily related to protection, as Dr Blacklow will discuss. However, individuals with pre-existing antibody who are reinfected with Norwalk virus characteristically develop a serological response after the reinfection, at least in the controlled conditions of a volunteer study. Thus, although theoretically perhaps one could

be reinfected without showing an antibody response, until this is established, we should still insist on the requirement to detect a serological response, as discussed previously, in attempting to establish an aetiological association of an agent with enteric disease.

Caul: The classification of small round viruses was put forward by Dr Appleton and myself as an interim measure, but it has worked well in the UK in the past 2–3 years for the collection of epidemiological data. I personally hope that people will now refrain from grouping agents like Wollan, Ditchling, cockle, or Parramatta agent with the Norwalk group, where they clearly don't belong.

Chiba: May I add some comments on the Otofuke agents described by Professor S. Urasawa's group? (Taniguchi et al 1979.) They have obtained good evidence for this virus being a single-stranded DNA virus (K. Taniguchi et al, unpublished paper, US–Japan Working Conference on Rabies, Arboviruses including Dengue, Korean Hemorrhagic Fever and Viral Gastroenteritis, Oiso, November 1980). If this is true, it would create a new virus family. The particles we named as the Sapporo agent seem to belong to the same group, in terms of size, morphology and immunological relatedness, at least in a one-way cross (Kogasaka et al 1981). Similar agents have been frequently associated with outbreaks of gastroenteritis in all age groups, throughout Japan. Perhaps we should pay more attention to this group of viruses. They may have been described under different names.

Woode: Dr Appleton referred to horizontal transmission of viruses in humans. This is of interest to those of us working on bovine calicivirus-like agents. We don't have evidence of food-borne calicivirus spread in calves and it looks much more like the traditional rotavirus horizontal transmission, via the faecal–oral route. Would those who work on the human agents like to discuss the degree of horizontal transmission between infected and uninfected people, other than the food source of infection? Do they readily move through a population, or are they restricted?

Appleton: Transmission among adults appears to require very close contact, as within a family, and where standards of hygiene are good the possibility of respiratory transmission must be considered. Children confined within one school classroom or nursery group must also be considered as close contact.

Cubitt: In all the outbreaks that we have looked at, both of strains of caliciviruses, and of small round structured viruses, we have had evidence of secondary transmission within families. Transmission of the virus seems to require close contact; generally infections are relatively mild and therefore the affected people will not visit their doctors, so unless you are doing a follow-up study in collaboration with epidemiologists you are unlikely to detect secondary cases.

Bishop: You have evidence of infection with viruses other than small round viruses, I believe?

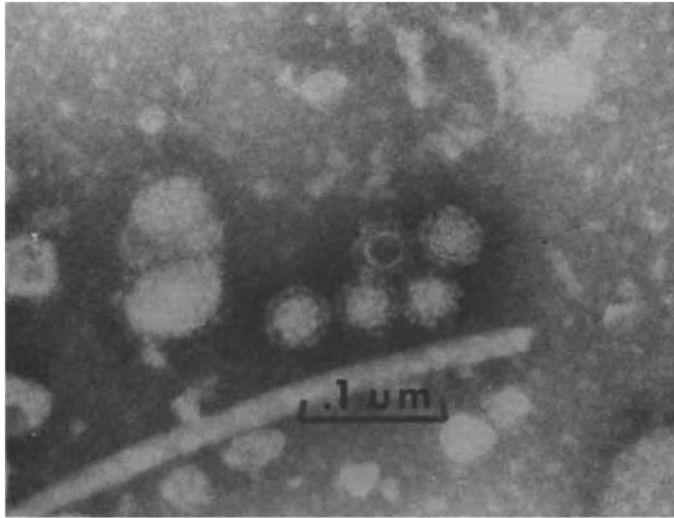

FIG. 1 (*Saif*). Electron micrograph of a small round structured virus from a weanling pig with diarrhoea.

Cubitt: This is preliminary work. In January 1986 there was a large reunion for catering staff in a firm in southern England, attended by about 200 people, aged about 30 up to 65 years. Between 24 and 48 hours after this meal, people started developing symptoms of diarrhoea and vomiting. Specimens were collected and some were sent to the Epsom Public Health Laboratory and some to us. Epsom found rotavirus in their faecal specimens: we also found rotavirus. Of 15 specimens sent to the Central Middlesex Hospital, five contained rotavirus. We have run these rotaviruses in ELISA tests, latex tests, and on polyacrylamide gel electrophoresis. They are group A rotaviruses with a long RNA profile. The Communicable Diseases Surveillance Centre set up a food analysis to establish the source of the outbreak. It became evident that the source was contaminated fruit salad. On questioning, they found that the person who was peeling the grapes and extracting the pips had a recent history of gastroenteritis in the family. We have still to do serological studies on this virus.

Saif: We have recently found a problematical small round structured virus from weanling pigs with diarrhoea. We passed the filtrate into gnotobiotic pigs and have produced mild diarrhoea in them. Using convalescent-phase antiserum in indirect immmunofluorescence we have seen immunofluorescence in small intestinal epithelial cells, particularly in the duodenum. The particle is about 35–42 nm in diameter, when examined without antiserum present (Fig. 1). When antiserum is present we see fuzzy particles with an indistinct periphery. The virus is similar to what people called mini-reos or mini-rotas, or

'fuzzy wuzzies' (Middleton et al 1977, Spratt et al 1978, Flewett 1978). It is not a rotavirus; there is no cross-reactivity in all our standard rotavirus assays. Nor is it related to our calicivirus, since pigs immune to our enteric calicivirus (Saif et al 1980) are still susceptible to infection and disease.

Chiba: It looks very like the Otofuke agent or Sapporo agent. They show a similar appearance to your virus, in the presence of antibodies of low titre.

Bridger: I have worked with two strains of bovine calici-like viruses (named Newbury agents), one of which probably is a calicivirus, and the other falls into Hazel Appleton's group of small round structured viruses; but I have never seen anything quite like Dr Saif's 35–40 nm particle.

Saif: I also have been working with a bovine calici-like virus that cross-reacts with your Newbury agent and, to me, morphologically this new particle is not the same.

Bishop: Where would Dr Appleton and Dr Caul classify this virus?

Appleton: This is a small structured virus, and fits into that group of agents within our classification scheme.

Kapikian: This resembles the classical 'mini-reovirus' described by Middleton et al (1977). I had never seen one until recently when, in studies of stool specimens by electron microscopy with Drs Ticehurst and Purcell, we saw a virus-like particle that appeared morphologically identical to that shown by Dr Saif. It looked like a mini-rotavirus or mini-reovirus, whatever that means!

Flewett: We need another name, though, because this is so misleading—the 'mini-reos' or 'mini-rotas' are not related to reoviruses or rotaviruses.

Greenberg: Dr Saif, your virus doesn't have double-stranded RNA, does it, like a reovirus? Did you put it on a gel?

Saif: We put it on a PAGE gel and couldn't see any bands, using the same techniques as for rotavirus dsRNA.

Greenberg: Then probably you should not use the term 'mini-reovirus'!

Bishop: Our emphasis has so far been on the involvement of bivalve molluscs as a source of virus in food-borne infections and illness. Has anybody any experience of other foods being involved?

Appleton: Evidence is accumulating for the involvement of foods other than shellfish in the transmission of viral gastroenteritis, but the epidemiology is more difficult to establish. People are likely to remember eating molluscs, particularly in the UK where this is not an everyday food. Meals involved are often cold buffets, but by correct use of questionnaires food specific attack rates can be established and other foods have clearly been incriminated. Transfer is presumably passive from infected food handlers, and the infectious dose is probably very low. Because of the way in which molluscs feed they can concentrate viruses within their tissues; therefore the infectious dose may be higher than with other foods. The attack rate in shellfish outbreaks is certainly very high.

Cubitt: So far in 1986 we have looked at eight large outbreaks, some of which

involved several hundred people. One series of episodes was in an hotel, where the implicated food was melon in one outbreak and, in the second, vermicelli added to soup. The link between the two outbreaks was one of the chefs, although the outbreaks were a month apart. In both outbreaks a small round structured virus was found to be the cause; and we have serological evidence that the same virus was causing the two episodes.

Bishop: Does this imply that the infective dose is very low, or that there is gross contamination of some foods?

Cubitt: We have done a volunteer study with human calicivirus, strain UK4, at the Common Cold Research Unit at Salisbury. The dilution of the inoculum given to volunteers by intranasal drops (because we were looking for respiratory involvement) was 10^{-4}, so we reckoned the dose was between 100 and 1000 particles per volunteer. Of four volunteers, three developed mild to moderate symptoms, so the infective dose must be very low.

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The candidate caliciviruses

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Abstract. The Caliciviridae are a family of small (35–40 nm) RNA viruses with a characteristic cupped morphology. They are unique in possessing only a single major structural polypeptide, of M_r 60 000–71 000.

The use of electron microscopy to investigate diarrhoeal diseases has revealed viral particles with the size and structure of the caliciviruses in the faeces of humans, domestic and farm animals, birds, reptiles and insects. *In vivo* experiments indicate that they are species specific and have confirmed that they replicate in the gut, which often results in the host developing diarrhoea and failing to thrive.

Biochemical characterization of these agents has been hampered by a failure to produce sufficient yields of virus *in vitro*. However, fluorescence and radiolabelling experiments indicate that the human, canine and chicken viruses replicate in the cytoplasm and possess an RNA genome. A major structural polypeptide (M_r 60 000–71 000) has been identified in the human, canine and insect viruses. Diagnosis of the candidate caliciviruses is dependent on electron microscopy and fluorescence labelling, with the exception of the human agents, for which radioimmunoassays have been developed.

There is little epidemiological information on these agents but there is increasing evidence that the human caliciviruses are a common cause of outbreaks of diarrhoea and vomiting in infants, adults and the elderly.

1987 Novel diarrhoea viruses. Wiley, Chichester (Ciba Foundation Symposium 128) p 126–143

In the past decade the use of electron microscopy for the study of diarrhoeal diseases has revealed numerous viruses in the faeces of many different animal species. Among these are particles which resemble those of an important family of animal pathogens, the Caliciviridae (Table 1).

The aims of this chapter are to:

(1) Collate the data that are available to support the view that these faecal agents should be considered as candidate caliciviruses, according to the criteria laid down by the International Committee on the Taxonomy of Viruses.
(2) Present the evidence that they are a cause of diarrhoeal disease.
(3) Summarize the epidemiological data available for the faecal agents that affect humans, namely human caliciviruses (HCV) and Norwalk virus.

TABLE 1 Characterized and enteric candidate caliciviruses

	1st report	*Origin*
Characterized caliciviruses		
Vesicular exanthema of swine (VESV)	1932	USA
Feline calicivirus (FCV)	1957	USA, Australia
San Miguel sealion virus (SMSLV)	1973	USA
Enteric candidate caliciviruses		
Norwalk virus	1972	USA
Human calicivirus (HCV)	1976	UK
Newbury agents of cattle	1978	UK
Porcine enteric calicivirus (PEC)	1980	UK, USA
Amyelosis chronic stunt virus (ACSV) (insect)	1981	USA
Chicken calicivirus (CCV)	1981	UK
Canine calicivirus (CaCV)	1985	USA

Extensive review articles on the Caliciviridae have been written by Studdert (1978) and Schaffer (1979), and on the candidate viruses by Cubitt (1985).

Properties of the virus particle

Size

The characterized members of the Caliciviridae have a diameter of 35–40 nm. This apparently wide range of values is partially accounted for by differences in techniques for the preparation and staining of virus particles. There are also inherent errors resulting from the difficulty of gauging the furthest points of an object and a frequent failure to calibrate instruments or to incorporate internal standards of size. The estimated sizes of several candidate caliciviruses are shown in Table 2. The majority fall within a range compatible with their classification within the Caliciviridae, but measurements of HCVs and Norwalk viruses obtained from faecal extracts have consistently been found to be smaller (means of 31 nm and 27 nm, respectively). However, when HCVs were obtained from cell cultures and measured, using crystalline catalase as an internal standard of size, they were found to be significantly larger (mean of 35 nm). These results suggest that factors such as the degree of hydration and the effect of proteolytic enzymes may have a significant influence on the dimensions of the particles.

The discrepancies associated with the measurement of small round viruses indicate that size is an unreliable criterion for placing them in a particular group. This is emphasized by the anomaly that size has been given as evidence in support of classifying Norwalk virus both as a candidate parvovirus and as a candidate calicivirus.

TABLE 2 Estimated sizes of caliciviruses

Virus	*Source*	*Size range (nm)*	*Mean ± 1 SD*
FCV K1	Cell culture	34–40	36.8 ± 1.5
CaCV	Cell culture	32–38	34.8 ± 1.7
HCV UK1	Cell culture	31–40	34.9 ± 1.9
HCV	Faeces	28–34	30.5
Norwalk virus	Faeces	25–27	NA
CCV	Excreta	30–39	36.0 ± 0.9
PEC	Faeces	NA	30
Newbury 1	Faeces	NA	33
ACSV	Gut	NA	38
ACSV	Excreta	NA	38 and 28

See Table 1 for names of viruses.
NA, not available.

Morphology

One of the features cited as evidence for classifying the caliciviruses as a family distinct from the Picornaviridae was their characteristic surface, which is formed by 32 'cupped' depressions arranged in icosahedral symmetry. When viruses are viewed along their two-, five- and threefold axes of symmetry they have a characteristic appearance (Fig. 1), four hollows arranged as a cross, a ten-pointed sphere and a 'Star of David', respectively.

The appearances of two strains of a characterized calicivirus, feline calicivirus strains K1 and F9 are shown in Fig. 2, alongside those of several candidate viruses. This comparison illustrates the problem of relying entirely on morphological criteria to recognize caliciviruses: some strains of virus from the same host species display the typical appearance, whereas others appear 'fuzzy' (Fig. 2a, b). A similar situation exists with the HCV strains, UK1 and UK4 (Fig. 2c, d) and has been noted with the cattle viruses, Newbury agents 1 and 2. Image enhancement by the Markham rotation technique can sometimes reveal that the fuzzy particles have the characteristic appearance of the Caliciviridae (Fig. 3). However, this technique is too time-consuming to be used routinely.

The appearance of some caliciviruses is known to be affected by proteolytic enzymes; these include some strains of FCV, and amyelosis chronic stunt virus (ACSV), which is degraded completely from a rough 38 nm particle to a smooth 28 nm form. Observations of some HCV strains indicate that they also are degraded proteolytically to a smooth form which is indistinguishable under the electron microscope from an enterovirus (Fig. 4).

A further problem often encountered by diagnostic laboratories is that particles have been damaged or totally disrupted by freezing the faecal

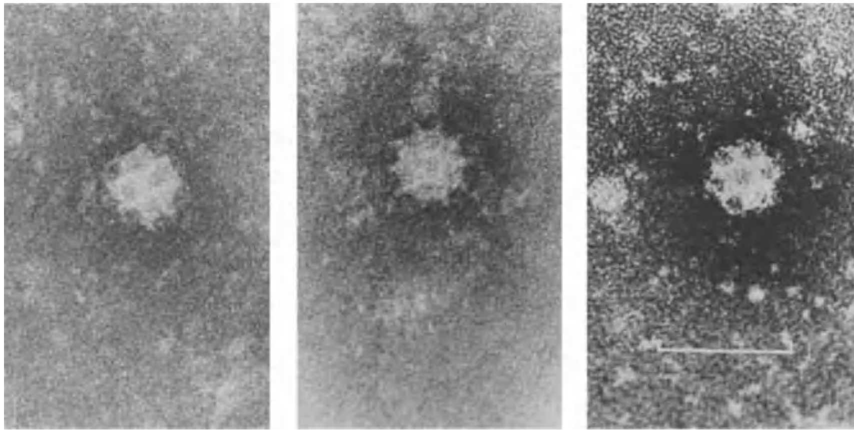

FIG. 1. Electron micrograph showing calicivirus morphology, when particles are viewed along the two-, five- and threefold axes of symmetry. Size bar, 50 nm. (Reproduced from Cubitt et al 1979 by permission of *Journal of Clinical Pathology* and *British Medical Journal.*)

samples. It is therefore advisable to hold samples at 4°C before their examination by electron microscopy.

Another factor which influences the appearance of the virus is the presence of coproantibodies, which can totally mask their surface morphology, making it impossible to distinguish caliciviruses from other small viruses.

Physicochemical properties

A knowledge of the physiochemical properties of caliciviruses is essential if one is to obtain pure preparations of the candidate viruses from faecal extracts. It is therefore surprising that there is so little information recorded in the literature (Table 3).

The Caliciviridae are not disrupted by chloroform, ether, or detergents. They have sedimentation coefficients between 170S and 183S in sucrose and a buoyant density of 1.36–1.39 g cm^{-3} in caesium chloride. These properties have been utilized in the extraction of human, chicken and canine viruses from faecal extracts. A procedure for purifying HCV is as follows:

(a) Faecal samples containing typical calicivirus particles are emulsified in 5 ml of 0.5% Zwittergent-314 prepared in Medium 199.

(b) The emulsion is mixed with an equal volume of chloroform or ether and spun in a bench centrifuge for 10 minutes; the supernatant is then removed.

(c) The extract is layered onto a preformed 5–20% linear sucrose gradient and spun at 39 000 r.p.m. for 40 minutes in a Beckman SW 50:1 rotor. (When

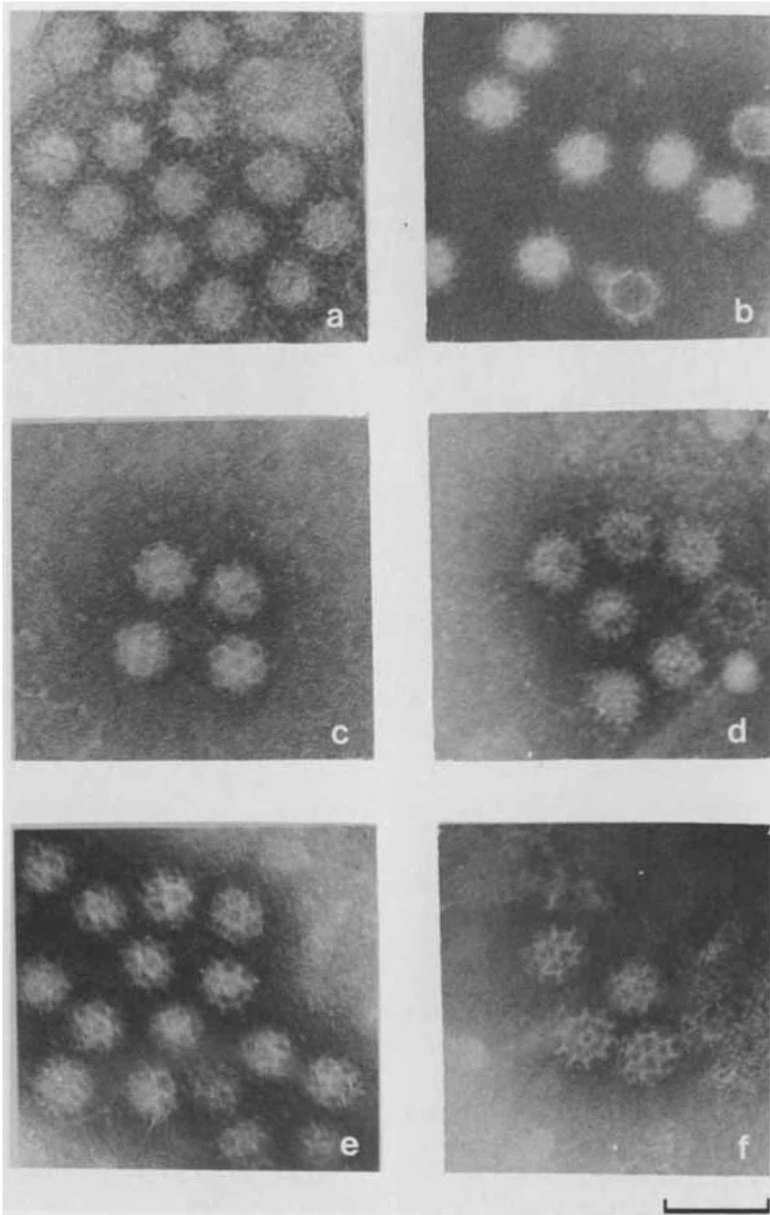

FIG. 2. Appearance of caliciviruses stained with 2% potassium phosphotungstic acid, pH 6.4. Size bar, 50 nm. a, FCV KI; b, FCV F9; c, HCV UK1; d, HCV UK4; e, CaCV; f, CCV.

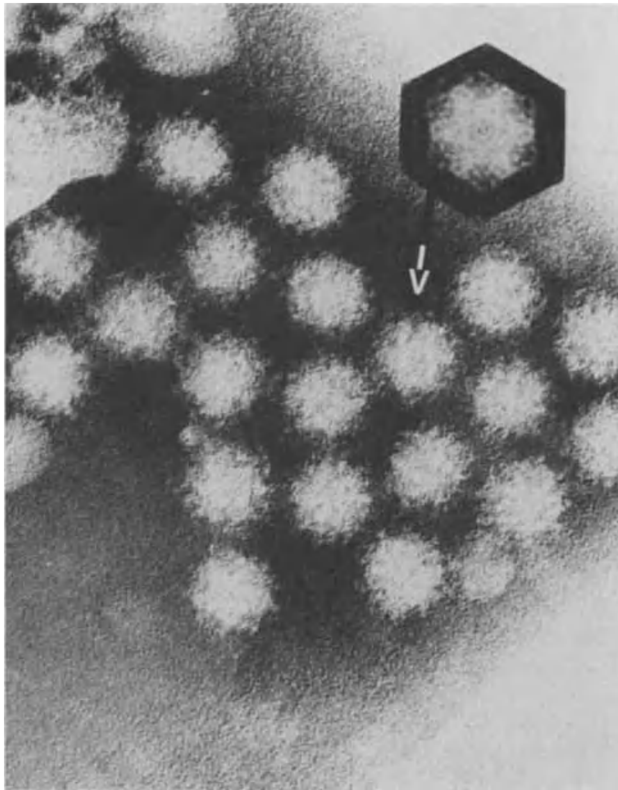

FIG. 3. Electron micrograph of HCV UK4 stained with 2% potassium phosphotungstic acid. Insert shows Markham rotation, $N = 6$, of the arrowed particle and reveals the characteristic 'Star of David' morphology.

poliovirus was used as a marker [160S], some HCV strains were found to form two distinct peaks [190S and 167S]. The 167S peak may contain defective interfering particles; an analogous situation is known to exist with some strains of FCV.)

(d) The fractions containing the greatest numbers of virus particles are banded on a caesium chloride gradient. HCVs have a buoyant density of 1.38–1.40 g cm^{-3}.

As there is a considerable loss of virus particles as a result of disruption in caesium chloride, alternative gradients should be used, such as glycerol/potassium tartrate or the iodinated polysaccharide Metrizamide (Nyegaard UK, Birmingham). Metrizamide has the additional advantage that it is non-ionic and can be prepared in isotonic solution, which enables the fractions containing virus to be inoculated directly onto cell monolayers without the need for dialysis. The density of HCV in Metrizamide is 1.09–1.10 g cm^{-3}.

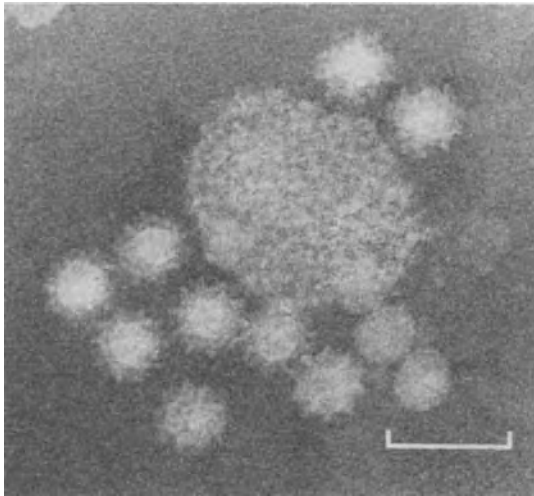

FIG. 4. Electron micrograph of proteolytically degraded HCV showing typical, partially degraded and smooth particles. Size bar, 50 nm.

TABLE 3 Physicochemical properties of caliciviruses

Virus	*Density in CsCl (g cm^{-3})*	*Sedimentation coefficient in sucrose*	*Resistant to lipid solvents*
FCV	1.36–1.40	181S	Yes
HCV	1.38–1.39	193S	Yes
Norwalk virus	1.38–1.39	ND	Yes
CCV	1.38–1.39	ND	Yes
CaCV	1.34–1.35	ND	Yes

ND, no data.

Nucleic acid

Caliciviruses possess one molecule of infectious positive-sense single-stranded RNA which is probably polyadenylated at the 3′ terminus. The lack of success in propagating many of the candidate viruses *in vitro* has meant that the nature of the nucleic acid is not yet known. However, radiolabelling with [^{3}H]uridine in the presence of actionomycin D has shown that CaCV, HCV and CCV possess an RNA genome and can replicate in cell culture. Amyelosis chronic stunt virus (ACSV), purified from the guts of infected insect larvae, contains a single major species of RNA with a sedimentation value of 36S.

Protein

Caliciviruses are believed to be unique in that they possess only a single major structural polypeptide, of M_r 60 000–71 000. A single polypeptide with an M_r within this range has been identified in three candidate viruses—HCV, Norwalk virus and ACSV. The protein composition of the other caliciviruses is not yet known.

Antigenic properties

Immuno-electron microscopy (IEM) and neutralization tests indicate that there are many distinct serotypes of VESV and SMSLV, but there is considerable cross-reactivity among strains of FCV. However, complement fixation and immunofluorescence tests show that all strains of FCV share a group antigen and that VESV and SMSLV are related.

Studies with several strains of HCV showed no cross-reaction with several serotypes of VESV, SMSLV, FCV and CaCV when tested by immunodiffusion, immunofluorescence, IEM or staphylococcal radioimmune precipitation. IEM studies indicate also that the HCV strains are antigenically distinct from viruses isolated from pygmy chimpanzees, pigs, cattle, walruses, mink, chickens and insects.

IEM tests with HCV and Norwalk viruses obtained from patients involved in outbreaks of diarrhoea and vomiting have shown that this test is strain specific. Four strains of HCV have been identified in the United Kingdom and a further antigenically distinct strain has been identified from Japan. Tests with Norwalk viruses have produced similar results.

When the five strains of HCV and a strain of Norwalk virus were tested in a radioimmunoassay, four of the five HCV strains gave a strongly positive result, suggesting that the test is detecting a group antigen. Preliminary results of immunofluorescence studies on HCV indicate that it also is detecting a common antigenic determinant.

IEM tests are ideal for investigating the cause of outbreaks of diarrhoea, provided that the virus and acute- and convalescence-phase samples of sera are available. Samples are often obtained too late to detect the virus, so there is a need to develop and evaluate serological tests for group antigen.

Replication of caliciviruses

Immunofluorescence and radiolabelling experiments with [^{3}H]uridine and [^{35}S]methionine have shown that HCV, CCV and CaCV can replicate *in vitro*, provided that trypsin is included in the medium. Fluorescent foci are confined to the cytoplasm and can be detected eight hours after infection. Affected cells

become refractile, round up and detach into the medium. Trypsin can produce similar effects and it is therefore essential to include uninfected cell controls.

HCV, CaCV and FCV can be propagated in a dolphin kidney cell line NBL-10 (American Tissue Culture Collection, CCL-78). Unfortunately, stocks of this line are already at a high passage number and there is a limited supply. Although the cells are initially susceptible to infection, after further repeated passages (more than four) they become refractory. HCV has also been propagated and passaged in primary embryonic kidney (HEK). However, not all HEK cells are susceptible to infection and fetal organs are not readily available.

Biological aspects

Host range

Natural infections with the candidate caliciviruses suggest that each one is highly species specific, although there is some epidemiological evidence to suggest that one strain of HCV may have originated from an infected dog. This is of interest, as early studies with VESV showed that it infected dogs and recently a number of strains of FCV have been isolated from dogs with symptoms of diarrhoea.

Numerous experiments have been done with HCV and Norwalk viruses in an attempt to provide an animal model for the study of their pathogenicity. The results indicate that chickens, mice, guinea-pigs, rabbits, kittens, puppies, piglets, calves, baboons and various monkeys fail to develop illness. Some rhesus monkeys and rabbits seroconverted when fed with HCV and a chimpanzee fed with Norwalk virus shed antigen in its stools and responded serologically.

In vivo transmission experiments

HCV and Norwalk viruses. Transmission experiments at the Common Cold Research Unit in the UK and in the USA have shown that virus administered by the nasal–oral route can produce symptoms of nausea, vomiting, diarrhoea, pyrexia and abdominal pains in *adult* volunteers. The severity of illness ranged through inapparent or mild to moderately severe symptoms. The incubation period was 24–72 hours; symptoms lasted for one to two days and excretion of virus particles in the faeces closely paralleled the duration of illness. These findings are indistinguishable from those observed in studies of calicivirus outbreaks, other than that symptoms were less severe. It is probable that the small doses of virus administered to volunteers and their age accounts for this difference.

Histopathological studies of volunteers infected with Norwalk and Hawaii agents indicate that the virus affects the jejunum, resulting in broadening and blunting of the villi. The epithelial cells remain intact but the microvilli are reduced in length. Similar studies with HCV have not yet been done.

Porcine enteric caliciviruses. Studies in the UK and in the USA have shown that gnotobiotic piglets fed by the oral route developed a profuse pale diarrhoea, two to four days after exposure. Affected animals became anorexic and failed to thrive.

Histopathological examination revealed villous atrophy in the small intestine. The presence of viral antigen in epithelial cells could be demonstrated by immunofluorescence.

Newbury agents of cattle. Young calves fed with Newbury agents 1 or 2 developed symptoms consisting of pale loose faeces and anorexia, one to three days after infection. The severity of the diarrhoea was similar to that caused by bovine rotavirus, and it lasted for one or two days. Small numbers of virus particles could be detected in faecal samples collected 1–4 days after infection.

Histological studies showed that infection was confined to the anterior half of the small intestine. Virus replicates in enterocytes on the sides of the villi. As these cells degenerate the villi become stunted, which results in malabsorption of xylose for a few days until the damage is repaired.

Chicken calicivirus. Day-old chicks fed with CCV produced loose droppings and developed sticky vents, 3–4 days after infection. Many of the birds died and those who survived failed to thrive. Virus particles were detected in excreta 7–9 days after exposure. Examination of material from birds that died within a few days of challenge revealed virus in homogenates of intestine.

Canine calicivirus. Studies in the USA and UK have shown that puppies fed with cell culture extracts containing CaCV remained healthy. Virus was isolated from the throat and faeces of some animals, one to three days after exposure. All the puppies seroconverted, which indicated that they had become infected.

Amyelosis chronic stunt virus. A diet contaminated with ACSV proved lethal to first instar navel orange worms. Older larvae failed to thrive and eventually died. The majority of the particles found in the frass (excrement) were smooth degraded particles. Characteristic rough particles were found in granular haemocytes.

Epidemiology of caliciviruses

Mammalian, avian and insect candidate caliciviruses

All these agents have been identified by groups in either the UK or USA. The majority were detected by electron microscopic examination of faecal samples obtained from young creatures presenting with diarrhoeal diseases. There are no data available on their wider geographical distribution, their prevalence in different age groups, or their economic importance as pathogens.

Human calicivirus and Norwalk viruses

An electron microscopic survey into the causes of infantile gastroenteritis in North-West London showed that HCV accounted for about 5% of cases of identified viral diarrhoeas; rotavirus accounted for 71%; adenovirus, 14%; astrovirus, 7%; and Norwalk-like agents, 4%.

Surveys in other countries have shown similar results. Sporadic cases of HCV infection in infants have been recorded throughout Europe (including Scandinavia) and North America, and in Saudi Arabia, India, Japan and Australia.

Serological studies using HCV UK1 and Japanese strains of HCV indicate that these strains are prevalent in populations throughout the world, including Africa and South-East Asia, where diarrhoeal diseases are a major cause of morbidity and mortality.

A more detailed survey of HCV infections in England and Wales showed that high levels of antibody, presumably maternally acquired, were present in sera obtained from neonates. This antibody appears to afford some protection against infection, as only 6/269 (2%) of sporadic cases of HCV infection were reported among this age group; two of these neonates were known to be suffering from Severe Combined Immune Deficiency disease. The vast majority (84%) of sporadic cases occurred in children aged between three months and five years and in the elderly (over 70 years).

A survey using the HCV UK1 strain and sera collected from infants in the UK, Japan and Saudi Arabia reflected this age distribution of infection during childhood (Fig. 5).

Recent studies have provided increasing evidence of two different patterns of HCV infection. Some strains, such as HCV UK1 and Japan, commonly affect infants, causing symptoms of vomiting and diarrhoea, sometimes accompanied by fever and upper respiratory tract infections. The incubation period is 48–72 h and the duration of illness is from one to eleven days. Transmission of the virus appears to be by the faecal–oral route. In these studies, most adults had high levels of humoral antibodies to these strains and remained unaffected, even when in close contact with sick children.

Other strains, such as HCV UK3 and UK4, affected individuals of all age groups; attack rates in adults were often $> 60\%$. Symptoms of illness were sometimes more 'flu-like' (fever, malaise, aching limbs and nausea) than gastrointestinal. The incubation period was 1–3 days and the duration of illness usually short, only one or two days. The UK3 strain may have been a zoonotic infection originating from an infected dog. Studies of outbreaks of HCV UK4 have shown that the vehicle of infection was raw oysters and, in other episodes, contaminated cold foods. Epidemiological investigations suggest that the virus can be spread by food handlers and indicate that there may be 'carriers' who periodically excrete virus. Such a situation is known to be an important element in the spread of feline calicivirus.

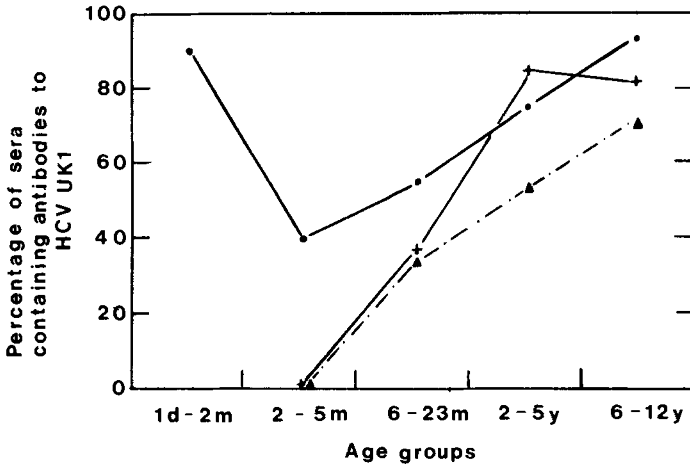

FIG. 5. Prevalence of antibodies to HCV strain UK1 in sera from various populations at different ages. (d, day; m, month; y, year). ●—●, United Kingdom; +—+, Saudi Arabia; ▲-·-▲, Japan.

The second pattern of infection is indistinguishable from that reported for Norwalk viruses, which commonly cause community outbreaks of diarrhoea and vomiting among adults. As with HCV UK4, outbreaks have been associated with the consumption of contaminated shellfish, water and cold foods.

Although neither HCV UK4 nor Norwalk viruses cause a severe illness they are of economic importance because of the very high attack rates in adults, which result in a significant loss of working days. This is particularly evident when outbreaks have occurred in works canteens or after large receptions and banquets.

Conclusion

The use of electron microscopy in a few research centres has proved invaluable in identifying candidate caliciviruses in diarrhoeal specimens. There is now a need to develop alternative diagnostic tests, such as enzyme-linked immunosorbent assays (ELISA), which can be used throughout the world.

Limited success in propagating HCV and CCV in cells by incorporating trypsin in the medium suggests that further studies on the value of proteolytic enzymes may lead to higher yields of virus.

There is convincing experimental evidence that caliciviruses can cause diarrhoeal disease in the young of many species. Further studies are needed to establish their prevalence and importance as pathogens, particularly in countries where morbidity and mortality due to diarrhoeal diseases are highest. This

may be of particular importance when strategies are being developed for the vaccination of populations against infantile gastroenteritis.

Human caliciviruses and Norwalk viruses are now emerging as a major cause of food- and water-borne disease in the populations of the USA and UK. Their significance in other countries is as yet unknown.

Acknowledgements

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DISCUSSION

Bridger: As I mentioned (p 124), we have worked with two strains of what we call bovine calici-like virus, or Newbury agents. One strain did seem to be a calicivirus morphologically, as it showed 10 spikes on the five-fold axis of symmetry. The second one is more difficult to classify. I can't say definitely that it falls into the small round structured virus category (SRSV), but seeing Dr Cubitt's evidence with adding antibody to known caliciviruses, I would like to say that the small round structured virus that we have studied is often clumped and its morphology is difficult to determine. I don't think it is clumped with antibody, because in experimental infections in gnotobiotic calves we see these clumps on the first day of viral excretion; but there appears to be something masking the structure. We have done cross-protection experiments between these two strains in cattle and found no protection between them (Bridger et al 1984). In a survey of calf enteritis in the UK that we did in 1982–1983, we showed that calici-like viruses were associated with 26% of diarrhoeal outbreaks, often in association with other pathogens (Reynolds et al 1986).

Greenberg: Dr Cubitt, do you conclude that Norwalk virus and Snow Mountain agent don't quite make it into the calicivirus family? Norwalk and Snow Mountain agent have been frequently associated with epidemics in adults or in older children, whereas the true enteric caliciviruses have been most frequently

associated with epidemics in young children. Do these two groups really have different epidemiological patterns? Can one separate them into two different types of viruses?

Cubitt: It certainly appears that there are two quite different epidemiological patterns. The HCV 'UK4' viruses have been clearly associated with large outbreaks of food poisoning due to cold foods, or shellfish harvested from contaminated water. In these outbreaks there is a high attack rate among all age groups, including adults. On the other hand there are outbreaks in the community caused by caliciviruses with classical morphology (strains UK1, UK2) particularly affecting infants, young children and the elderly, which are spread by person-to-person transmission. In these episodes, although nurses, parents and teachers were in close contact with infected children, they remained well and were shown to have pre-existing antibody to the strains causing the outbreaks (references in Cubitt 1985).

Greenberg: So in your classification there are two groups: caliciviruses and calicivirus-like viruses. Is this a useful differentiation?

Cubitt: I was trying to emphasize that electron microscopy is a useful tool, but we must start looking at other methodologies, and it is important that research groups collaborate on this. We have shown by our collaboration with Professor Chiba's group in Japan that his radioimmunoassay is very useful; again, our collaboration with Dr Blacklow's group is giving very interesting results. When this joint study is completed, maybe we can give you the answers.

Greenberg: In several studies on hepatitis A, and in your studies on the seroepidemiology of caliciviruses, one finds antibody mainly in adults and not in children. One interpretation is that people are being exposed to the virus late in life. However, with hepatitis A, when this epidemiological pattern is seen, an alternative explanation frequently given is that there was an exposure long ago, when the population was young, and that the virus has since disappeared from that population. That explanation will give you the same picture.

Chiba: I would like to show you a couple of figures so that you can see how frequently human calicivirus can be associated with outbreaks of infantile gastroenteritis. We have done a long-term study on the causative agents of outbreaks of gastroenteritis in an orphanage in the city of Sapporo. Over the ten-year period from 1976 to 1985, we have done electron microscopic examinations of stool specimens from 18 outbreaks (Fig. 1). Except for one outbreak, viruses or virus-like particles were detectable. The results can be summarized as shown in Fig. 2. This figure emphasizes that calicivirus was associated with five of the 18 outbreaks, being the second most prevalent virus after rotavirus.

Bishop: Have your caliciviruses been compared with UK strains?

Cubitt: The Japanese strains seem to be serologically distinct from calicivirus strains obtained from outbreaks in the UK.

Kapikian: You gave us an elegant demonstration of the use of immuno-

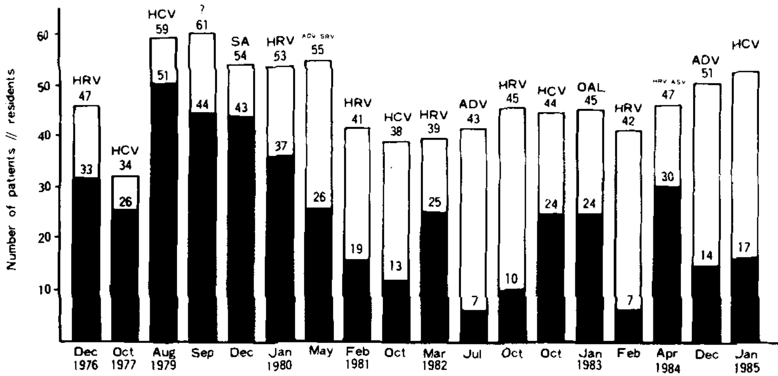

FIG. 1 (*Chiba*). Outbreaks of gastroenteritis in an orphanage in Sapporo, 1976–1985. HRV, rotavirus; HCV, calicivirus; ADV, adenovirus; SA, Sapporo agent; OAL, Otofuke agent-like; ASV, astrovirus; SRV, small round virus.

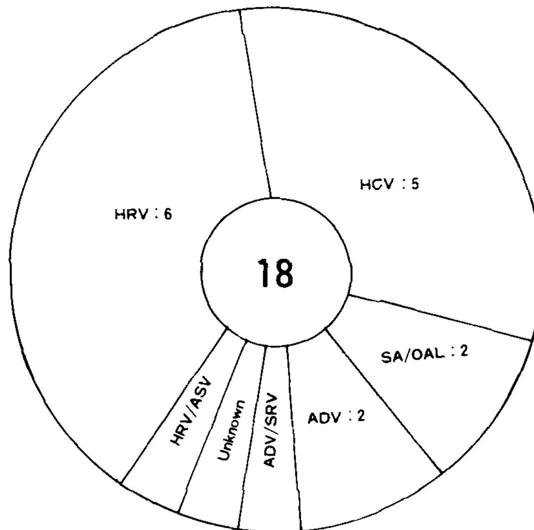

FIG. 2 (*Chiba*). Frequency of viruses associated with 18 outbreaks of gastroenteritis in an orphanage in Sapporo, 1976–1985.

electron microscopy (IEM) to classify the different particles. With respect to the volunteer study in which the virus was given intranasally, did you imply that transmission was by the respiratory route? Couldn't the virus suspension have been swallowed after the intranasal administration?

Cubitt: Yes. We were looking to see if there was respiratory involvement and we didn't give the virus as an oral challenge, but as drops through the nose; so

some of it would have gone into the gut. Interferon levels were studied by Dr David Tyrrell's group at Salisbury; we also took nasopharyngeal washings for culture but were unable to demonstrate any evidence of respiratory tract infection.

Kapikian: You showed the existence of five different human calicivirus serotypes, by IEM, and by Dr Chiba's radioimmunoassay method, four of the five were related. What is the solid-phase IEM method that you use?

Cubitt: We add 1% agarose to the wells of a microtitre plate. Various dilutions of the patient's sera prepared in serum-free maintenance medium (it is essential to have calcium and magnesium ions, to prevent spontaneous clumping of the virus) are then incorporated in the agarose. Partially purified calicivirus is added to the wells and a carbon formvar coated grid inverted over each drop. We incubate at 37 °C for 2 h or, for better results, overnight at 4 °C. The grids are then removed, stained with 2% KPTA, pH 6.4, and examined in the electron microscope. The end-point is taken as the highest dilution at which antibody can clearly be seen on the virus. The control grid must show free particles. We frequently find prozone effects resulting in difficulty in finding virus at serum dilutions of 1:20 to 1:40, whereas strongly positive reactions can readily be detected at dilutions of 1:320. I therefore think that it is essential to have serial dilutions of serum in these immuno-electron microscopy tests.

Flewett: Professor Chiba, were your outbreaks in children related to eating shellfish?

Chiba: No, I don't think so.

Bishop: Have you any reason to believe that any food vehicles are involved? In an orphanage this would be more likely than in the community as a whole; or do you feel that these agents are spread from person to person?

Chiba: I would say the latter. This orphanage has four separate rooms, according to the children's age, and usually an outbreak moves in sequence from one room to another.

Bishop: David Cubitt hinted at the potential for zoonosis with caliciviruses, and that human infection was perhaps derived from a dog in one instance. Can you elaborate on that?

Cubitt: In this outbreak (Humphrey et al 1984) a dog which vomited just prior to the outbreak was shown to have antibodies to the UK3 strain of HCV which was found to be the cause of the illness among the old people.

There have been several attempts, mostly unsuccessful, to infect animals with HCVs (Cubitt 1985). We have demonstrated seroconversions in young rhesus monkeys, and Elisabeth Kjeldsberg in Scandinavia demonstrated seroconversion in rabbits (Kjeldsberg & Mortensson-Egnund 1983). There is also evidence that San Miguel sealion virus might infect man (Smith et al 1978a).

Horzinek: Is it still correct to assume that the vesicular exanthema of swine epidemic was caused by the feeding of carcases of sealions to pigs, showing that caliciviruses can pass the species barrier?

Cubitt: Yes, this was the hypothesis of Smith et al (1978b), that VESV originated in fish; it has also been suggested that chicken calicivirus (CCV) may have entered the UK because chickens were fed on fishmeal.

Horzinek: But there is no serological relationship between San Miguel virus and the human isolates?

Cubitt: So far as we know, there is no serological relationship between any of our human caliciviruses and the characterized or candidate 'enteric' (animal) caliciviruses (Cubitt 1985).

Blacklow: Does the cytoplasmic fluorescence seen in HEK cells infected with HCV tend to be an incomplete event, or is the fluorescence able to be passaged serially in HEK cells?

Cubitt: It doesn't passage at all well. It seems to be similar to what we have been hearing for the other novel diarrhoea viruses; we are getting a replicative cycle but poor yields of virus. We are doing further studies, using the cell systems I looked at before, but trying various proteolytic enzymes to see if we can find a better alternative to the Difco trypsin, which varies from batch to batch. Propagation in the dolphin cell line works if you can get the cells, as I said.

Flewett: We had a batch of commercial semi-purified trypsin which was probably contaminated by a bovine rotavirus. Crystalline trypsin should therefore be used.

Horzinek: Aching limbs was a symptom in one patient affected with UK strains of HCV. This is interesting, because Niels Pedersen found a calicivirus in cats which causes the 'limping kitten' syndrome, where kittens have joint pains for two to four days and then recover (Pedersen et al 1983).

Cubitt: I hadn't read that. It is interesting that the feline calicivirus, although principally a respiratory tract infection, has been reported to infect dogs and cause gastroenteritis in them.

Hall: Do these caliciviruses actually grow in shellfish?

Appleton: The shellfish concentrate viruses from polluted water, because of the way in which they feed, drawing water in over their gills. Some work done with enteroviruses in the Republic of Ireland shows that not only does the virus pass over the gills and into the gut system, but after a day or two it is absorbed into the tissues (K. Collins, personal communication). But there is no evidence of replication in the shellfish.

Hall: Is there a long period of survival in the shellfish?

Appleton: Yes. There has been much work on the depuration of oysters. All bacterial contaminants have gone within 24–48 hours, but enteroviruses are still present after six weeks.

Kurtz: You mentioned the transformation of caliciviruses from a 'rough' form to a 'smooth' form. Isn't this rather unusual?

Cubitt: It is due to the virus being uncoated. The paper by Hillman et al

(1982) provides convincing evidence that there is a change from a rough to a smooth form as the result of proteolytic digestion.

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Immunobiology of Norwalk virus

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Abstract. Clinical immunity to Norwalk virus in inoculated human volunteers appears to be unusual for gastroenteritis viruses, as certain individuals are repeatedly ill on long-term virus rechallenge and others remain persistently well. In these volunteers there is a paradoxical inverse correlation between the pre-challenge serum (and jejunal fluid) Norwalk antibody level (measured by radioimmunoassay) and resistance to illness, suggesting that non-immunological factors, perhaps genetic, may be important in determining resistance. Most reported naturally occurring Norwalk disease outbreaks in developed nations also show that humoral antibody fails to correlate with immunity to infection. The unusual pattern of clinical immunity to Norwalk virus indicates a need for caution in the development of vaccines against this agent as well as a need for additional information on its immunobiological characteristics. The virus is known to contain a single protein, like the caliciviruses. Recently we have found evidence for at least a one-way serological cross-relatedness between Norwalk virus and human calicivirus. Twelve of 20 paired sera from ill patients in outbreaks due to calicivirus strain UK4 seroconverted to Norwalk virus by radioimmunoassay and two of eight paired sera from UK2 outbreaks showed seroconversion. Future studies of outbreaks caused by various calicivirus strains should be designed to correlate acute-phase serum antibody titres to Norwalk virus with clinical susceptibility and immunity to infection.

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Introduction

The Norwalk-like virus group

Norwalk virus is a 27 nm diameter, non-enveloped, round particle of unclear substructure that is responsible for outbreaks of gastroenteritis among older children and adults (Cukor & Blacklow 1984, Kaplan et al 1982). It is the first-discovered and most extensively studied of a group of poorly defined, non-cultivatable small viruses that share properties of morphology, density, and derivation from epidemics or family outbreaks of gastroenteritis (Cukor & Blacklow 1984). In addition to the failure of the virus to undergo detectable

replication in cell culture, there are no animal models available for the study of Norwalk virus disease. Consequently, most information on the immunobiology, epidemiology and pathogenesis of Norwalk virus infection has relied upon data and clinical reagents collected from human volunteer studies. Such studies either have not been done for other small viral particles (e.g., Otofuke, 'small round virus') or have been limited in scope (e.g., Hawaii, Montgomery County, Snow Mountain). Therefore, at the present time our knowledge of Norwalk virus greatly exceeds that for the other agents and Norwalk can be considered as the prototype strain.

Assays for detection of Norwalk virus

The virus can be visualized in diarrhoeal stool by immuno-electron microscopy (IEM), using convalescent-phase serum from a human volunteer as the source of antibody (Kapikian et al 1972). The IEM procedure can also be adapted to estimate antibody levels to the virus in human serum. In practical terms, however, IEM is a cumbersome assay that is unable to permit the rapid testing of large numbers of samples necessary for understanding the immunobiology, epidemiology and pathogenesis of infection. Fortunately, radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) techniques have been developed for this purpose (Greenberg et al 1978, Blacklow et al 1979, Herrmann et al 1985). They rely for their critical reagents on human volunteer sera and stools derived from individuals experiencing experimentally induced Norwalk illness. The RIA and ELISA tests are limited to a few research laboratories that possess small amounts of these valuable human reagents.

Epidemiology

Forty-two percent of 74 outbreaks studied of acute non-bacterial gastroenteritis in the United States from 1976 to 1980 have been attributed to Norwalk virus, on the basis of RIA antibody seroconversions in at least 50% of serum pairs from each outbreak (Kaplan et al 1982). An additional 23% of the 74 outbreaks were ascribed arbitrarily to 'Norwalk-like' agents, based on seroconversions that were detected but were seen in less than 50% of serum pairs studied from an outbreak. The designation of this 'Norwalk-like' category carries with it the assumption that the Norwalk immunoassay will detect a low frequency of heterologous antibody responses directed against other small gastroenteritis viruses. Regardless of interpretation, it is clear the Norwalk virus is a frequent cause of epidemic gastroenteritis. Furthermore, seroepidemiological studies indicate that infection is common worldwide. In the United States approximately two-thirds of adults possess serum antibody to the virus (Greenberg et al 1978, Blacklow et al 1979). Antibody is unusual during childhood but is rapidly acquired during late adolescence. In developing nations, however, serum antibody commonly appears during childhood (Cukor & Blacklow 1984).

Classification

The infectivity of Norwalk virus for volunteers remains stable after exposure to ether, acid (pH 2.7) and heat (60 °C for 30 minutes) (Cukor & Blacklow 1984). The virion, purified from faeces, has a buoyant density in caesium chloride of 1.38 to 1.40 g/cm^3. It also contains a single, 59 000 M_r protein which is similar to that found in caliciviruses (Greenberg et al 1981b). Unfortunately, a definitive classification of Norwalk virus cannot be made without determination of its nucleic acid type. For this, laboratory propagation will probably be necessary, inasmuch as the virus is shed in human stool in relatively low titre. With regard to morphology, it has been noted that human calicivirus loses its characteristic surface appearance in stool after storage at −70 °C, so that it resembles Norwalk virus (Humphrey et al 1984). Also, Greenberg and colleagues have observed a resemblance of Norwalk virus to calicivirus when Norwalk is visualized with little or no IEM-detectable coating antibody (Greenberg et al 1981b).

Clinical immunity to Norwalk Virus

Volunteer studies

Clinical immunity to Norwalk virus in inoculated human volunteers appears to be unusual for gastroenteritis viruses, as certain individuals are ill repeatedly on long-term homologous virus rechallenge and others remain persistently well (Parrino et al 1977). As shown in Fig. 1, when a group of 12 volunteers was inoculated with Norwalk virus and then rechallenged 27 to 42 months later, precisely the same six volunteers who became ill on the initial challenge became ill again on rechallenge. In contrast, those who were clinically well on the first challenge remained well on the second. With each illness, significant rises in serum antibody titre occurred, usually from baseline values that demonstrated pre-existing antibody to Norwalk virus. Volunteers who persistently resisted illness usually had low or absent serum antibody levels before and after exposure to the virus. Paradoxically, then, the presence of serum antibody (measured by either IEM or RIA) to the virus and the ability to generate it constitute risk factors for this illness. When local jejunal fluid antibody to the virus was measured in two separate studies, in Bethesda and Boston, a similar situation was noted in that the titre of pre-existing local gut antibody was markedly greater in volunteers who subsequently developed illness than in those who remained well (Blacklow et al 1979, Greenberg et al 1981a). In contradistinction to the response to long-term rechallenge, short-term clinical immunity exists when previously ill volunteers are rechallenged six to 14 weeks later.

These findings demonstrate an unusual pattern of clinical immunity that differs markedly from the types of responses that one traditionally associates with other common viral infections. In a retrospective study of serum antibody

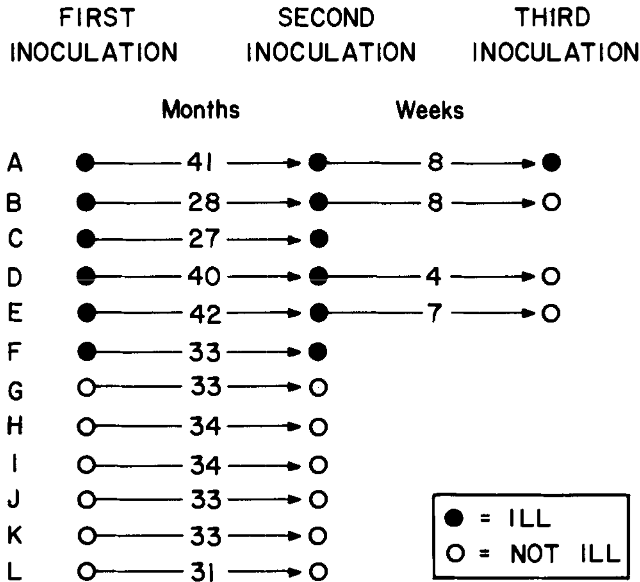

FIG. 1. Sequence of Norwalk virus inoculation studies in 12 volunteers, shown individually by alphabetical letters. The numbers indicate the months or weeks between inoculations. The filled circles represent volunteers who experienced clinical illness, and the open circles those in whom clinical illness failed to develop. (Reprinted, by permission of *The New England Journal of Medicine*, from Parrino et al 1977.)

responses to Norwalk virus in inoculated volunteers, it has been noted that 10 of 13 subjects with pre-existing antibody became ill, whereas 17 of 25 lacking antibody did not ($P = 0.009$) (Blacklow et al 1979). These results support the concept that some individuals are susceptible to repeated infections with Norwalk virus whereas others are incapable of developing infection.

The reason for this unusual pattern of clinical immunity is not known. It is possible that non-immunological, genetically determined factors are the primary determinants of resistance to Norwalk virus, perhaps at the level of intestinal receptor sites. A limited number of inoculated volunteers have been studied for the histocompatibility loci A, B and D, but no correlation with resistance or susceptibility to Norwalk illness has been found (Cukor & Blacklow 1984). Another interpretation of clinical immunity to Norwalk virus is that repeated exposures to the virus are needed to generate an eventual immune response as well as concomitant illness. According to this immunopathological interpretation, resistant adult volunteers are not 'primed' because they have had fewer naturally occurring previous exposures to the virus than susceptible subjects. This hypothesis is consistent with the scarcity of antibody in young children and with its gradual appearance during early adulthood in developed nations.

Naturally occurring illness

Clearly, it is more difficult to study clinical immunity to Norwalk virus in the natural setting than in volunteers. However, some observations from outbreaks of Norwalk virus disease tend to support the results from human volunteers. Epidemiological studies of such outbreaks show a high degree of susceptibility to illness in adults, as is also the case for volunteers. About half of unselected adult volunteers develop acute gastroenteritis upon ingestion of the virus and, in some naturally occurring disease epidemics, illness rates have exceeded 90% (Cukor & Blacklow 1984, Kaplan et al 1982). Many of these ill patients have antibody to Norwalk virus in their acute-phase serum samples, so it is clear that humoral antibody does not, by itself, protect against naturally occurring illness. This also suggests that recurrent illness may be a common event in nature.

An analysis of published Norwalk disease outbreaks in which detailed epidemiological and virological studies are provided indicates that the majority of ill persons have pre-existing antibody to the virus. Some of these reports have commented on not only the presence but also the high titre of pre-existing antibody. These outbreaks have occurred in diverse locations, including Australia, Michigan, Florida, Maryland and New Jersey (Linco & Grohmann 1980, Koopman et al 1982, Gunn et al 1982, Jenkins et al 1985, Griffin et al 1982). One report has even noted apparent familial clusters of disease resistance and susceptibility among equivalently exposed individuals in a water-borne outbreak associated with lake swimming (Koopman et al 1982). Data are not generally available, however, where pre-existing antibody titres in ill and virus-exposed asymptomatic persons are compared. Reported comparisons have usually been made between symptomatic individuals and 'control' subjects (some of whom may have been exposed to the virus and others not). Such a summary comparison of 38 Norwalk virus outbreaks indicated no apparent differences between acute-phase geometric mean antibody titres in ill and 'control' subjects (Kaplan et al 1982). The findings from one study of Panamanian Indians isolated on two remote islands are at variance with the other reports of Norwalk illness, in that disease protection seemed to be associated with the presence of pre-existing antibody to Norwalk virus (Ryder et al 1985). The reason for this difference from other studies is not known.

Implications for vaccine development

The pattern of clinical immunity seen with Norwalk virus, particularly as demonstrated in volunteer studies, indicates that immunity to the virus is not long lasting and that bouts of illness throughout life would seem very possible. Furthermore, the unusual pattern of immune responses to the virus indicates a need for caution in developing vaccines. If clinical immunity is short-lived with wild-type Norwalk virus, it is probably unreasonable to expect that long-term

protection will be conferred by a vaccine strain. Possibly, vaccination could be considered for those requiring relatively short-term protection, such as travellers to developing nations where Norwalk virus is known to cause some cases of travellers' diarrhoea. However, it is clear that additional information is needed on the immunobiological characteristics of Norwalk virus before disease prevention can be addressed. Recent studies, designed to analyse some aspects of the immunobiology of Norwalk virus, are outlined in the following section.

Immunological relatedness of Norwalk virus and human calicivirus

General comparison of the two viruses

As detailed above under 'classification', Norwalk virus and human calicivirus share some features of their morphology, density and protein composition. These findings suggest that the two viruses may be closely related. A detailed discussion of human caliciviruses is presented elsewhere in this volume (Cubitt 1987). For the purpose of discussion of our recent studies of their relatedness to Norwalk virus, it should be noted that the epidemiology of infection with these two viruses shows some differences as well as similarities. British and Japanese studies indicate that, unlike Norwalk virus, calicivirus gastroenteritis is common in infants and young children, and the prevalence of calicivirus antibody is also high in young children (Cubitt & McSwiggan 1981, Chiba et al 1979, Sakuma et al 1981). Data from Japan indicate that the presence of serum antibody to calicivirus may correlate with resistance to illness, which is also unlike the situation with Norwalk virus (Nakata et al 1985). On the other hand, both viruses often produce epidemics of acute gastroenteritis, often affecting adults and those in closed institutional settings, and sometimes spread by a food-borne route, such as the ingestion of contaminated shellfish (Kaplan et al 1982, Gill et al 1983).

Studies of the serological relatedness of Norwalk virus and calicivirus

We have obtained Norwalk antibody titres on 43 pairs of acute- and convalescent-phase serum samples collected from four separate English outbreaks of human calicivirus gastroenteritis, defined by the morphology of the virus in stools. Serum samples were tested, under code, for RIA antibody titres to Norwalk virus by our blocking test, previously described. IEM antibody titres to calicivirus were also obtained on the same 43 serum pairs. Thirty-two paired sera were evaluated from the outbreak in Tower Hamlets (London) previously described (Gill et al 1983), as well as three paired sera from an outbreak in Colchester. Both these outbreaks have been ascribed to type UK4 calicivirus. Two type UK2 outbreaks were also evaluated: three paired sera were tested from an outbreak in Harefield, and five from an outbreak in Portsmouth.

Seventeen acute- and convalescent-phase serum pairs from symptomatic patients in the Tower Hamlets outbreak were studied. Seroconversion to Norwalk virus developed in ten (59%) and seroconversion to calicivirus occurred in thirteen (76%). Fifteen serum pairs were also evaluated from asymptomatic patients in the outbreak, all of whom remained well, presumably due to their failure to eat contaminated oysters, which were felt to have been the vehicle by which infection was initiated. One of the fifteen (7%) well persons developed a low level seroconversion to Norwalk virus, and a different individual seroconverted to calicivirus (7%).

The Tower Hamlets outbreak provides evidence for at least a one-way serological cross-relatedness between Norwalk virus and human calicivirus type UK4. Further support for this relatedness is seen in the study of an outbreak in Colchester in which two out of three persons seroconverted to Norwalk virus (67%), while all three (100%) seroconverted to calicivirus. It should also be noted that Norwalk RIA antibody titres in patients with calicivirus infection in both outbreaks who showed seroconversion to Norwalk virus were of a magnitude similar to those observed in well-defined outbreaks of Norwalk disease.

The reciprocal geometric mean titre (GMT) of acute-phase Norwalk serum antibody for the 17 ill patients at Tower Hamlets was 313.19, and the GMT for the 15 asymptomatic patients was 48.53. Unfortunately, inasmuch as the asymptomatic individuals were presumably not exposed to the virus on this occasion, no valid comparisons can be made of the association between pre-existing antibody titre and susceptibility to infection. It is interesting, nonetheless, that ill persons had considerably higher GMTs than a similar population of individuals who were present at the social functions where the outbreak originated. Future studies of outbreaks due to various calicivirus strains should be designed to correlate acute serum antibody titres to Norwalk virus with clinical susceptibility and immunity to infection.

Seroconversion to Norwalk virus developed in two of eight patients from outbreaks in Harefield and Portsmouth, both ascribed to the UK2 strain of calicivirus. In contrast, all eight patients seroconverted to calicivirus. Thus, there may be a difference in the degree of antibody reactivity to Norwalk virus antigen between sera collected from calicivirus outbreaks due to different viral strains: 12 of 20 (60%) patients exposed to calicivirus UK4 seroconverted to Norwalk virus, as compared with two of eight (25%) patients in UK2 outbreaks. These results are summarized in Table 1.

Some of the specimens showing seroconversion to calicivirus and Norwalk virus have been reacted by S. Chiba in his RIA test for calicivirus antibody (Nakata et al 1985) and most have failed to demonstrate antibody rises (data not shown). The reason for this discrepancy is not known, but may reflect strain differences between caliciviruses or different reactive antigens used in the RIA procedures for Norwalk virus and calicivirus.

TABLE 1 Summary of seroconversions[a] to Norwalk virus and human calicivirus detected among symptomatic patients in four UK gastroenteritis outbreaks due to calicivirus

Outbreak	*Calicivirus strain*	*Norwalk virus*[b] *(no. seroconverting/ no. tested)*	*Calicivirus*[c] *(no. seroconverting/ no. tested)*
Tower Hamlets[d]	UK4	10/17	13/17
Colchester	UK4	2/3	3/3
Harefield	UK2	2/3	3/3
Portsmouth	UK2	0/5	5/5

[a] Seroconversion is defined as a fourfold or greater rise in antibody titre between acute-phase and convalescent-phase serum samples.
[b] Seroconversion to Norwalk virus measured by RIA.
[c] Seroconversion to calicivirus measured by IEM, using homologous virus strain.
[d] In addition, 15 asymptomatic individuals were tested; one seroconverted to Norwalk virus and another seroconverted to calicivirus.

To date, we have not studied whether a two-way serological cross-relatedness exists between Norwalk virus and human calicivirus, namely, by examining large numbers of paired sera from Norwalk outbreaks for their reactivity with calicivirus antigen. Paired sera from an asymptomatic chimpanzee that developed antibody to Norwalk virus after experimental virus inoculation have not reacted in the calicivirus RIA test of S. Chiba (Nakata et al 1985).

Implications of Norwalk virus relatedness to calicivirus

Our studies of the relatedness of Norwalk virus and calicivirus indicate that several other poorly defined, small viruses that share characteristics of their derivation and also morphological, biophysical and, in some cases, biochemical properties, may also share immunological properties. It has been known for some time that some pairs of agents (e.g., Norwalk and Montgomery County) are closely related, perhaps identical, when examined by IEM (Blacklow & Cukor 1981). This technique would be expected to measure shared antigens on the surface of the virion which could reflect very close serotypic relatedness. In contrast, our current immunoassay techniques, available for only a few of these small viruses, may reflect a broader serological reactivity with inner or soluble components of the virion contained in human stool. For example, as described above under 'Epidemiology', some gastroenteritis outbreaks show a detectable but less than 50% seroconversion rate to Norwalk virus, and these outbreaks have been arbitrarily called 'Norwalk-like' (Kaplan et al 1982). Support for this concept is provided by our recent finding of RIA seroconversions to Norwalk virus in two of 21 patients (10%) in an outbreak of Snow Mountain agent gastroenteritis in which six of 11 (54%) tested patients seroconverted to Snow

Mountain by ELISA (C. Guest et al, unpublished work 1986). Both of the patients seroconverting to Norwalk virus also seroconverted to the Snow Mountain agent. The latter has been called 'Norwalk-like' and, as is the case for Norwalk, possesses a single protein of molecular weight characteristic of caliciviruses (Madore et al 1986).

Further studies are clearly needed of the relatedness between Norwalk virus, calicivirus, and other small viral agents associated with gastroenteritis.

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DISCUSSION

Kurtz: Did the volunteers who remained well excrete virus?

Blacklow: Not as measured by radioimmunoassay.

Kurtz: Do you think there is a difference between the two populations, susceptible and non-susceptible, in terms of genetic make-up, which might explain why non-susceptible subjects, with their relative lack of antibody to Norwalk virus, do not become ill?

Blacklow: We have very little information on that. The volunteers all came from the same geographical area and were about the same age. Their histocompatibility antigens showed no correlation with disease resistance or susceptibility, as I mentioned.

Kurtz: It would appear from David Cubitt's paper and your joint paper that whatever the names currently given to these viruses, there are two epidemiologically distinct patterns here: first, the morphologically typical calicivirus causing infection in children and, second, a group of viruses associated with outbreaks in adults where there is a high attack rate and illness occurs in people who already have antibody. There appears to be some cross-reacting antibody; even if UK4 and UK2 are different strains, following infection with them a one-way antibody response to the Norwalk agent develops. Evidently there is a group of viruses causing these adult outbreaks which in the electron micro-

scope do not have the typical appearance of the caliciviruses that cause illness in small children.

Recently there has been a lot of Norwalk-like illness in Britain in adults and children. Many viruses have been seen, all of which look like the Norwalk agent, not like a typical calicivirus.

Kapikian: The new information from Dr Blacklow and Dr Cubitt is very striking: I didn't expect that individuals with a seroresponse to a calicivirus would also have a serum antibody response to the Norwalk virus. Of course, this doesn't establish that the Norwalk virus is a calicivirus, but it is strong presumptive evidence. With regard to this, however, Dr Blacklow showed that of the 20 individuals with known infection with the UK4 calicivirus, 16 developed a seroresponse to the homotypic UK4 strain and 12 of the 20 also developed a seroresponse to the Norwalk virus. However, for the UK2 strain of calicivirus the relationship was less striking, since of the eight individuals with known infection with the UK2 strain, each developed a seroresponse to the homotypic UK2 strain but only two of the eight developed a seroresponse to the Norwalk virus. I had the impression from Dr Cubitt's paper that the UK4 strain lacked the classical appearance of a calicivirus, i.e. without the surface hollows, whereas the UK2 strain appeared to be a classical calicivirus morphologically. With this in mind, it seems that the correlation between seroresponses to Norwalk virus and UK2 virus in UK2 virus-infected individuals is not statistically significant, whereas that for UK4 virus-infected individuals is significant. So the evidence that the Norwalk virus is a calicivirus is still incomplete, since the UK4 virus may be a Norwalk-related agent, because it resembles it morphologically, whereas the UK2 virus may be a true calicivirus not related to Norwalk virus.

Cubitt: The HCV UK4 strain lacked the classical calicivirus morphology until it was enhanced by Markham rotation.

There is one more piece of information regarding the relationship of Norwalk and HCV; in 1980 I sent sera to NIH from our UK1 calicivirus outbreaks and no serological responses were found in Dr Greenberg's Norwalk RIA test, so HCV UK1 and Norwalk virus are apparently antigenically distinct.

Chiba: I have a question for Professor Blacklow concerning the possible antigenic relation between Norwalk virus and human calicivirus. We have never obtained any results suggesting an antigenic relatedness between those two viruses, either by IEM or by RIA. As mentioned earlier by David Cubitt, our RIA has been shown to detect the common antigen of morphologically distinctive human caliciviruses but not Norwalk virus. In addition, according to Dr Shuji Nakata, who now works with Dr Mary Estes in Houston, RIA for Norwalk virus cannot detect human caliciviruses (personal communication). How can you explain the difference in your results, taking all these findings into consideration?

Blacklow: The findings may be accounted for on the basis of the different

kinds of reagents used in the immunoassays. Your assays rely on hyperimmune animal serum. Our assays use human serum reagents. Maybe there are other differences between the assays as well.

Turning to Dr Kapikian's comment on the UK2 strain, although the number of Norwalk seroconversions was not as great as against the UK4 strain, nonetheless there were two of eight individuals in UK2 outbreaks who seroconverted to Norwalk virus. In one of the UK2 outbreaks it was 2/3 persons and in the other, 0/5, which combined to make 2/8 (Table 1).

Greenberg: In the long run, the genomic material of all these enteric viruses has to be identified, and that will be the conclusive proof of their relatedness or lack of relatedness.

Bishop: Is there any evidence of relationships between animal and human caliciviruses?

Cubitt: No; using a wide range of tests (Cubitt 1985) we have failed to find any evidence of an antigenic relationship between animal and human caliciviruses.

Hung: Are the diarrhoeal diseases from Norwalk virus and caliciviruses distinguishable clinically?

Blacklow: The clinical illness in the outbreaks due to calicivirus seems to be similar to Norwalk virus when infections occur in the same age group, such as outbreaks among older individuals (Cubitt et al 1981).

Chiba: It is rather difficult to say, however, because the age at which outbreaks occur seems different between the two viruses.

Kurtz: During 1986 we have seen a lot of Norwalk-like illness, often as endemic infections on hospital wards, as I mentioned, and often there appeared to be a double illness, rather than a biphasic illness, in the patients. A first illness occurred after an approximately 48 hour incubation period, and lasted a day or two, with vomiting and some diarrhoea; this was followed after an interval of 2–3 days by a second bout of the same type of illness. I don't know if this has been noticed in any of the Norwalk outbreaks in the past?

Greenberg: It is not something that I recall.

Blacklow: That has not been seen in experimentally infected volunteers.

Appleton: We have seen this pattern in gastroenteritis outbreaks where we have found parvoviruses. We have followed sequential specimens from some adult patients, and seen a rise in the number of virus particles excreted, reaching a maximum three to five days after onset of symptoms, and then gradually falling. Occasionally a patient has developed a second episode of illness perhaps a week later and we have observed a corresponding increase in virus excretion coinciding with the symptoms.

We need to sort out the age distribution of infections with Norwalk and the caliciviruses. Does Dr Chiba find the calicivirus infections invariably in young children? This is not what we are finding in the UK, where we see these virus infections in all age groups.

Chiba: I have never experienced adult cases of calicivirus infection, maybe simply because we have been concentrating on outbreaks of infantile gastroenteritis. We have probably been missing outbreaks in older children and adults. Actually, an increasing number of outbreaks of gastroenteritis associated with small round viruses in school children and adults have recently been reported from various parts of Japan. We have to do further tests to identify these viruses and classify them as Norwalk virus, calicivirus or Otofuke agent.

Blacklow: Norwalk virus affects school-age children and adults in developed societies. Serum antibody prevalence studies show the relatively late appearance of antibody during adolescence. However, prevalence studies in developing countries show antibody appearing earlier, in the three- to six-year-old age group (Cukor et al 1980).

Kapikian: Although Norwalk virus in Western countries does not cause significant disease in infants and young children, in studies by Black et al (1982) in Bangladesh and/or Ryder et al (1985) in Panama, seroresponses to Norwalk virus were documented in infants and young children and such infections could be associated characteristically with mild diarrhoeal illness. In addition, by the fifth year of life, most children possessed serum antibody to Norwalk virus. Studies in Guatemala by Mata et al (1981) showed that in infants, repeated diarrhoeal episodes caused by various pathogens resulted in severe growth retardation and precipitated severe malnutrition. This was induced by decreased food consumption during episodes of diarrhoea, coupled with acute weight loss and alterations in absorption, secretion and metabolism (Mata et al 1981). Histopathological lesions develop in the jejunum in adult volunteers after experimental Norwalk or Hawaii virus infections. These lesions have been described in challenged volunteers who developed illness and in certain volunteers who did not develop clinical manifestations (Agus et al 1973, Dolin et al 1975, Meeroff et al 1980, Schreiber et al 1973, 1974). It is conceivable that such a mucosal lesion in the jejunum of infants and young children could also play a role in this cycle of malnutrition.

Caul: We have studied several outbreaks of Norwalk-like gastroenteritis in the south of England, where a high degree of secondary spread occurred. This secondary spread is not easily shown to be due to faecal–oral contamination. Bearing in mind your detection of Norwalk virus in vomit, Dr Kapikian, we wonder whether a true respiratory phase is involved (i.e., excluding the possibility of vomit transmitting the infection). Have you seen this at all? And have you done any volunteer studies, giving nasal secretions?

Blacklow: This rapid secondary spread is something that those working with Norwalk virus have often noted, and one wonders if there is another route of transmission besides faecal–oral contamination. In the early Norwalk volunteer studies, three individuals ingested orally throat washings derived from volunteers experiencing Norwalk virus-induced disease. None of them developed the illness (Dolin et al 1972).

Greenberg: I have one piece of information (personal communication) from

Mike Osterholm, an epidemiologist in Minnesota. In one epidemic among schoolboys in a classroom, a child vomited in one corner of the room and there was a gradient in attack rates that fell off as the distance from the index case increased. That is my only evidence for something other than faecal–oral transmission.

Holmes: This is reminiscent of arguments about the spread of rotavirus in the Marshall Islands outbreaks (Foster et al 1980). It comes down to the problem of defining respiratory spread. David Cubitt covered this point nicely when he said he gave volunteers samples of calicivirus through the nose, but had no evidence of replication in the respiratory tract; it was the same as if it had been given by mouth. If an aerosol of any of these viruses with a fairly small infectious dose is created, whether it goes in via the mouth or nose it has a good chance of going through the alimentary tract and causing a primary intestinal infection. It is true respiratory spread only if the virus multiplies in the upper respiratory tract. Chuck Mebus looked for that in bovine rotavirus infections, and nobody since has convincingly demonstrated it. 'Air-borne spread' is probably a better term for infection via swallowed aerosols: 'respiratory' spread is a confusing term.

Woode: We have this problem with rotavirus in piglets, where vomiting occurs and thus there is possible pharyngeal contamination. With the bovine and swine coronaviruses, the enteric strain replicates well in the lung and respiratory tract to quite high titre but is non-pathological at that site. Some people feel that the respiratory route might be important in the epidemiology of TGE virus. As there are respiratory caliciviruses, perhaps the enteric caliciviruses can also replicate subclinically in the respiratory tract.

Holmes: I would certainly accept these as examples of respiratory spread.

Bishop: This is relevant to the question of the spread of these small viruses. Many small viruses are involved in food-borne outbreaks. Other groups may be spread predominantly by person-to-person contact by the faecal–oral or respiratory routes.

Flewett: Dr Lizbeth Kraft (1966) had evidence of air-borne transmission of mouse rotavirus from mouse box to mouse box: she could prevent it by fitting air filters. I don't think rotavirus infection of the respiratory tract has been shown in suckling mice. Dr Coelho working with us a few years ago sectioned whole baby mice and she found fluorescence only in the intestinal tract (Coelho et al 1981).

Hung: Dr Blacklow, have you detected local immunity (sIgA) to Norwalk virus in patients?

Blacklow: We have looked at small intestinal secretions for their blocking activity in a radioimmunoassay procedure and blocking activity has been noted, indicating the presence of antibody in the gut lumen. The blocking activity has not been defined as to immunoglobulin class, so we can't say that it is secretory IgA (Blacklow et al 1979).

Flewett: In your volunteers who already had antibody and developed disease

when they were given the Norwalk agent, was there evidence that these people had sensitized lymphocytes or activated macrophages—in other words, is there any evidence of cell-mediated immunity playing a part?

Blacklow: We have not looked at that. The peripheral blood counts of volunteers in earlier Norwalk studies showed no depression in lymphocyte numbers during the acute phase of the illness, but specific cell-mediated immunity studies were not done.

Bourne: The observations on Dr Blacklow's disease-susceptible group are consistent with what is known of the kinetics of the intestinal humoral response. It is also recognized that cell-mediated immunity (CMI) is involved in mucosal defence and that serum antibody does not always correlate with intestinal defence (Newby & Stokes 1984).

The other interesting point concerns possible genetic influences on receptor sites for viruses. This warrants further research. There is evidence with bacterial infections that receptor sites can be influenced by immune responses which stimulate crypt cell division, resulting in the appearance of immature enterocytes on villi that are more sensitive to the effects of enterotoxin. *E. coli* adhesion may also be influenced in this way. Dietary change can also influence these receptors (Stokes et al 1986).

Wadell: In that context, Dr Blacklow said that the volunteers were from the same group. Were they of the same blood group?

Blacklow: Most of the volunteers did not have their blood group determined.

McCrae: Even with the abortive infections (mentioned in connection with atypical rotaviruses), is there any way of looking at the CMI response? Can you use the abortively infected cells as targets, for instance? It looks as if the CMI response is more important than the humoral response, with many of these viruses.

Blacklow: That may be the case, but the problem, conceptually, is how to do a virus-specific CMI study with Norwalk virus when there is no animal model system available and the virus cannot be cultivated *in vitro*, in order to obtain suitable target cells for study. Unfortunately, one cannot get even an abortive single-cycle replication *in vitro* with Norwalk virus.

Bishop: Is there any direct evidence of which cell is infected in humans?

Blacklow: Two groups have taken small intestinal biopsies from infected volunteers. The results showed a histopathological lesion of the proximal small intestinal mucosa, with epithelial cell damage and crypt hypertrophy, but small viral particles were not seen by electron microscopy in these damaged villi (Agus et al 1973, Schreiber et al 1973). We did immunofluorescence studies using convalescent human serum, known to have antibody to Norwalk virus, but these studies failed to reveal viral antigen in villous tissue.

Bishop: Did you look in the stomach contents or stomach epithelium?

Blacklow: We did not do immunofluorescence studies of stomach epithelium, but biopsy specimens from the stomach were morphologically normal (Widerlite et al 1975).

Saif: With the porcine enteric calicivirus the primary site of replication and also of pathological changes is in the duodenum, which is very different from most of the other enteric viruses we work with, where the distal part of the small intestine is infected (L.J. Saif, unpublished work).

Bishop: Have you seen caliciviruses in duodenal epithelial cells?

Saif: We are looking there now.

Greenberg: One reason why antibody doesn't appear to be effective in protecting against Norwalk virus might be the fact that the virus replicates in the duodenum, where there is either gastric acid or a large amount of bicarbonate, and there are many proteolytic enzymes, which might make it an unsuitable place for an antibody molecule to interact with an antigen. As I remember, you did jejunal biopsies, Dr Blacklow: did you also do duodenal biopsies?

Blacklow: Biopsies were done at the duodenal–jejunal junction.

Hall: Which site of the duodenum did you sample, Dr Saif?

Saif: We collected our small intestinal segment about 15 cm caudally to the pyloric valve.

Hall: My experience of a number of enteropathogens, both viruses and bacteria, is that the duodenum is very resistant, both to infection and to damage.

Flewett: Human deaths from small round structured viruses must be rare. The very first calicivirus that we found was from the small intestine of a baby of a few months old, who died from an infection that ran through the family. Very recently we received specimens taken from an elderly woman who died from an enteritis. We didn't find anything in the small intestinal contents, but we did find calicivirus/Norwalk-like particles—small round structured viruses—in the large intestine. I don't know what that proves!

Hung: Could I ask Dr Cubitt whether human calicivirus produces cytopathic effects *in vitro*?

Cubitt: Yes. The human calicivirus is very like the canine calicivirus, in that individual cells round up, become refractile and bud off into the culture medium. If it is a continuous cell line, the cell sheet closes up and looks perfectly normal.

Mathan: We recently studied an epidemic of acute gastroenteritis in a village near Vellore with a population of 1375 (Patel et al 1985). There were two peaks of incidence, in January–February 1982 and from June to August 1982. The second peak was due to *Shigella flexneri* infection. From 15 out of 18 patients and two out of eight control stool samples collected during the January peak, we cultured Echovirus type 11 in colonic tumour-derived continuous cell lines. Both of the control children from whom the virus was cultured developed diarrhoea within 49 hours. Antibody responses of the patients clearly indicated Echovirus type 11 infection.

I mention these results to make two points. First, in addition to the viruses already discussed, Echovirus type 11, one of the enteroviruses, appears to be associated with epidemics of acute diarrhoea. Secondly, colonic tumour-

derived, differentiated continuous cell lines are suitable for the field isolation of enteroviruses. This was particularly so since by electron microscopy I could visualize viruses in only five of the 15 patients from whom virus was cultured.

Blacklow: It is interesting that in 1958, Eichenwald reported a diarrhoeal outbreak due to Echovirus type 18 in a newborn nursery in the USA (Eichenwald et al 1958). This is clearly an uncommon occurrence.

Kurtz: I find that human embryo fibroblasts are the most sensitive cells in which to grow Echovirus type 11, but I don't have Professor Mathan's colonic cell line.

Bishop: I wonder how adventurous people have been in using cell lines to try to grow the caliciviruses, of either animal or human origin? Is there a body of opinion as to what they will not grow in, and how long is that list?

Woode: True animal caliciviruses of the vesicular exanthema group grow in Vero cells preferentially, but we failed to get our bovine enteric candidate virus to grow in those cells.

Bishop: David Cubitt has been resourceful enough to go to the dolphin, but how many other species have been tried?

Cubitt: I have tried many of the cells listed in the Flow catalogue, but without success! (Cubitt 1985.)

Kapikian: In our laboratory, numerous investigators have tried with a myriad of techniques to propagate the Norwalk virus, but unfortunately without success.

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Toroviridae: a proposed new family of enveloped RNA viruses

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Abstract. The proposed family Toroviridae is characterized by enveloped, peplomer-bearing particles containing an elongated tubular nucleocapsid of helical symmetry. The capsid may be bent into an open torus, conferring a biconcave disk or kidney shape on the virion (largest diameter 120–140 nm), or straight, resulting in a rod-shaped particle (dimensions 35 × 170 nm). Morphogenesis occurs by the budding of preformed tubular nucleocapsids through membranes of the Golgi system and of the rough endoplasmic reticulum. Berne virus, which is proposed as the family prototype, contains a single strand of infectious positive-sense RNA, of M_r about 7.0×10^6, which is polyadenylated. The RNA is surrounded by the major nucleocapsid phosphoprotein (about 20 kDa) which, in turn, is enveloped by a membrane containing a major 22 kDa protein and a 37 kDa phosphoprotein. The viral peplomers, measuring about 20 nm in length, carry determinants for neutralization and haemagglutination; the peplomers are formed by an *N*-glycosylated protein in the 75 to 100 kDa range. Six (to seven) subgenomic polyadenylated RNAs have been identified in infected cells, with M_r values of 2.6, 1.2, (1.0), 0.55, 0.35, 0.27 and 0.22×10^6. Torovirus replication requires some synthetic activity of the host cell. All toroviruses identified so far cause enteric infections and are probably transmitted by the faecal–oral route. Serological relationships between the equine, bovine and human viruses have been demonstrated.

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In 1972, a virus was isolated during routine laboratory diagnostic work from a horse under observation at the surgery clinic in Berne, Switzerland. 'Berne virus' (laboratory designation of the strain: P138/72) was not neutralized by diagnostic antisera against notorious equine viruses and was shown to possess a unique morphology and substructure (Weiss et al 1983).

In 1982, Woode et al reported studies with an unclassified virus isolated from diarrhoeic calves in Breda, Iowa, USA. 'Breda virus' was propagated in gnotobiotic calves and caused agglutination of rat erythrocytes; indications for

the existence of two serotypes were obtained from haemagglutination inhibition experiments. More recently (Beards et al 1984) particles resembling Berne/ Breda viruses in morphology were described in the stools of children and adults with gastroenteritis in Birmingham, England and Bordeaux, France; these particles were shown to be related to Berne and Breda virus by means of immuno-electron microscopy.

These observations indicate that Berne/Breda-like viruses occur in different species including man and that they may be pathogenic. The present review is intended to summarize and discuss the available and partly unpublished data in the framework of this symposium on Novel Diarrhoea Viruses; more detailed reviews are given elsewhere (Horzinek et al 1986a, Weiss & Horzinek 1986c). In particular, we report on the unique physico-chemical characteristics of Berne virus, which is the only isolate studied in culture so far. On the basis of the available information the establishment of a new family of animal viruses was proposed at the 6th International Congress of Virology at Sendai, Japan. Pending approval by the International Committee on the Taxonomy of Viruses in 1987 in Edmonton, Canada, the name *Toroviridae* has been coined for the new family (Horzinek 1984, Horzinek & Weiss 1984).

Resistance to environmental conditions

Thermal inactivation of Berne virus proceeds at a linear rate in the 31–43 °C range. Compared to transmissible gastroenteritis virus of swine (TGE virus), a coronavirus, it appears that Berne virus is more readily heat-inactivated: at 39 °C TGE virus lost only about 1 log of infectivity within 24 h (Laude 1981), whereas a decrease of more than 5 logs was observed with Berne virus. Storage at temperatures lower than −20 °C preserves the infectivity, whereas at 4 °C appreciable loss occurred between 92 and 185 days. Freeze-drying or desiccation at 22 °C causes only insignificant losses.

For a lipid-containing RNA virus, Berne virus possesses an unusual stability to extreme hydrogen ion concentrations. Whereas ortho- and paramyxoviruses, rhabdo-, retro- and arenaviruses, are inactivated at pH values of less than 5.0 and some togaviruses even at pH values as high as 6.0 (see Horzinek 1981), Berne virus retains its infectivity titres at pH 2.5 (Weiss & Horzinek 1986a). This behaviour resembles that of non-enveloped enteric viruses, such as entero- or reoviruses, and may indicate that Berne virus has adapted to passage through the gastrointestinal tract. Again, TGE virus is not acid resistant and its pathogenic potential is explained by the lack of hydrochloric acid in the stomach of young piglets (J.M. Aynaud, personal communication). Berne virus, however, can infect horses 10 months of age, as we have shown by seroepidemiological methods in a stud farm (Weiss et al 1984).

Berne virus infectivity is slightly enhanced by treatment with trypsin or chymotrypsin and also with low concentrations of pronase (Weiss & Horzinek

1986a). This effect is unlikely to be due to dispersion of aggregates but probably reflects an intrinsic property of the virion. Enhancement of infectivity by proteolytic activation is well documented for other enteric viruses such as bovine coronavirus, reoviruses and astroviruses.

It has been demonstrated that Berne virus contains essential lipids, as infectivity is abolished by treatment with organic solvents (Weiss et al 1983). These lipids do not seem to be readily accessible to phospholipase C and sodium deoxycholate, however, since viral infectivity is hardly affected by these agents. The resistance to deoxycholate is especially noteworthy and may again reflect the biological requirements of an enteric virus, which must withstand the emulsifying action of gall bladder secretions (Weiss & Horzinek 1986a).

Morphology

Since most reports of toroviruses are based on ultrastructural observations of particles in stools or faeces, the negatively stained virion has been extensively described (Woode et al 1982, Weiss et al 1983, Moussa et al 1983, Beards et al 1984). Torovirions are pleomorphic particles, spherical, oval, elongated or kidney-shaped in form, and measuring 120 to 140 nm in diameter. In preparations of purified virions we have observed a sausage-like internal structure with transverse striations (estimated periodicity about 4.5 nm) which appeared tightly attached to the membrane and did not leave the particle when the membrane was damaged. Depending upon the preparation, virions are either bald or studded with projections (peplomers) extending out from the envelope.

There is some controversy about the morphology and dimensions of the peplomers. We have described them as 'drumsticks' consisting of a thin stalk carrying a distal spherule (total length about 20 nm). The particles in human faeces were described as carrying peplomers 7–9 nm in length. Occasionally what appeared to be a second ring of smaller peplomers was seen, partly superimposed upon the first (Beards et al 1984). The longer peplomers are believed by Woode et al (1982) to be of doubtful specificity and have been reported only occasionally on particles in samples of human faeces.

The thin-section morphology of toroviruses has been studied so far only with Berne virus (in infected equine cells: Weiss et al 1983, Weiss & Horzinek 1986b) and Breda virus (in gut epithelium from infected calves: Pohlenz et al 1984, Fagerland et al 1986). In Berne virus-infected cells, densely staining spherical, elliptical and elongated particles were seen accumulating at the cytoplasmic membrane and in vacuoles. At higher magnification, a clear distinction can be made between an electron-lucent envelope and a dense, elongated core; especially in cross-sections, the core shows a conspicuous light centre. Enveloped rod-like and crescent-shaped cores are prevalent in the extracellular space or in cytoplasmic vacuoles. Twin circular structures which we interpret as cross-sections through a hollow, tubular nucleocapsid (core) bent into an open

torus are regularly seen; they are surrounded by a tightly fitting membrane. From a statistical evaluation of virion diameters measured in thin-section electron micrographs we concluded that the particle is not spherical in shape (Weiss et al 1983).

In cells of the intestinal mucosa of Breda virus-infected calves, elongated enveloped virions with rounded ends were detected, of average dimensions 35 × 80 nm. The particles were described as being pleomorphic and varying in length (Pohlenz et al 1984, Fagerland et al 1986). The crescent shape described above for Berne virus was not reported.

The morphological data are best explained by assuming a helical nucleocapsid, tightly coiled into a hollow tube which is either straight or bent into an open torus. The virion would consequently have the shape of a biconcave disk—not unlike an erythrocyte. Alternatively, the envelope may follow the smaller curvature of the torus, thereby creating a sausage or kidney shape (Fig. 1). Further support for an elongated tubular nucleocapsid of helical symmetry comes from the observation of circular cross-sections with an electron-lucent centre, of enveloped bacilliform particles, and of convoluted strand structures in the cytoplasm and notably also in the nucleus of infected cells.

A protean particle morphology—bacilliform, kidney-shaped or discoidal—is quite uncommon in virology; different orientations of the particles with respect to the electron beam further contribute to the heterogeneity of the images. This is certainly the main reason why electron microscopists were reluctant to accept the pleomorphic structures as viral in nature, when encountering them in, for example, faecal specimens.

Morphogenesis

In equine dermis cells infected with Berne virus, particles are detected for the first time 10 h after infection. Virions are encountered in all parts of the Golgi system and, infrequently, in the rough endoplasmic reticulum. Budding has been observed at intracytoplasmic membranes, generally those of the Golgi system. This is not unlike the situation in other enveloped RNA viruses, such as bunya-, corona- and rhabdoviruses. However, Berne virus displays unique features in its budding: a rigid, tubular capsid which had been preformed at a site different from the place of budding is incorporated into the virion. Preformed nucleocapsids are also encountered in cells infected with paramyxoviruses which, however, show a different development (budding at the peripheral cytoplasmic membrane, sideways attachment of the capsid to the membrane). Another important feature of Berne virus morphogenesis is a change which the capsid undergoes during budding. We have the impression that the loosely coiled, electron-lucent nucleocapsid becomes progressively straight and electron dense as it enters the bud. It can be speculated that this is due to the interaction of the nucleoprotein with a putative matrix polypeptide. This is in

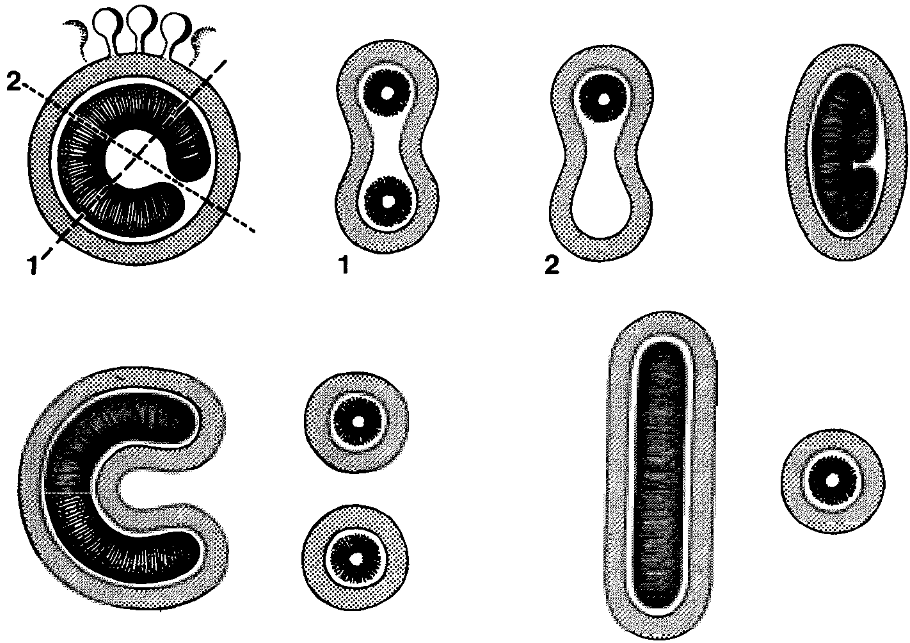

FIG. 1. Schematic drawings of Berne virus particles which explain the variations in morphology observed by electron microscopy. The biconcave disk shape with a toroidal tubular nucleocapsid (upper row) is evident from transverse sections, which may cross the capsid in a single virion twice (1) or only once (2). The same projections of a curved and straight bacilliform particle, respectively, are shown below.

contrast to the situation in rhabdoviruses, where budding is coordinated with the coiling of a helical nucleoprotein strand into a tubular nucleocapsid structure (Weiss & Horzinek 1986b).

Within the cytoplasmic vesicles and cisternae, virions are rod-like or bacilliform in shape. Only particles which have been released or are about to be released possess the characteristic torus form. Consequently, it has to be postulated that further morphogenetic events occur during the transition of intravesicular virions to the extracellular state. These observations are supported by information on the morphology of Breda virus, where bacilliform viruses have been encountered in thin sections through infected enterocytes (Pohlenz et al 1984, Fagerland et al 1986) whereas torovirions of the characteristic shape are prevalent in faecal samples (Woode et al 1982). Consequently, the biconcave disk, 'torovirion' form which we have described cannot be considered as an artefact of the *in vitro* cultivation of Berne virus.

Viral capsids have been encountered in the nucleus; it is not known whether this represents a dead-end stage in virion assembly, or has some correlation with

the dependence of virus replication on nuclear functions, described previously (Horzinek et al 1984).

The capsid

A 20K polypeptide accounts for about 84% of the protein mass and is thus the most prevalent polypeptide in the Berne virion. It was detected in density gradient fractions with maximum infectivity at 1.16 g/ml but also in an intermediate peak (ρ = 1.11 g/ml), which contains about 10% of the infectivity of the virion peak (Weiss et al 1983). The 20K protein is part of a substructure that can be liberated from the virion by treatment with Triton X-100 and subsequently pelleted through sucrose of virion density. In infected cells, it is present in a component of nucleoprotein density (1.36 g/ml) in caesium chloride, containing hot trichloroacetic acid-soluble phosphate label, indicative of nucleic acid. In blotting experiments, the 20K protein was the only RNA-binding polypeptide species detectable in the infected cell lysate; its isoelectric point is in the neutral to basic range, as indicated by two-dimensional polyacrylamide gel electrophoresis (M.C. Horzinek & J. Ederveen, unpublished observations). These properties qualify the 20K species as the main protein constituent of the nucleocapsid. Two additional polypeptides (19K and 17K) are regularly detected in extracts of infected cells. As we have shown by Cleveland digestion, they share oligopeptides with the 20K protein and are interpreted as its fragments. The nucleocapsid of Berne virus was demonstrated after ether treatment as a flexible bacilliform structure showing conspicuous transverse striation (Horzinek et al 1985).

The nucleocapsid proteins of most enveloped RNA viruses are in the 30K to 60K M_r range; exceptions with low M_r are the Flaviviridae, equine arteritis virus, and three genera of the Bunyaviridae family. The 20K protein of Berne virus is phosphorylated, as are the nucleocapsid proteins of corona- and rhabdoviruses. The demonstration of a 20K nucleocapsid protein further supports our proposal that Berne virus is a representative of a new family which is definitely not 'coronavirus-like' (Horzinek et al 1984).

The envelope

Second in abundance (about 13% of the virion protein mass) is a 22K protein which occurs in non-infectious material of low density (1.07 g/ml), present in media from Berne virus-infected cultures. Treatment with Triton X-100 results in its conversion into more slowly sedimenting material, which suggests that the 1.07 g/ml structure is membranous in nature. When virions are treated in the same way, material with a similar sedimentation behaviour is generated. Another phosphorylated polypeptide of 37K is also associated with the viral envelope, since it is present in both the low density (1.07 g/ml) peak and the

virion peak; it is far less abundant than the 22K protein and lacks RNA-binding properties. Phosphorylated envelope proteins have been identified e.g. in some rhabdoviruses.

The peplomer

Two virus-specific glycosylated polypeptide species with M_r values of about 200K and in the 75–100K range, respectively, have been recognized in Berne virus-infected cells in experiments using [^{3}H]glucosamine and [^{3}H]mannose as labels. In purified virions only the smaller glycoproteins are found. They are heterogeneous in their electrophoretic behaviour and relative abundance, with major bands of 96K and 75K, and a minor 86K species. Concanavalin A becomes bound to the virion surface, as shown by a reduction in infectivity. Analysis using sodium dodecyl sulphate–polyacrylamide gel electrophoresis, blotting to nitrocellulose filters, and glycoprotein identification with concanavalin A and horseradish peroxidase confirmed that the viral glycoprotein signals coincide with the maximum of infectivity and haemagglutinating activity in an isokinetic sucrose gradient. Haemagglutination was recently found to be caused also by Berne virus (Zanoni et al 1986). Polyclonal immune rabbit serum and a neutralizing and haemagglutination-inhibiting monoclonal antibody raised against Berne virus recognized both the 96/86/75K and the '200K' glycoproteins. A concentration-dependent inhibition of synthesis of infectious virus by tunicamycin was noted. However, particles are released from the cells in small quantities which lack the glycoproteins; these are also absent from cytoplasmic extracts, but a novel polypeptide of about 150K is identified instead. Translation of poly(A)-selected intracellular RNA from infected cells in a rabbit reticulocyte cell-free system also resulted in the appearance of a high M_r polypeptide (170K). Using pulse-chase labelling and radioimmunoprecipitation we showed a precursor–product relationship between the intracellular '200K' and the virion glycoproteins (Horzinek et al 1986b). These experiments support the notion that the peplomer is constructed from post-translationally processed glycopolypeptides. The presence of a novel 150K protein in tunicamycin-treated cells and of a 170K protein in *in vitro* translation lysates indicates the size of the non-glycosylated primary translation product. The numerical variations reflect the imprecision of the M_r determinations in the high weight range, rather than true differences. The antigenic relationship between the non-structural '200K' and the virion glycoprotein is further supported by earlier radioimmunoprecipitation experiments where a rabbit antiserum raised against purified Berne virus recognized both polypeptides in infected cell lysates (Horzinek et al 1985). The polydispersity of the structure is reminiscent of the matrix protein E1 in coronaviruses.

In addition to these experiments with Berne virus, the Breda virus serotype 2

was studied with respect to its sedimentation and density properties in sucrose gradients and to its structural polypeptides. Virus freshly prepared from faeces of experimentally infected gnotobiotic calves sedimented at 350S and showed a buoyant density of 1.18 g/ml; these values compare well with those of Berne virus (400S and 1.16 g/ml, respectively). Efficient purification of the Breda viruses could be achieved by a two-step method, involving pelleting followed by isokinetic and isopyknic sucrose gradient centrifugation. Radioiodinated purified virus showed polypeptides with M_r values of 105K, 85K, 37K and about 20K. Mouse immune serum raised against Breda 2 virus recognized the polypeptides of the homologous virus, but also the two highest M_r proteins of Breda 1 virus in radioimmunoprecipitation. The same serum inhibited haemagglutination of the heterologous serotype to a low, but significant, degree and efficiently neutralized the infectivity of Berne virus. These observations support our conclusion that the polypeptides in the 75–100K range represent the peplomeric proteins of toroviruses (Koopmans et al 1986).

The genome

On the basis of sedimentation analyses, two classes of particles were distinguished in the supernatants of cell cultures infected with Berne virus. The 380S particle population consisted of infectious virions which contained a single species of polyadenylated RNA with an M_r of 7.0×10^6. This RNA is infectious when assayed under hypertonic conditions. The other particle population sedimented at about 50S, banded at a density of 1.11 g/ml and contained three polyadenylated RNA species with M_r of 0.35, 0.28 and 0.22×10^6. Dot-blot hybridization proved that the viral genome and the three small RNAs are of the same polarity as mRNA. The 50S particles are not infectious, nor do they direct synthesis; interference with virus multiplication was not observed (M.C. Horzinek et al, unpublished observations).

After one cycle of replication, cells infected with purified 380S particles also contained the four RNA species and additional molecules of M_r 2.6, 1.2/1.1 and 0.68×10^6; the latter RNA was detected in actinomycin D-treated cells in which the co-migrating 18S rRNA had been inhibited. The novel phenomenon of virus-like, mRNA-containing particles which are neither defective interfering viruses nor satellite viruses may be another peculiarity of toroviruses (M.C. Horzinek et al, unpublished observations).

The replication of Berne virus is reduced more than 1000-fold by actinomycin D, when the drug (0.1–1.0 μg/ml) is added during the first 8 h after infection; α-amanitin (25 μg/ml) produces a similar though less pronounced effect. UV irradiation of the cells for ≥5 s before infection leads to a dramatic decrease in the production of extracellular virus under conditions in which the replication of Semliki forest virus is unaffected (Horzinek et al 1984).

Epidemiology

In a horizontal study of Berne virus-seropositive mares and their offspring, a decline in maternal neutralizing antibodies and a sudden synchronous seroconversion in all foals were observed, again without clinical symptoms. The virus is widespread in the Swiss horse population and has been so during the last decade; rises in antibody titres were noted in 9% of paired sera sampled at random. Positive reactions were also obtained in serum neutralization tests and enzyme-linked immunosorbent assays (ELISA), using small numbers of horse sera from Germany, France and the USA. The results of neutralization tests and ELISA were correlated in 83% of random samples tested; 13% were neutralization-positive and ELISA-negative and in 4% the inverse was observed. These latter results may indicate that more distantly related toroviruses occur in the horse (Weiss et al 1984). Preliminary studies using an ELISA with Breda virus serotype 2 as antigen have shown that more than 90% of adult cattle in The Netherlands are seropositive; maternal antibody in calves usually disappears at about seven weeks of age and actively acquired antibodies emerge at about six months (U. van den Boom & M.C. Horzinek, unpublished results).

Neutralizing activity against Berne virus was found in the sera of other ungulates (cattle, goat, sheep and pig), laboratory rabbits and two species of wild mice (*Clethrionomys glareolus* and *Apodemus sylvaticus*). Inconclusive results were obtained with feline and human sera; those from dogs and foxes (*Vulpes vulpes*) were consistently negative (Weiss et al 1984).

Concluding remarks

The discovery and partial characterization of a new group of animal viruses by workers in Ames, Berne and Utrecht has shown that classical virology still has its surprises—even in the 1980s. Characterization of this group has reached a stage that safely allows us to propose family status for toroviruses. In itself, it may not be too exciting to add a family to viral taxonomy and classification, even less so since toroviruses—although widespread in nature—apparently are not very dramatic in their pathogenicity. However, study of the organization of the genome, its transcription and translation, and of virion morphogenesis may permit comparisons to be made. Toroviruses will be the fourth family of enveloped viruses where the genome is of messenger polarity (in addition to the Toga-, Flavi- and Coronaviridae); they may contribute to our insight into strategies used by RNA viruses in general, perhaps even into their molecular evolution.

Acknowledgements

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DISCUSSION

McCrae: You indicated that there is a single glycoprotein in the peplomer. Since you detect three bands, why do you conclude that there is not more than one glycoprotein? You start off with a large precursor.

Horzinek: It is really a matter of semantics. The three glycoproteins that are processed from the 200K precursor are all recognized by the same monoclonal antibody, so they must bear the same epitope, unless there is aggregate formation during immune precipitation; we were unable, however, to find the large protein in Western blots.

McCrae: Does that monoclonal recognize the unglycosylated precursor?

Horzinek: We don't know yet whether the *in vitro* product is also recognized.

McCrae: What is happening to the other 80K of protein that doesn't end up in the final product? That is a lot of protein to encode that just disappears, if you are saying that the protein component of the three glycoproteins is the same and they differ only in their glycosylation.

Horzinek: It may not be just glycosylation that accounts for the differences in size. The post-translational cleavage might be taking place at different sites and rates. We also sometimes find discrete smaller products (50K, 70K), which can be brought down by polyclonal antisera (not by monoclonal sera), so these may be the remainder of the 200K protein which is not incorporated into the virion.

McCrae: You said that Berne virus appears to bud everywhere in the infected cell. What is the composition of the glycoprotein? Is it endoglycosidase H resistant, or is the predominant budding site on the far side of the Golgi?

Horzinek: I can only say that it is *N*-glycosylated and that tunicamycin inhibits virus infectivity, but we can get particles from infected cells that also contain an unglycosylated backbone protein of 170K. We have not used endoglycosidases yet to further characterize the peplomer protein.

Bishop: You are culturing Berne virus in mule skin cells. Are these fibroblasts?

Horzinek: They look fibroblastic, but the cytoskeleton of these cells hasn't been properly studied.

McCrae: Do you have any idea what the nuclear phase of the virus is? Is the genome capped?

Horzinek: We don't know yet, but one speculation is that there is capping in the nucleus.

McCrae: Are you sure that the slowly sedimenting particles are not satellite viruses? Are they part of the same genome, and not a completely separate passenger virus?

Horzinek: We have prepared cDNA now to viral RNA which picks up the whole genome as well in hybridization, so we know that these species are Berne virus specific. The three smallest messenger RNAs are enveloped and packed in particles that look like virions but are not infectious; they are not interfering with the infectivity of standard virus, either.

Kapikian: I gather that there has been only one isolate of Berne virus ever made, in 1972, and that this agent is not associated with a disease, but that antibody to it is found in the serum of different animal species, as you described. Are intensive efforts being made to isolate this virus, from horses?

Where would you look for it—in the stool, or in the throat?

Horzinek: The virus was isolated from a rectal swab, and is most probably an enteric virus, as is Gerald Woode's antigenically related Breda virus. I think Berne virus is mainly an enteric virus, and it may be an enteric pathogen.

Kapikian: Has Berne virus been administered to horses experimentally, to see whether it is pathogenic?

Horzinek: This was done, but at a time when we didn't know that we should aim for enteric infection, so it was given parenterally, and the only thing the horses did was to seroconvert (Weiss et al 1984). It has not been given orally to foals, because it is so difficult to find seronegative mares.

Kapikian: Has anyone systematically examined stools from foals that have diarrhoea for the presence of these agents?

Horzinek: This has not been done, but the seroepidemiology has shown that the foals of course have maternal antibodies; these antibodies wane and fall to zero within 3–4 months after birth. An antibody-negative period follows, then the whole herd suddenly seroconverts to high titres. No symptoms have ever been seen during this period of seroconversion (Weiss et al 1984).

Kapikian: So it appears to be a 'virus in search of a disease'? (See Huebner 1957.)

Horzinek: In horses, yes!

Bridger: Would you predict that many coronaviruses have been misdiagnosed, and are in fact toroviruses?

Horzinek: Yes, I would.

Woode: There are reports of coronavirus-like agents in foal diarrhoeas, and we are now looking for toroviruses in horses.

Flewett: As I recall, Berne virus was isolated from a foal that was also infected with *Salmonella lille* and had had a haemorrhagic enteritis.

Horzinek: It had pseudomembranous enteritis and some miliary changes in the liver.

Flewett: So there were pathogens present to account for the symptoms recorded at the time, even without the torovirus.

Kapikian: Why was that initial specimen cultured?

Horzinek: The late Franz Steck did this, because the surgery clinic regularly had virus isolated from sick horses. It was a routine procedure that resulted in the isolation of the new agent.

Kapikian: Did you think it was a coronavirus at first?

Horzinek: Gerald Woode should tell you the whole story!

Woode: When I visited Dr Steck in Berne in 1981, we compared electron micrographs of Berne and Breda virus. It was immediately clear to us that they were similar. As Breda virus was pathogenic, research was reactivated on Berne virus, isolated 10 years earlier and thought to be avirulent.

Horzinek: The virus strain P138/72 was in the freezer for 10 years; when Franz Steck came to Holland, he told me about it and asked whether I would

like to characterize it, because he thought it could be a toga-like virus, and I was engaged at that time in the characterization of non-arthropod-borne togaviruses such as equine arteritis virus.

Greenberg: You said that the search for antibodies in humans was inconclusive. Can you expand on that?

Horzinek: We have not found human antibodies using Berne virus neutralization, but Dr Flewett has some relevant results on this.

Flewett: The short answer is that these antibodies probably do exist in humans, as I shall describe after Gerald Woode's paper (see p 183).

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Breda and Breda-like viruses: diagnosis, pathology and epidemiology

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Abstract. Breda virus serotype 1 (Iowa) was isolated from a severe outbreak of neonatal calf diarrhoea in which 15% of the diarrhoeic animals died. Two further isolates, one from Iowa and one from Ohio, are antigenically related to the first isolate but belong to the Breda virus serotype 2 group by haemagglutination inhibition and by immuno-electron microscopy (IEM). The majority (88.5%) of cattle are serologically positive for Breda virus by enzyme-linked immunosorbent assay (ELISA). The diagnostic methods of choice should include a combination of electron microscopy, IEM, haemagglutination inhibition or ELISA, in order to identify all serotypes of Breda virus. Serotypes 1 and 2 infect and replicate in crypt and epithelial cells of the small intestine, from the mid jejunum region posteriorly through to the descending colon. Virus replicates in and is released from cells before the appearance of microscopic lesions and clinical signs. Diarrhoea starts within 24–72 hours of infection and death can occur within 36–48 hours after the onset of diarrhoea. The morphology of Breda virus, determined by negative staining and from ultrastructural studies of infected cells, is unique and probably diagnostic. Little is known about the epidemiology, other than that transmission is faecal–orally and may require close contact. Large quantities of virus are released in the faeces.

1987 Novel diarrhoea viruses. Wiley, Chichester (Ciba Foundation Symposium 128) p 175–191

For the purpose of this review, the terms Breda and Breda-like viruses will be restricted to those isolates that have been shown to be morphologically similar and antigenically related to the Breda virus isolated in Iowa in 1979 from a diarrhoeic neonatal calf (Woode et al 1982). These viruses include three from calves in the USA (Woode et al 1985), one from a calf in France (Moussa et al 1983), human isolates (Beards et al 1984) and the equine Berne virus (Weiss et al 1983). Most of the information on the diagnosis, pathology and epidemiology of this group of viruses is restricted to studies on the Breda virus in the USA; in

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particular, little information is available yet on their epidemiology.

In the past 16 years at least 14 pathogenic viruses have been discovered and associated with diarrhoea in calves, including different serotypes of rotavirus, Breda virus, calicivirus-like agents, and astrovirus; several of these viruses are also found in humans and other animals (Woode et al 1985). Commonly, two or more unrelated viruses, together with enteropathogenic bacteria and the protozoan *Cryptosporidium,* are found to be present in a particular epizootic and frequently in the same animal. Much effort has been directed to separating the agents present in mixed infections and determining their respective pathogenicities. Breda viruses are no exception to this. Breda virus serotype 1 (Iowa) appeared to be free of other viruses, but both of the Breda virus serotype 2 strains (Ohio and Iowa) were contaminated with a rotavirus. Breda virus 2 (Iowa) was obtained free of rotavirus by the use of a gnotobiotic calf previously immunized with rotavirus (Woode et al 1985).

Diagnosis

The characteristics of this proposed family of viruses (the Toroviridae) have been described by M.C. Horzinek et al (this volume). The distinct morphology and the presence of a haemagglutinin are the two most useful properties for making the diagnosis. In addition, the presence of at least one common antigen has permitted the use of enzyme-linked immunosorbent assays (ELISA) and immunofluorescence, and of the weaker cross-reactions shown by virus neutralization and immuno-electron microscopy (IEM), for antigenic identification. The haemagglutinin and the neutralizing antigens are mainly serotype specific and therefore not reliable as diagnostic tools, except for the particular serotype(s). The morphology, although superficially similar to that of coronaviruses, is sufficiently distinct for the experienced observer to be able to predict the presence of a Breda or Breda-like virus in faecal samples. The tendency of the virus in negative-stain electron microscopy (EM) to appear kidney- or sausage-shaped, occasionally spherical, usually with relatively short peplomers (envelope projections), distinguishes it from coronaviruses. However, in some preparations it is difficult to differentiate it from coronaviruses and, in these examples, IEM is the method of choice (Woode et al 1982, 1985, Beards et al 1984). Many animals excrete low titres of Breda virus in the faeces, in which the particles are difficult to find. The sensitivity of the EM method can be improved by the use of antibody, either by IEM or when antibody is bound to the grid to 'capture' the virus (Fig. 1). IEM with antiserum to one serotype may identify different serotypes of Breda virus, but the cross-reactions are not optimal.

The haemagglutination–haemagglutination inhibition (HAHI) of rat or mouse erythrocytes, with faecal or intestinal preparations, is a useful diagnostic method, as calves excrete the haemagglutinating (HA) antigen for 3–5 days at high titre. However, as the haemagglutinin is strongly serotype specific, HAHI is a poor method for identifying new serotypes (Woode et al 1982, 1983, 1985). In

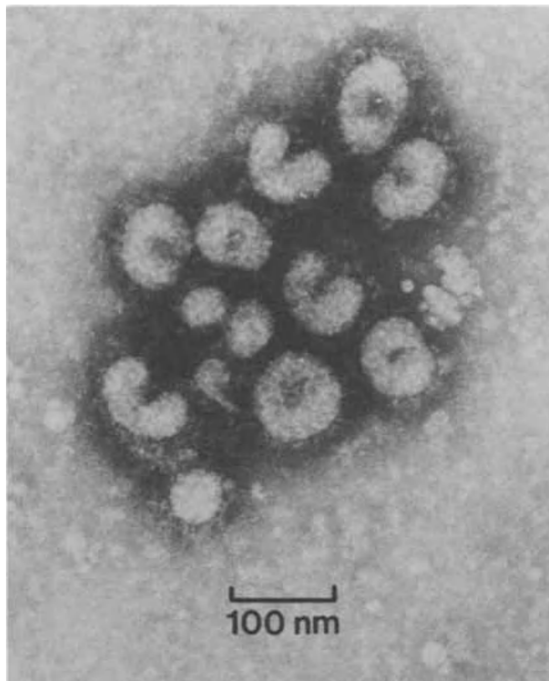

FIG. 1. Virus–antibody aggregates observed after incubation of Breda virus serotype 2 (Ohio) with antiserum to Breda virus serotype 2 (Iowa). (Courtesy *American Journal of Veterinary Research*.)

this laboratory, faecal preparations are titrated with a 1:40 dilution of one of the following sera: antiserum to bovine coronavirus; or antiserum to Breda virus 1 or 2; or fetal bovine serum. The titre with fetal bovine serum is contrasted with that obtained with each of the antisera, and a reduction of the HA titre by 5 $\log_2$ or greater by one of the antisera is diagnostic.

ELISA tests on faecal preparations (to measure antigen) and on serum (to measure antibody) can be used diagnostically. The common antigen of the Breda virus group cross-reacts by ELISA between Breda virus serotypes 1 and 2, and cross-reacts with Berne virus (Weiss et al 1983, Woode et al 1985), although the homologous reactions are 8–10-fold higher in titre, and the test can be useful for serotyping.

Immunofluorescence (IF) techniques can be used to demonstrate the common antigen of Breda group viruses. Intestinal sections from experimentally infected calves, taken posteriorly from the mid jejunum through to the descending colon, are positive for viral antigen in the epithelium of villi and occasionally of crypts, just before and for 24–72 hours after diarrhoea commences. Infected cells remain for longer in the large intestinal epithelium, particularly of the spiral colon and, as for the bovine coronavirus, the large

intestine should be selected preferentially for calves necropsied at an indeterminate time after the onset of diarrhoea.

The USA isolates of Breda virus have not been adapted successfully to cell culture, despite repeated attempts, so this method is not suitable for routine diagnosis.

From the serological evidence that 88.5% of cattle have antibody to Breda virus, these infections are apparently common (Woode et al 1985), yet few field isolates have been obtained. Failure to demonstrate this virus in more cases of disease might result from one or more of the following: the low titres of virus excreted by some calves (Woode et al 1982, 1985); strains of virus with low virulence, where infections do not result in overt disease, as appears to be the case with the Berne equine virus (Weiss et al 1984); confusion of Breda virus and bovine coronavirus by EM examination; reliance on the HAHI test for identification of the virus in faeces, which will miss those Breda viruses with a different or low titre HA antigen; or the presence of strains of Breda virus which may not agglutinate rat or mouse erythrocytes but would react with those of another species, just as Berne virus agglutinates human, rabbit and guinea-pig erythrocytes, but not mice or rat red cells (Zanoni et al 1986).

Pathology and pathogenesis

Breda virus 1 (Iowa) was isolated from a severe outbreak of diarrhoea in calves in which six out of 39 (15.4%) died, and where other pathogens were not associated significantly with the epidemic. Experimental studies with one isolate from this outbreak demonstrated mild to severe diarrhoea both in colostrum-deprived, conventionally reared calves and in gnotobiotic calves. In studies of rotavirus, *Escherichia coli* and Breda virus infection, variation between animals in the clinical severity of infection has been shown to confuse one's interpretation (Woode et al 1982, 1985, Runnels et al 1986). However, the results in different experimental animals do reflect the field observation that severity varies considerably between individuals. Breda virus serotype 2 (Ohio) in older calves produced a relatively mild infection, but serotype 2 (Iowa) can cause a life-threatening disease in some experimentally infected calves.

Diarrhoea begins 24–72 hours after infection and the animals may demonstrate abdominal discomfort, uncontrollable shivering of the body and limbs, and varying degrees of anorexia. Most severe clinical signs occur 24–48 hours after the onset of diarrhoea, with signs of dehydration and weakness.

The diarrhoea in gnotobiotic calves is usually greenish-yellow or brown, watery with mucus, rather than the brilliant yellow of rotavirus infections. The lesions in the large intestine probably contribute to the colour and consistency, by reducing or preventing the reabsorption of water and bile salts.

Breda virus produces a 15–65% reduction in the rate of absorption of D-xylose, in contrast to the 75–100% reduction observed in rotavirus infections

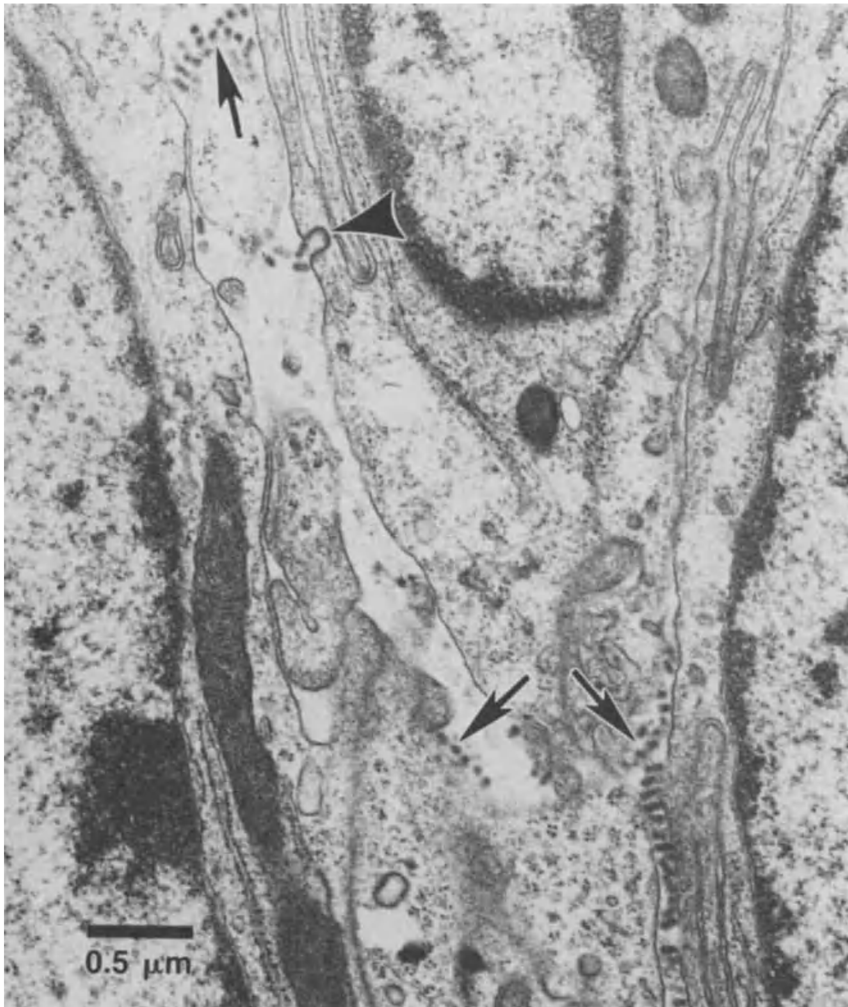

FIG. 2. Basolateral aspect of mid jejunal epithelial cells from a calf infected with Breda virus serotype 2 (Iowa). Numerous virions are in the intercellular spaces (arrows). Arrowhead points to a site where a coated vesicle has apparently fused with the plasma membrane, with the subsequent release of virions. (Courtesy *Journal of General Virology*.)

(Woode et al 1978, 1982, 1985). These differences are consistent with the apparent normality of the upper 30–50% of the small intestine in Breda virus infections, compared with the atrophy usually observed in this region with rotavirus infections.

Post-mortem examinations of the intestine show little macroscopic evidence of infection except for thinness of the intestinal wall. Histological lesions are

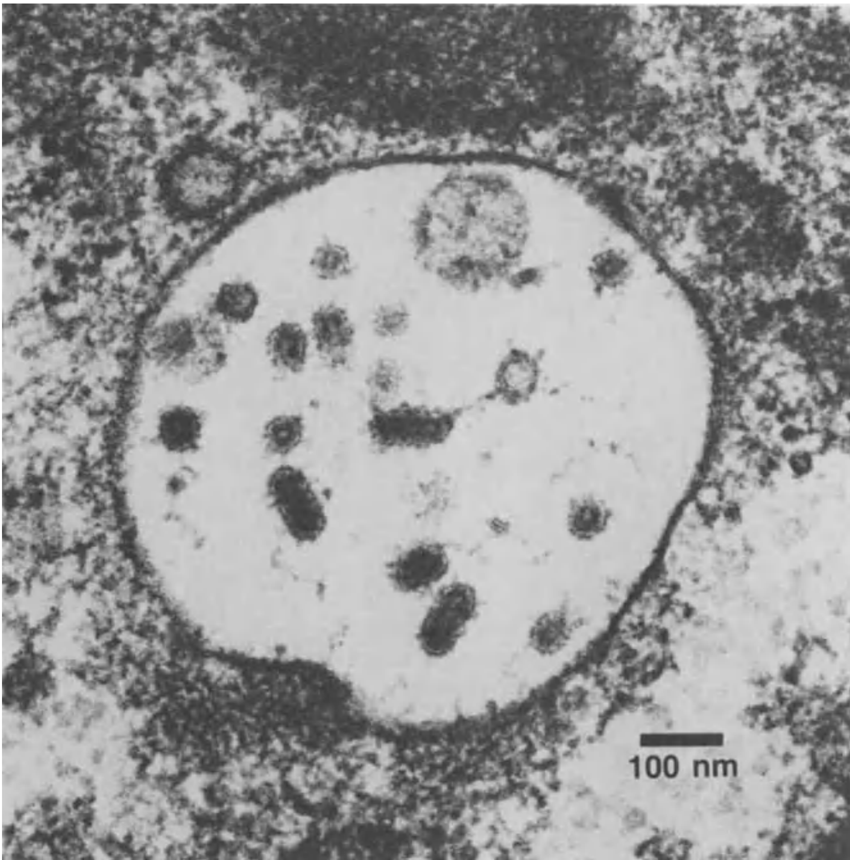

FIG. 3. Typical Breda virions seen in longitudinal and cross-section in a cytoplasmic vacuole of the villous epithelial cell. Taken from the lower jejunum of a calf infected with Breda virus serotype 2 (Iowa). (Courtesy *Journal of General Virology*.)

limited to the mid jejunum through to the lower small intestine, and all parts of the large intestine to the descending colon (Woode et al 1982, Pohlenz et al 1982, 1984, Fagerland et al 1986). Viral infection can be demonstrated in the crypt and villous epithelial cells by IF and EM and includes the M cells of the Peyer's patch epithelium (Woode et al 1984). A few hours later, necrosis of the cells results in atrophy of the villus, which become covered by flattened or cuboidal cells. An increased frequency of mitotic figures is observed in the crypts and some villi are fused at their distal tips. These fusion sites are usually bound on both sides by IF-positive cells, but occasionally only the cell on one side of fused villous sites is IF positive, implying that the virus in or on cell membranes may be one of the causes of the fusion of villi. Focal necrosis occurs in both the surface epithelium and crypt epithelium of the caecum and colon.

From our immunofluorescence and ultrastructural studies it appears that

infection and the release of virus can involve all or the majority of the epithelial cells of a villus in the small intestine in the absence of histological or ultrastructural lesions and before the onset of clinical signs. Shortly thereafter the infected epithelial cells become necrotic, and villous atrophy develops, in conjunction with the commencement of diarrhoea. Infected cells become rounded and vacuolated and slough into the intestinal lumen. Apart from the presence of virus in the Golgi complex, the cells appear normal (Fig. 2). A few hours later the cells demonstrate: an expanded cytoplasmic volume; duplication of the Golgi complex with vesicles; distension and fragmentation of the cytocavity network; large autophagolysosomes that contain debris, damaged organelles and viral particles; and irregular cell surfaces, with shortened microvilli and disintegration of the subsurface cytoskeleton and some irregular mitochondria. Tubules are observed in the nucleoplasm and cytoplasmic matrix or lysosomes; in the cytoplasm these tubules may be associated with virions or incomplete viral particles. The tubules are of the same dimension as the internal structure (possibly the nucleocapsid) of the virus (Woode et al 1982, Pohlenz et al 1982, 1984, Fagerland et al 1986). Complete virions are elongated with rounded ends, with a highly electron-dense core containing an electron-lucent central channel. The virions most closely resemble bacilliform rhabdoviruses, although Breda virus is smaller (Fig. 3) (Fagerland et al 1976). This morphology is similar to that described for Berne virus (Weiss & Horzinek 1986).

Epidemiology

Little is known of the epidemiology of Breda or Breda-like viral infection. The virus probably spreads faecal–orally. Breda virus 1 (Iowa) appears to be a relatively labile virus, as the infectivity of faecal material was lost after a few days at 4 °C and after several months at −70 °C. In contrast, Breda virus 2 (Iowa) and Berne virus appear to be relatively stable, and can probably survive for some time in faeces in cold conditions. In outbreaks of disease, there is great viral contamination of the ground, buildings, feeding utensils, and farm workers' hands and clothing, and the infection spreads rapidly among the calves, with diarrhoea commencing as early as 1–3 days after birth (G.N. Woode, unpublished work).

Whether Breda virus infection carries over from one calving period to another, in apparently immune adult populations, is unknown. In the summer months the virus would not be expected to survive outside the host in infected buildings, or on pasture. It is probable that with most enteric viral infections the presence of continuously infected adult cows, or the constant recirculation of infection among adults, explains the persistence of the virus in the herd, as has been described for bovine coronavirus and rotavirus (Crouch & Acres 1984).

There is little information on the role of other hosts in the spread of the disease. Despite the fact that many species of animals and humans possess antibody to the Berne–Breda virus group, there are no reports of cross-infection between animal species. In one study (G.N. Woode, unpublished work) we

attempted to infect gnotobiotic lambs with Breda virus 1 and 2 (Iowa), but there was no evidence of virus replication or disease induction in this host species.

Discussion

All the requirements thought necessary to prove that Breda virus serotypes 1 and 2 are pathogenic have been met. The viruses appear to be antigenically and morphologically distinct from all other known viruses of cattle and, together with Berne virus of equines, because of their unique morphological and biochemical properties, they have been proposed as members of a new family, the Toroviridae. Although the Breda agents have not been cultured, the fact that they can be passed serially in calves, and increase in titre, confirms their infectious nature.

The development of *in vitro* culture techniques and of improved diagnostic methods is now needed. A search for these agents in humans and animals should be pursued, so that we can assess their importance as disease-inducing viruses.

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DISCUSSION

Flewett: An outbreak of haemolytic uraemic syndrome occurred in 1984 in the West Midlands (Beards et al 1984). The sera and faeces eventually came to us for examination. Some fringed particles were seen in a couple of faecal specimens, all the same size and quite abundant. I had heard about Gerald Woode's Breda virus and wondered whether ours could be anything like it. He sent me Breda virus convalescent serum, and it agglutinated the particles in the faeces. I showed the pictures at a meeting in France, at which Dr Françoise Lamouliatte was showing similar particles in faeces from children in Bordeaux. She had paired sera. Gerald Woode sent me Breda 1 and Breda 2 virus in faeces and we mixed them with the acute and convalescent sera. There seemed to be agglutination. Since then, we have had only one more good faecal sample, although there have been other reports of similar particles in Rotterdam; they were seen in about seven children with haemorrhagic diarrhoea.

Our good sample came from a child with diarrhoea. We did not get a convalescent serum to study. This sample was also full of rotaviruses and on electron microscopy we saw very nice Breda-like particles. This was the only human specimen that haemagglutinated rat erythrocytes; the haemagglutinin was neutralized by Breda 2 serum better than by Breda 1 serum. We don't know whether the agent caused the diarrhoea, because this specimen also contained rotaviruses. Since then, using the Breda antigens and some Berne antiserum from Marianne Weiss, we showed that the Berne serum also agglutinated the 'human' particles. They showed a clear internal torus form after storage for three or four weeks.

We have made an ELISA test using Breda antigen. We used an old Breda 2 virus suspension which had lost its peplomers and therefore banded at 1.16 g/ml; this fraction reacted best in the ELISA test. With a fresh preparation, with the peplomers still in position, the virions banded at 1.14 g/ml and this band reacted with our sera at that density, so the test was probably all right. We also got some reaction in the ELISA at a density of 1.07 g/ml. We looked at various human faecal specimens for evidence of Breda virus; a number gave us

a positive ELISA test, but when they were fractionated in a density gradient, only the fraction of 1.07 g/ml reacted; the 1.16 g/ml fraction did not. So we suspect that this was a non-specific ELISA reaction.

We have looked at blocking antibodies in humans and several animal species, all UK specimens. Most of our cattle were antibody positive and most pigs were negative, though one herd of swine had good antibodies; so did one flock of sheep. We didn't find anything in goats. We had one doubtful human serum but the blocking was not good; the other 92 human sera tested were negative; these were mostly from veterinary workers and a few blood donors. We had reckoned that if this was a zoonosis, we might get more zoonotic cases among veterinary workers than among the general population, but this was not found. We also looked at human faeces for the haemagglutinin; a number had titres of 1:50 and above against rat erythrocytes, but in none of those was the haemagglutination neutralized by Breda 1 or Breda 2 antiserum.

This is as far as we have gone. If we have genuine human cases of this torovirus infection, they must be rare. Every time we found anything like toroviruses in human faeces, another pathogen was also found. In the haemolytic uraemic syndrome, it is almost certainly *E. coli* producing Vero cell toxin. The one HA-positive faeces was also full of rotaviruses. It may be, if these are genuine infections, that for the torovirus to grow in man, another pathogen is needed, to prepare the intestinal tract for these viruses.

Caul: We have looked at a few well-characterized animal coronaviruses and, from the electron micrographs that we have seen, the peplomers of the toroviruses are quite different from those of coronaviruses, with the possible exception of the bovine coronavirus, which is unusual anyway, in that this coronavirus has a double fringe of projections. In the coronaviruses that we have examined the projections are clearly resolved and widely spaced and their attachment to the particle by means of a very thin stalk is often very clear. That was not quite so evident on Dr Woode's micrographs of Breda virus; so I think the morphological difference between coronaviruses and toroviruses is quite clear, with the exception of the bovine coronavirus.

Woode: Yes, although I think these toroviruses are a difficult group of viruses to diagnose.

Snodgrass: In order to diagnose Breda virus and coronavirus, do you do the haemagglutination test directly on a faecal suspension?

Woode: Yes, we run faecal samples routinely against rat red cells. We first titrate faecal suspensions in the presence of antibody-free fetal calf serum. All the positive samples are re-run against a 1:40 dilution of a coronavirus Breda 1 or 2 antiserum.

Bridger: What percentage of the faecal samples that give a high haemagglutination titre are diagnosed as Breda virus?

Woode: Most (70–80%) of the faecal samples causing haemagglutination are coronaviruses.

Bridger: With haemagglutination assays on faeces, do you find higher levels of background than 1:8 to 1:16?

Woode: Perhaps 10% of faecal samples have a high titre of haemagglutinin, which is not blocked by antiserum to coronavirus or Breda virus.

Snodgrass: Is the sensitivity of haemagglutination satisfactory for the diagnosis of coronaviruses?

Woode: I would say that haemagglutination–haemagglutination inhibition (HAHI) has a very close relationship with the results from electron microscopy (or immunofluorescence of gut sections). We like the HAHI method because we can pick up the three viruses (Breda virus 1 and 2 and coronavirus) and then determine the antigenic specificity, which is less laborious than immuno-electron microscopy on many specimens.

Wadell: When you miss specimens, is it because they don't agglutinate rat erythrocytes, or something else?

Woode: All known bovine Breda viruses possess a haemagglutinin. We isolate Breda virus infrequently, despite the serological evidence that the infection is common (more than 88% of cattle have serum antibody). Calves vary considerably, both in the duration of virus excretion and in the virus titre produced, and for these reasons isolation rates may be artificially low.

Wadell: This is useful to know, because there is a problem with using haemagglutination for screening purposes. In the adenovirus system we have one genome type, the Ad19 prototype, which does not agglutinate dog and guinea-pig erythrocytes and is not isolated as a pathogen. The 19a genome type that causes keratoconjunctivitis agglutinates dog and guinea-pig cells and so do Ad8 and Ad37—totally different serotypes that also cause keratoconjunctivitis. So if you screened by using these erythrocytes, you would miss the non-pathogenic genome type.

Woode: I agree.

Horzinek: Berne virus does not agglutinate rat erythrocytes, but it does agglutinate my human blood group O red cells (Zanoni et al 1986).

Saif: We have studied Breda virus (type 2) in gnotobiotic and conventional calves, mostly in older calves (3–4 weeks of age). We have produced only very mild diarrhoea with Breda virus but, in contrast to Dr Woode's results, we have seen more prolonged shedding of the virus, at as high titres as with rotavirus. We usually detect shedding by immuno-electron microscopy for up to 10–14 days. This prolonged shedding is very different from what we have seen with some of the other enteric viruses studied.

With regard to finding other field isolates of Breda virus, during one winter (1984–1985) we had what was apparently a small outbreak in our own dairy calves, when they were only 4–5 days old. They had a mild, 2–3-day diarrhoea. We collected about 10 faecal samples from that herd. Breda virus-like particles were present in about six of the 10, and they all reacted with our Breda virus antisera. So in that situation Breda virus was perhaps the cause of the illness.

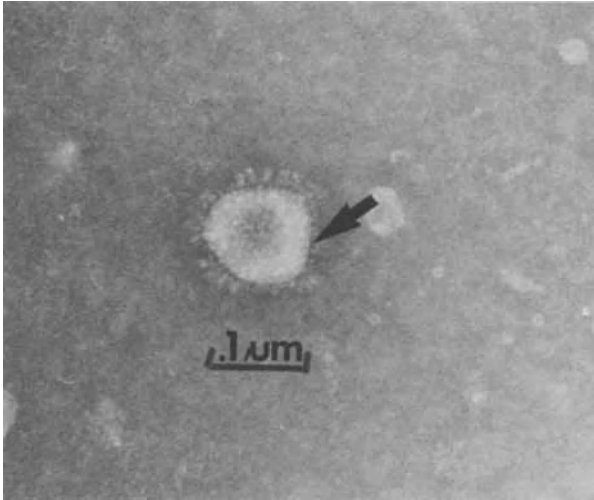

FIG. 1 (*Saif*). Immuno-electron micrograph of Ohio bovine Breda virus incubated with hyperimmune anti-coronavirus serum. Although Breda virus resembles coronavirus morphologically, no evidence of cross-reactivity is evident. The arrow points to the shorter layer of virus peplomers.

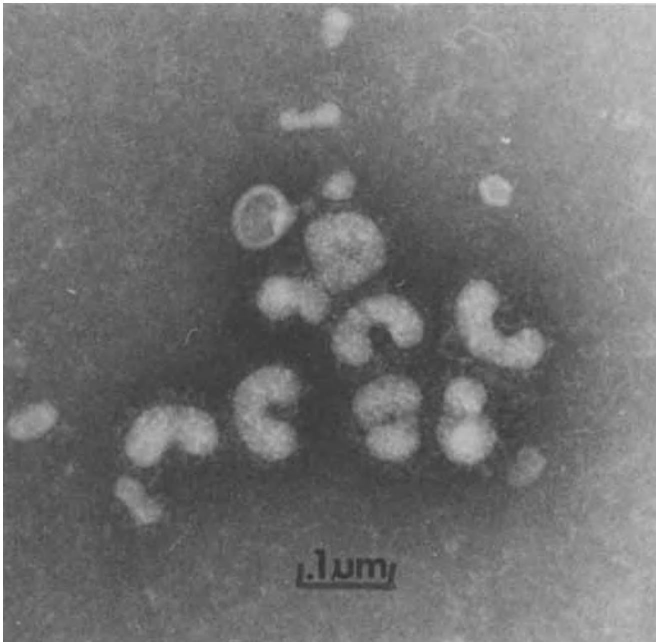

FIG. 2 (*Saif*). Immuno-electron micrograph of typical aggregates of kidney bean-shaped Breda virus particles seen after incubation of Breda virus with hyperimmune antiserum to Breda virus.

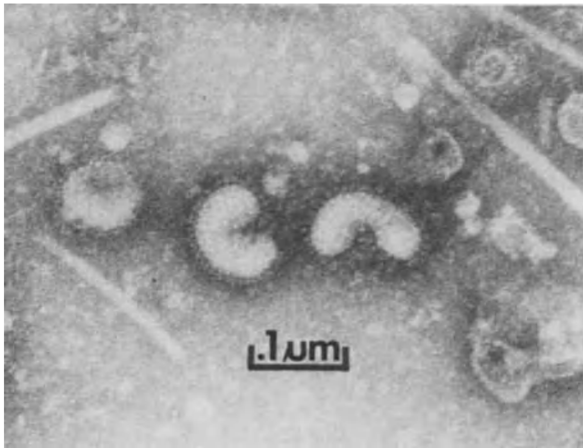

FIG. 3 (*Saif*). Electron micrograph of kidney bean-shaped pig Breda virus-like particles. Only short peplomers were evident on these particles.

We looked at the morphology of Breda viruses. Fig. 1 shows the Ohio calf Breda virus, and emphasizes how difficult it can be to distinguish Breda viruses from coronaviruses. There are preparations where we do not see the typical cigar-shaped or kidney bean-shaped Breda virus particles (shown in Fig. 2), and I would hesitate to say on the basis of morphology whether it is a Breda virus or a coronavirus. We always use immuno-electron microscopy with specific coronavirus or Breda virus antiserum to distinguish the two viruses. In some preparations one can see particles with both long and short (arrow, Fig. 1) peplomers.

We have also seen these Breda virus-like particles in stools from weanling pigs with diarrhoea (Fig. 3). In this micrograph we see the typical kidney bean appearance but with only the short peplomers. We have reacted this pig Breda virus-like sample with antisera to Breda virus serotypes 1 and 2; by immuno-electron microscopy we couldn't demonstrate cross-reactivity. We did find clumping or agglutination when we reacted the Breda virus-like sample with convalescent-phase antiserum from pigs in the herd. So there could be other Breda viruses in nature which don't cross-react with the Breda 1 and 2 antisera. We tried to pass this Breda virus-like sample in gnotobiotic pigs, unsuccessfully. There are probably differences in the stability of these viruses, because this virus was stored at only −20 °C, not −70 °C, and thus may have lost infectivity.

*Horzinek:*The stability of toroviruses seems to vary. Breda 1 is apparently very labile; Berne virus on the contrary is stable to trypsin and chymotrypsin and this treatment in fact increases infectivity in culture. It is stable in the pH range between 2.5 and 9.7, which for an enveloped virus is considerable stability, and also stable to sodium deoxycholate treatment. This stability makes sense for an enteric virus, which wants to survive in the gut; but there is

no other biological membrane known that withstands 2% deoxycholate treatment.

Hung: The coronavirus-like or Breda virus-like particles are very elusive, in our view. During the epidemics of the Chinese rotavirus (ADRV), a coronavirus-like particle was the first thing that came to our attention, and confused us for about three months. We found plenty of particles, morphologically very similar to coronavirus, but afterwards we classified it into four kinds, morphologically. Some are very bizarre, very big, with rarified projections; some had one row of projections, and some of them had a double layer, or three layers. We made a comparative study with normal stool randomly collected from normal subjects in Beijing. We found coronavirus-like particles in stool from many diarrhoea patients, as well as in normal individuals, with no difference in morphology or frequency, so I am doubtful about an aetiological role of such particles in human beings. Sometimes the particles agglutinate spontaneously. Initially some of my colleagues thought that they were agglutinated by IgA, but we found similar aggregation of particles in faeces of normal individuals, so we are rather sceptical about reports describing coronavirus-like particles solely by electron microscopy.

Bishop: This focuses on the need to decide whether 'fringed' particles seen in faeces are or are not infectious agents.

Kapikian: I agree with Dr Hung that probably the most perplexing question when examining stools by electron microscopy is the characterization of these fringed objects, which I record in my lab. notes as 'coronavirus-like-like'. The second 'like' signifies that they are not true coronaviruses morphologically. We have also seen objects that resemble Breda virus. My scepticism about such objects arises from the examination of stools from volunteers administered Norwalk virus or rotavirus or from individuals with hepatitis A, because I have observed such fringed objects in stools from individuals with each of these documented infections. Thus to me, these objects constitute background material. Of course, the particles you describe are clearly viruses, but from the standpoint of human disease, it is practically impossible to make a diagnosis of their presence in stools on the basis of morphology by electron microscopy. It is of interest that electron micrographs from an article by Hinkle & McCarty (1978) showed vesicles prepared from mitochondria, and disrupted membranes of *E. coli* which formed inside-out vesicles, and both were reminiscent of some of these approximately 120nm fringed objects observed by electron microscopy of stools (Hinkle & McCarty 1978). So caution should be the byword when observing such objects in stools, followed by scientific evaluation.

Caul: Of course, the inverted mitochondria do look different from the coronavirus-like particles, and have been shown to be different.

Kapikian: However, there are now certain reports of similar fringed particles in the stool which are described as coronaviruses, but without a follow-up characterization.

Mathan: By immuno-electron microscopy with patient sera we have found antibodies to coronavirus-like particles (CVLP), with some evidence of antigenic variation. Gel electrophoresis of CVLP purified from stool samples showed a pattern different from classic coronaviruses, but this may be related to the breakdown of proteins in the faecal filtrate. Morphologically, CVLP are quite distinct from mitochondrial vesicles.

Kapikian: To further examine the question of the significance of the Breda virus, which is an agent that appears to be similar to a fringed particle observed by Dr Flewett in human stools, and since the Breda virus described by Dr Woode is related antigenically by IEM to the fringed particle from humans described by Dr Flewett, and the Berne virus described by Dr Horzinek is also related to Dr Flewett's fringed particle, is there any possibility of obtaining sufficient Breda virus antigen from a calf excreting an abundance of this particle, and developing a simple complement fixation test with this antigen and appropriate antiserum? You could then take a series of paired acute and convalescent sera from a longitudinal or cross-sectional study of viral diarrhoea and test those that were negative for rotavirus infection for a seroresponse to the Breda antigen. This would be a simple seroepidemiological study that attacks the problem from the other side, from examination of sera rather than stools. This could yield important information, and if seroresponses were detected one could then examine the stools of these patients by electron microscopy for Breda-virus-like particles.

Horzinek: There is no problem with growing Berne virus to high titre and concentrating it, so the antigen can be obtained. We have tested several toroviruses and there seems to be cross-reaction at the level of the peplomer, which would make IEM possible, using a broadly cross-reactive serum. I would suggest that if one sees particles that one cannot identify, one should make thin sections of an ultracentrifugation pellet and look for 23 nm circular cross-sections through the nucleocapsid tube with a 6–9 nm light centre; they are unique to toroviruses, with the exception of rhabdoviruses, which also show this morphology (but a larger diameter).

Kapikian: That approach is reasonable, but considerably more difficult than testing sera against an antigen, and thus could be carried out in only a few laboratories. However, many laboratories have stored paired sera from children with gastroenteritis, and over 50% of these patients fail to yield an aetiological agent for their illness. Sera from such cases of gastroenteritis would be ideal to test for antibody rises to a Breda-like antigen from one of these sources. Since we cannot import Berne virus into the USA, some of the sera could be sent to Dr Horzinek; or Dr Woode might be able to furnish us with the Breda antigen.

Woode: We developed an ELISA using purified Breda virus from bovine gut. On the assumption that if humans can be infected with Breda virus, those of us working on the virus were most likely to have antibody, we tested our own sera. The main problem with human sera is the high incidence of antibody to

bovine protein, so we tried to block the reaction with virus-negative faecal material mixed with the sera, and then reacted the sera with the viral antigen. Many of us did have antibody; it varied from the pathologist who had no or low titre antibody, to one of my collaborators from Costa Rica who had high levels. We were left wondering whether we had controlled the test well enough to be sure that the human sera weren't reacting with bovine protein contaminating the virus.

Kapikian: With paired sera you might get round this problem, if the titre changes with the convalescent serum.

Horzinek: We ran radioimmunoprecipitation and neutralization tests on paired sera from patients with the haemolytic uraemic syndrome, obtained from the Centers for Disease Control in Atlanta. There was no rise in antibody.

Flewett: This is not surprising, because these viruses are unlikely to have anything to do with the haemolytic uraemia syndrome.

Holmes: Roger Schnagl semi-purified fringed particles from Australian Aboriginals and did a decoration-type IEM on the particles with various human sera. He seems to be able to measure antibodies to these viral particles that bind around the fringes (Schnagl et al 1986). This may be the first sign of an immune response to these particles and supports the suggestion that they may be viruses. Sera from areas where the particles are frequently seen had high antibody titres, whereas sera from Melbourne had none.

Greenberg: Is the genome size of Berne virus (5.7×10^6 Da) small enough for it to migrate into polyacrylamide gels? Would another way of looking for Berne/Breda agents be to look at silver-stained gels of faecal specimens for large molecular weight genomic material that isn't adenovirus or rotavirus? People are doing polyacrylamide gels of faecal material all the time and if the genome could be detected by silver-staining PAGE, that might be another way to look for these agents.

McCrae: I don't think most of the RNA would go into the gel—not a single-stranded nucleic acid.

Horzinek: Does coronavirus RNA go into the gel? It is about the same size as Berne virus RNA.

McCrae: Coronavirus RNA would not run into a 7.5% gel, which is what most people are running. On that topic, do these viruses cross-hybridize? Since you have cloned nucleic acid to the Berne virus, does it actually hybridize with coronavirus RNA?

Horzinek: We haven't done that yet.

Woode: Dr Horzinek, what do you feel about the significance of the presence or absence of an eluting agent in Breda virus or coronavirus? Would such a factor usually be an enzyme?

Horzinek: I rather doubt whether elution is due to an enzyme; it may well be a mechanical phenomenon. We know that the fringe of surface projections can sometimes appear to be shaved, leaving projections which are half as long as

usual. The situation may be similar to that in coronavirus, where the S_1 and S_2 subunits of the peplomer gene product are held together by hydrogen bonds; treatment with urea removes the S_1 subunit from the virion (Cavanagh 1983). We could not find neuraminidase (sialidase) activity in preparations of Berne virus.

Woode: Breda virus 1 does not elute from red blood cells. The haemagglutinin is stable for weeks, and 24 hours after the test the cells are still agglutinated.

Horzinek: The phenomenon may depend on the peplomeric structure. The peplomers in Breda viruses 1 and 2 may just be different, through the processing of the 200K precursor protein; there definitely is a difference in the molecular weights of the peplomer protein(s) between the two serotypes (Koopmans et al 1986).

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Comparative pathology of infection by novel diarrhoea viruses

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Abstract. Examination of diarrhoeic faeces in the electron microscope often reveals viruses that are presumed to be enteropathogenic. Lesions caused by novel rotaviruses were similar to those of group A rotaviruses, but enterocyte syncytia were seen which are probably pathognomonic for novel rotaviruses. In adenovirus infection in piglets, mature enterocytes were infected and destroyed; intranuclear inclusion bodies were seen in infected enterocytes. Calici-like viruses infected mature enterocytes in calves and the lesions were similar to those described in humans infected with calici-like viruses; in both host species it was impossible to demonstrate virus particles in enterocytes examined in the electron microscope. The Breda virus infected villi and crypts in the lower small intestine and the surface and crypts in the large intestine; it was the only enteropathogenic virus to show this distribution of infection and lesions. Astrovirus infection in lambs was comparable to a mild rotavirus infection, but in calves the epithelium of the dome villi of Peyer's patches was infected. Parvovirus in cats and dogs infected and destroyed small intestinal crypt cells, causing dilated crypts and stunted villi; intranuclear inclusion bodies were prominent.

1987 Novel diarrhoea viruses. Wiley, Chichester (Ciba Foundation Symposium 128) p 192–217

The ability to examine, in an electron microscope, faeces from cases of diarrhoea, led to the discovery of rotavirus. A similar approach has been used to detect a number of viruses which are apparently enteropathogenic and which are discussed in this symposium.

Although a causal association between virus infection and diarrhoea may be indicated by finding virus particles in samples from a disease outbreak, it is necessary to confirm the pathogenicity of the virus by experimental reproduction of the disease and examination of the intestinal tract for the presence of lesions. This approach is possible in animals but is difficult or impossible in man. Some novel viruses which cause diarrhoea in man are represented by an equivalent virus capable of causing diarrhoea in animals; thus animal models can provide supportive evidence for pathogenicity in man and permit a better understanding of disease processes.

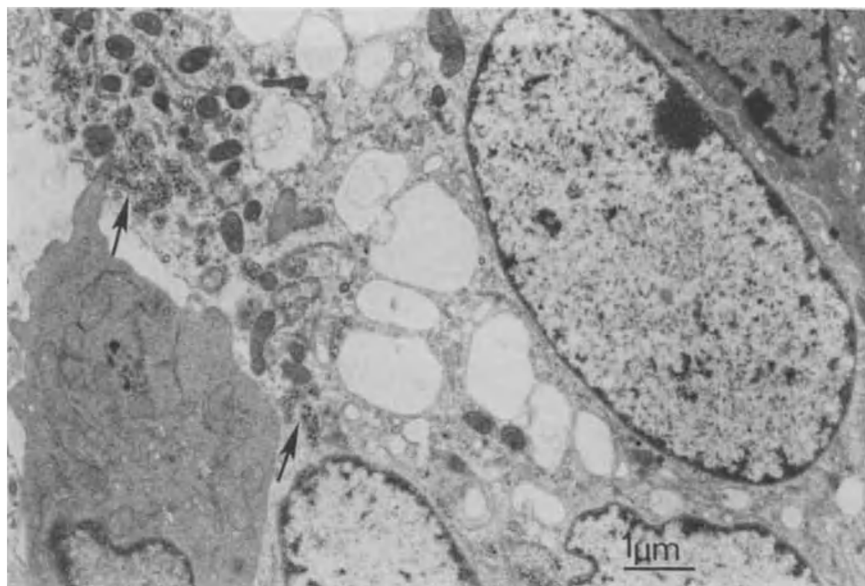

FIG. 1. Transmission electron micrograph of a virus-infected enterocyte syncytium on a villus in the mid small intestine of a piglet killed 24 hours after oral inoculation with a novel group B rotavirus (NIRD-1). The cytoplasm of the syncytium is electron lucent and vacuolated and three nuclei are visible; virus particles are visible in the rough endoplasmic reticulum (arrows). The dark cell (top right) is a normal cell with electron dense cytoplasm.

Novel rotaviruses

Viruses morphologically identical to rotaviruses, but lacking the group A antigen which characterized the original rotaviruses, have been associated with diarrhoea in man and animals; lesions have been described in pigs, lambs and rats. In the pig, infection was detected on the sides of villi throughout the small intestine (Askaa & Bloch 1984); in the sheep it was detected on the tips of the villi in the mid small intestine (Chasey & Banks 1986) and in the rat on the tips of the villi in the lower small intestine (Vonderfecht et al 1984). These differences could, however, reflect different stages in the development of lesions rather than differences in the preferred sites of infection.

Villi were stunted in all species and syncytia had formed on the surface of the villi by fusion of up to 20 enterocytes with loss of intercellular membranes. Fused cells were swollen with pale, vacuolated cytoplasm and short, disorientated microvilli. Mature virus particles were seen in dilated cisternae of the rough endoplasmic reticulum and viroplasm in the cytoplasm.

Lesions were not detected in the small intestine of one piglet killed 16 hours after oral inoculation with a faecal filtrate containing the novel group B rotavirus

FIG. 2. Scanning electron micrograph of villi in the mid small intestine of a piglet killed 24 hours after oral inoculation with a novel group B rotavirus (NIRD-1). An intact syncytium is present on one villus (single arrow), a ruptured syncytium on another (double arrows) and an area of exposed lamina propria on the tip of a third villus (treble arrows).

NIRD-1 but they had developed throughout the small intestine of another piglet killed 24 hours after inoculation (G.A. Hall, unpublished observations). In the upper small intestine, villi were shortened and groups of swollen enterocytes at the tips or on the sides of villi had pale vacuolated cytoplasm. Neutrophils were observed occasionally among these abnormal cells. In the mid small intestine, villi were shortened markedly and on their surface were seen syncytia of swollen, vacuolated, pale-staining enterocytes with swollen nuclei and abnormal microvilli. Virus particles were detected in these cells within dilated rough endoplasmic reticulum (Fig. 1). The tips of some villi were without epithelial covering or were covered by flattened enterocytes (Fig. 2). Identical, but less severe, lesions were seen in the terminal ileum.

The pathogenetic processes of classical and novel rotaviruses appear to be similar (Fig. 3a). Mature enterocytes become infected and exfoliate, producing stunted villi, maldigestion and malabsorption. The occurrence of syncytia on the surface of villi may prove to be a pathognomonic lesion of some novel rotaviruses because these lesions do not appear with group A rotaviruses or other viruses. There is, however, another report of syncytia, in association with a fringed virus-like particle measuring 80 nm in diameter, in calves (Mebus et al 1978).

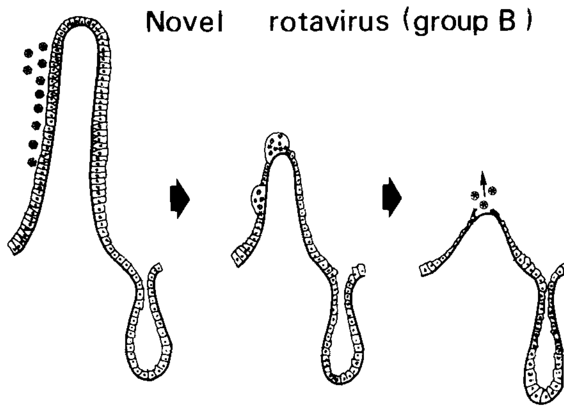

FIG. 3 (a) Postulated pathogenetic process of a novel group B rotavirus (NIRD-1). Mature enterocytes are infected, virus-infected syncytia form and disintegrate, and villi become stunted and crypts hypertrophied.

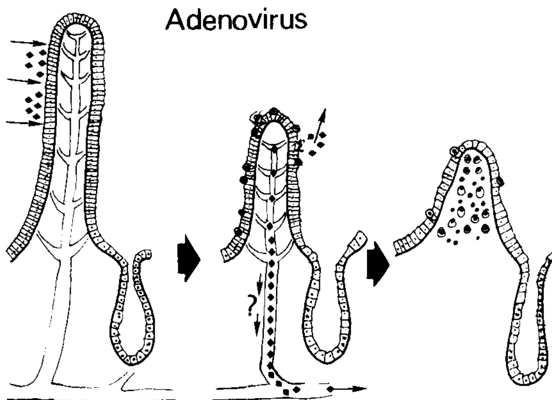

FIG. 3 (b) Postulated pathogenetic process of pig enteric adenovirus infection. Mature enterocytes are infected and intranuclear inclusions develop; enterocytes degenerate and rupture. Villi become stunted, crypts hypertrophy and the lamina propria is infiltrated by mononuclear inflammatory cells.

Adenoviruses

Adenoviruses have been associated with pneumonia and diarrhoea in several species, including human infants, turkeys and pigs. Adenovirus-induced diarrhoea appears to be most severe in turkeys, much less severe in infants and least severe in pigs. In pigs in Belgium, adenovirus diarrhoea is seen regularly as outbreaks of mild diarrhoea lasting 3–4 days and resulting in poor growth (R. Ducatelle, personal communication). Virus particles have been detected in

diarrhoeic faeces and often in normal faeces, usually at weaning. The pathogenesis of enteric adenovirus infection, which has not been described in infants, has been studied comprehensively in pigs, which may be the best animal model for the human disease.

The lesions in natural (Coussement et al 1981) and experimental (Ducatelle et al 1982) infections were identical. Stunted and normal villi were seen in the lower jejunum and ileum of pigs infected experimentally. Ten to 20% of enterocytes on the sides or at the base of stunted villi were infected, as judged by the presence of intranuclear inclusions, by immunoperoxidase staining, or by electron microscopic demonstration of virus particles in the nucleus or cytoplasm. The nuclei of infected enterocytes were enlarged, rounded and located near the brush border, and protruded into the lumen. In infected enterocytes microvilli were damaged and initially there was increased electron density of the cytoplasm. Later there were vacuoles in the cytoplasm and the luminal surface of degenerate cells ruptured, releasing cytoplasm into the lumen. The villus lamina propria was infiltrated with mononuclear inflammatory cells. Antigen, but not lesions, persisted for up to 45 days.

The pathogenetic process of enteric adenovirus is not well understood (Fig. 3b). Although mature enterocytes were the first cells to be infected in pigs, evidence from pigs and other species suggests that viraemia may occur, infecting other tissues. Detection of intranuclear inclusion bodies in enterocytes on the villi appears to be confined to adenovirus enteropathies.

Calici-like viruses

A number of small round viruses cause acute epidemic gastroenteritis in humans (Dolin 1978); in calves these are represented by calici-like viruses or Newbury agents (Bridger et al 1984). Similar viruses have been associated with diarrhoea in piglets (Saif et al 1980). The lesions produced in adult human volunteers by two calici-like viruses, Norwalk and Hawaii agents, have been investigated by means of gastric, jejunal and rectal biopsies (Schreiber et al 1973, 1974). Both agents caused vomiting, diarrhoea, xylose and fat malabsorption, and identical lesions. Jejunal villi were stunted and enterocytes covering the stunted villi were cuboidal and disarranged, lacked normal nuclear polarity, and were vacuolated; the vacuoles did not contain lipid. Numbers of intrapithelial lymphocytes and neutrophils were increased. Enterocytes on stunted villi had microvilli which were shortened and disorientated, and the mitochondria and smooth endoplasmic reticulum were swollen. Damaged enterocytes contained multivesiculate bodies and widened intercellular spaces. The lamina propria of the shortened villi was compressed and infiltrated by mononuclear inflammatory cells and neutrophils. Increased numbers of mitotic figures were seen in the crypts, which were lengthened, particularly late in the disease process. Concentrations of alkaline phosphatase and trehalase were reduced in biopsied tissue. Abnormalities were not detected in biopsies from stomach and rectum.

In calves, two antigenically distinct isolates of calici-like virus (Newbury agent SRV1 and SRV2) caused anorexia, increased faecal output, change in faecal colour and malabsorption of xylose; Newbury agent SRV1 caused more severe changes than SRV2 (Bridger et al 1984). The sequential pathology of gnotobiotic calves infected with Newbury agent SRV1 has been studied (Hall et al 1984). Lesions were restricted to the anterior small intestine and their severity varied markedly between calves killed at the same time after infection. Enterocytes on the sides of villi were shown to be infected by the immunoperoxidase method but virus particles were not seen in these cells by transmission electron microscopy. Virus-infected cells became degenerate and exfoliated (Fig. 4). Damaged cells were swollen, their microvilli were abnormal or absent, and intracellular organelles were damaged. The most severe lesions, seen one day after inoculation, consisted of severely stunted, fused, conical and leaf-shaped villi with, in some instances, exposure of the lamina propria at the tips. Remaining enterocytes were cuboidal or flattened and vacuolated; the vacuoles contained lipid. The lamina propria was compressed in stunted villi, appearing more cellular than in normal villi and containing nuclear debris. Macrophages, some degenerate, were numerous in lacteals. Villi were stunted three and four days after infection, but abnormal enterocytes were confined to the tips of the villi and normal columnar enterocytes were visible on the sides of the villi, towards the base. Abnormal enterocytes were swollen, contained lipid in vacuoles, varied in size, were abnormally arranged and were covered by distorted microvilli; many appeared to be sloughing from the villus tips (Fig. 5). Abnormal enterocytes were less numerous in the calf killed seven days after inoculation and virtually absent from the calf killed at 10 days.

Mean values of villus height and crypt depth are illustrated in Figs. 6 and 7. Crypts throughout the small intestine were lengthened after infection. This change may be interpreted, as it has been in other viral enteropathies, as evidence of an increased crypt cell production rate associated with a repair process. In the anterior small intestine, where damage was greatest, villi initially became stunted but returned to normal size by 10 days after infection. In the terminal ileum, where no damage was detected, increased villus height appeared to result from increased enterocyte production in the absence of enterocyte loss. Similar observations have been recorded in lambs infected with astroviruses (Snodgrass et al 1979); crypt depth lengthened throughout the small intestine, whereas villus height was unchanged in the upper small intestine but was reduced in the mid and lower small intestine. An interpretation of the observations in calves and lambs is that after virus-induced damage, which was restricted to part of the small intestine, there was a systemic stimulus to crypt cell mitosis throughout the small intestine. The hormone enteroglucagon has been postulated to be a stimulator of small intestinal mucosal growth, and blood levels are raised in calves after infection with enteropathogenic viruses but not after infection with non-enteropathogenic viruses (Hall et al 1985). Activity of β-galactosidase in mucosal scrapings was reduced in infected calves, as was

FIG. 4. Scanning electron micrograph of a villus in the anterior small intestine of a gnotobiotic calf killed 18 hours after oral inoculation with Newbury agent SRV1. Degenerate enterocytes on the surface of a stunted villus.

their ability to absorb xylose, and these changes correlated with a reduction in villus height. Measurements of xylose absorption in calves infected with enteropathogenic viruses (Hall et al 1985) suggested that the test was better able to detect malabsorption affecting the anterior small intestine than that affecting the lower small intestine and large intestine.

The pathogenetic processes of calici-like viruses in humans and calves appear similar (Fig. 8). In both, mature enterocytes were infected and destroyed, villi became stunted in the anterior small intestine, and there were loss of digestive and absorptive function. Minor differences were detected between lesions in

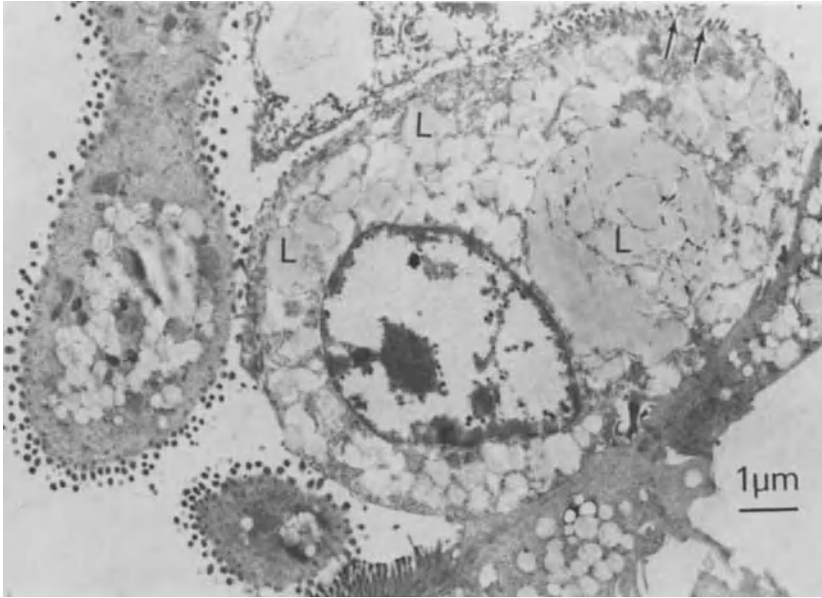

FIG. 5. Transmission electron micrograph of an enterocyte at a villus tip in the anterior small intestine of a gnotobiotic calf killed two days after oral inoculation with Newbury agent SRV1. The enterocyte has almost exfoliated, the microvilli are abnormal (arrows) and the cytoplasm contains abundant lipid (L).

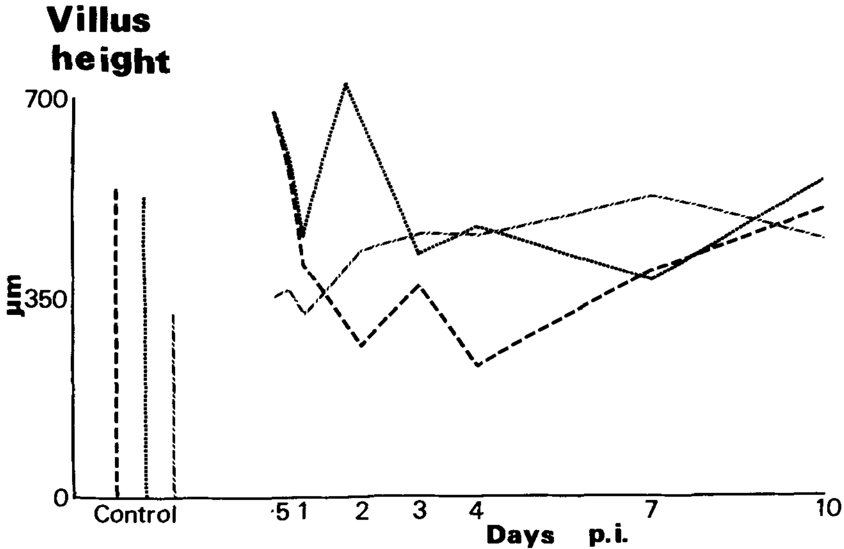

FIG. 6. Mean villus height (µm), at 10% (▬ ▬ ▬), 50% (▪▪▪▪▪▪) and 90% (▬/▬/▬) small intestinal length, in calves infected orally with Newbury agent SRV1. Each point represents mean measurements from two calves, except at Days 7 and 10, when single calves were studied.

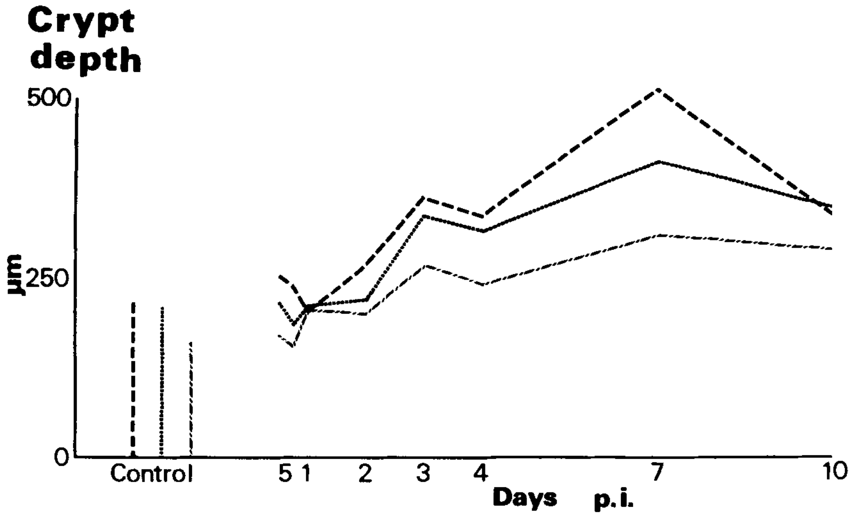

FIG. 7. Mean crypt depth (µm) at 10% (▪▬▬▬▪), 50% (▪ ▪ ▪ ▪ ▪), and 90% (▬▪▬▬▪▬) small intestinal length, in calves infected orally with Newbury agent SRV1. Each point represents mean measurements from two calves, except at Days 7 and 10, when single calves were studied.

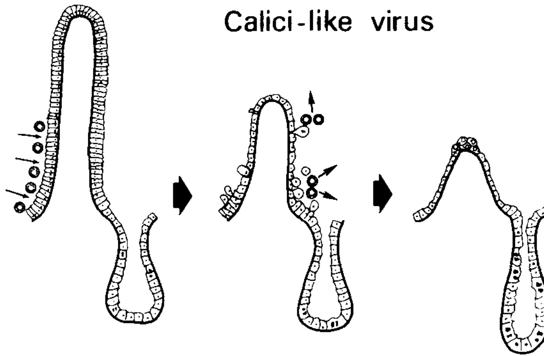

FIG. 8. Postulated pathogenetic process for calf calici-like virus (Newbury agent). Mature enterocytes are infected, they degenerate and exfoliate. Villi become stunted and crypts hypertrophied.

humans and calves; there was infiltration of the lamina propria and epithelium by neutrophils in humans, and intracytoplasmic lipid was present in enterocyte vacuoles in calves. The reasons for these differences are unclear but they are unlikely to reflect important differences between the pathogenetic processes.

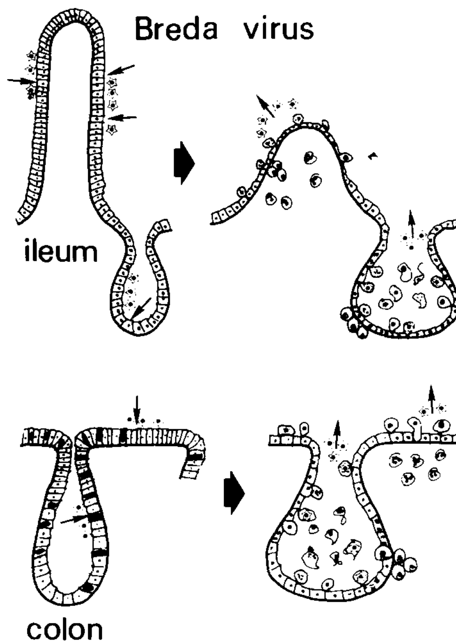

FIG. 9. Postulated pathogenetic process for Breda virus in calves. In ileum, caecum and colon, mature and crypt enterocytes are infected; they degenerate and slough. Villi become stunted and crypts dilated. Cellular debris accumulates in crypts and the lamina propria is infiltrated by mononuclear and neutrophil polymorphonuclear leucocytes.

Breda and Breda-like viruses

Breda and Breda-like viruses, isolated from calves, a horse and humans, were associated with diarrhoea in calves and humans. In gnotobiotic calves infected with Breda virus (Pohlenz et al 1982, 1984, Fagerland et al 1986) necrosis of crypt epithelial cells was seen in the mid jejunum and ileum. Foci of macrophages occurred in the lamina propria below the necrotic crypt cells, and the lamina propria was infiltrated mildly and diffusely with neutrophils. Later in the disease process there was degeneration, necrosis and exfoliation of enterocytes from crypts and villi in the lower small intestine and from the surface and in crypts of the caecum and colon. Villi were stunted and fused and crypts were dilated and contained cell debris. Epithelial cells covering the dome villi of the Peyer's patches were infected. The lamina propria was infiltrated with macrophages and polymorphs. Cytopathic changes in infected enterocytes included swelling, dilatation of the cytocavitary network, stunting of microvilli and vacuolation. Virions were detected in large autophagosomes in enterocytes and in phagocytosed material within macrophages.

The pathogenetic process in Breda virus infection (Fig. 9) resembles that of

coronavirus infection in calves, because similar lesions occur in both infections in the lower small intestine and large intestine. However, infection and necrosis of both villus and crypt enterocytes in the small intestine appears unique to Breda virus. Other viruses infect villus cells but not crypt cells, or vice versa.

Astroviruses

Astroviruses were detected in faeces in infantile diarrhoea (Madeley & Cosgrove 1975) and subsequently in faeces from animals. Studies in lambs (Snodgrass & Gray 1977, Snodgrass et al 1979, Gray et al 1980) showed that astroviruses infected only mature enterocytes and subepithelial macrophages in the small intestine, producing villus atrophy. Enterocytes containing intracytoplasmic vacuoles and inclusions and degenerate nuclei were seen. Infected enterocytes contained viroplasm and virus particles were detected along the microvilli and in lysosomes and autophagic vacuoles; virus particles were also detected in lysosomes in macrophages in the lamina propria.

An isolate from a calf in the United Kingdom was considered to be non-pathogenic for calves (Woode & Bridger 1978). Recently, however, an isolate from the United States, which was related antigenically to the UK isolate, was found to infect preferentially the epithelium covering the dome villi of the jejunal and ileal Peyer's patches, although diarrhoea did not occur (Woode et al 1984). Calves inoculated orally with mixtures of astrovirus and rotavirus or astrovirus and Breda virus 2 developed severe diarrhoea, and there was more extensive astrovirus infection of the dome epithelium. Immunofluorescent staining and electron microscopy demonstrated infection in M cells—the specialized epithelial cells on the surface of dome villi—and in absorptive enterocytes on dome villi. An exudate of sloughed epithelial cells, mononuclear inflammatory cells and eosinophilic cells was noted above the infected dome villi, and infected epithelial cells were changed from columnar to cuboidal; the epithelium contained mononuclear cells and eosinophilic cells and was fused occasionally to the epithelium of an adjacent absorptive villus, forming a bridge. Small numbers of virus-infected enterocytes were detected on absorptive villi at sites where fusion had occurred. Increased numbers of neutrophils and degenerate nuclei were detected in the lamina propria of dome villi. The germinal centres associated with infected dome villi were depleted of cells centrally, and the remaining cells were similar to macrophages, having eosinophilic cytoplasm and prominent nuclei.

Retrospective studies of two gnotobiotic calves infected with the UK isolate of astrovirus, and killed one and three days after inoculation (G.A. Hall, unpublished observations), confirmed in the calf killed at three days that infection appeared to be restricted to the epithelium of the dome villi; small numbers of virus-infected cells were detected on the surface of dome villi. Virus-infected enterocytes were not detected in the calf killed one day after inoculation. In both

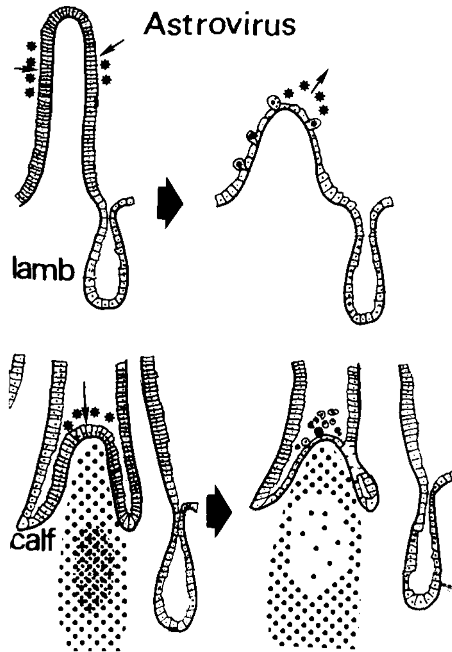

FIG. 10. Postulated pathogenetic process for astroviruses in lambs and calves. In lambs mature enterocytes are infected and exfoliate; villi become stunted and crypts hypertrophied. In calves the epithelium of dome villi is infected; enterocytes exfoliate and inflammatory cells emigrate on to the surface of the dome. The lamina propria of the dome is infiltrated by neutrophils and the germinal centres are depleted of lymphoid cells.

calves the epithelial cells covering most dome villi were columnar but dome villi covered by cuboidal epithelial cells were detected. Cells were seen exfoliated from the surface of infected dome villi and from the surface of dome villi in uninfected gnotobiotic calves. Mononuclear inflammatory cells and eosinophilic cells were observed within the epithelium of dome villi of both astrovirus-infected and uninfected gnotobiotic calves, and in uninfected calves neutrophils were numerous in the lamina propria of the dome villi.

The pathogenetic processes in lambs and calves infected with astroviruses appear to be distinct (Fig. 10). In lambs the lesions and pathogenetic process were not unique and were similar to those seen in rotavirus infection, though less severe. In calves, however, the epithelial cells of dome villi were infected. It is possible that damage to dome villi could limit their function and give rise to reduced immune responses in concurrent and consecutive infections; severe diarrhoea was seen when astrovirus infection occurred concurrently with rotavirus or Breda virus 2 infection (Woode et al 1984). The bovine and lamb astroviruses were found to be antigenically distinct (Woode et al 1984) and might

be distinct in other respects, having only a similar appearance when examined in an electron microscope.

Parvoviruses

Parvoviruses are worthy of inclusion in a discussion of novel enteric viruses—first, because they cause enteric disease of variable severity in domestic animals, from a very severe, often lethal, enteritis in kittens and puppies to a relatively mild disease in calves. Second, they may be represented among the collection of small round viruses that have been associated with diarrhoea in man. Third, their pathogenetic mechanism, which mimics that of radiation-induced damage, is different from that of most enteropathogenic viruses; and, fourth, the canine parvovirus appears to be truly novel, since there is no evidence for its existence before 1978 (McCandlish et al 1979).

In dogs infected experimentally with a canine parvovirus of faecal origin, the virus was assumed to have entered the body through the alimentary tract (the exact site was not identified), and spread to the thymus, and subsequently to the germinal centres of the lymph nodes, where it replicated. Viraemia occurred 2–4 days after infection. In weaned pups, the most actively dividing cells were small intestinal crypt cells, and these became infected and destroyed. Destruction of crypt cells resulted in the attenuation of surviving cells and the development of dilated crypts containing cell debris. Villi, deprived of their supply of maturing enterocytes, became fused and stunted, and the mucosa became flattened (Macartney et al 1984a,b). Severe damage to the small intestine caused a substantial loss of neutrophils into the intestinal lumen, resulting in panleucopenia.

The pathogenetic process in parvovirus infection is distinct from that of most enteropathogenic viruses (Fig. 11), but shows some similarities to adenovirus infections. Parvovirus infection is not restricted to the intestine and a range of tissues may become infected and damaged. The intestinal cell which becomes infected and destroyed is the dividing cell in the small intestinal crypt and not the mature enterocyte on the villus. The viraemia is an important part of the pathogenetic process and circulating antibody may neutralize virus in the blood and halt the disease process. Circulating antibodies are important, therefore, in protection against this enteropathy, whereas, in many of the other viral enteropathies, circulating antibodies are probably less important in protection than are luminal antibodies.

Conclusions

The lesions produced by novel enteropathogenic viruses in man are poorly described, but in many instances a comparable infection occurs in animals and this has enabled new insights to be obtained into disease processes. Most

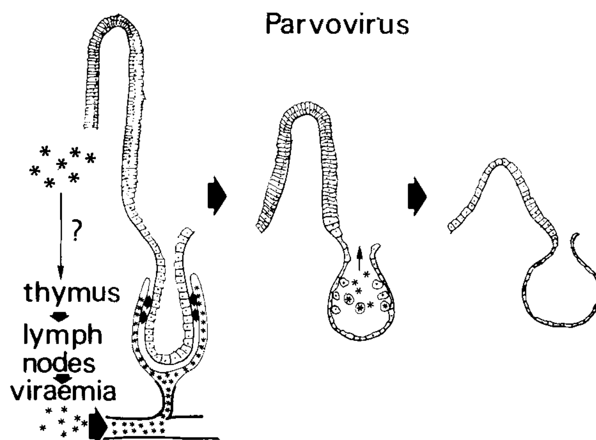

FIG. 11. Postulated pathogenetic process for parvovirus infection in puppies. Virus replicates in thymus and lymph nodes and crypt cells become infected after a viraemia. Degenerate crypt cells slough and crypts dilate; villi, deprived of new enterocytes, become stunted.

enteropathogenic viruses infect the intestinal mucosa from the lumen and the mature enterocyte is infected most frequently; this occurs with novel rotaviruses, adenoviruses, calici-like virus, Breda virus and lamb astrovirus. The Breda virus is distinctive because it infects both villus and crypt cells in the small intestine, together with surface and crypt cells in the large intestine. Astrovirus infection in calves is almost unique in infecting preferentially the epithelium of dome villi. The lesions produced by novel rotaviruses are distinguished by the formation of syncytia; adenovirus infection is distinctive because of the intranuclear inclusion bodies in villus enterocytes. The lesions of parvovirus in kittens and puppies are unique because the pathogenetic process originates in crypts and intranuclear inclusions are seen in crypt cells.

Although many of these novel viruses produce distinctive or pathognomonic lesions, pathology does not provide a practical diagnostic method and improved tests for detecting infection by these viruses are required, so that the importance of each virus in disease outbreaks may be determined.

Acknowledgements

The assistance of Mr K.R. Parsons with studies of astrovirus-infected and Newbury agent-infected calves, and of Mrs P. Bland with electron microscopy, is gratefully acknowledged.

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DISCUSSION

Hung: Are there differences between viral and bacterial diarrhoea, in their pathology and pathogenesis?

Hall: There are several major differences. Many bacteria have specific mechanisms by which they cause diarrhoea. These vary widely, and include the *E. coli* that produce enterotoxins which cause the gut to secrete fluids, the cytotoxins of the enteropathogenic *E. coli*, which are known to cause damage, and the salmonellas, which probably operate by yet other mechanisms. The mechanisms by which bacteria cause diarrhoea are very different from those of viruses, which basically involve killing enterocytes.

Woode: Is there evidence of group B rotavirus infection outside the syncytial area of the villi? That is to say, do you see single epithelial cells infected with this virus?

Bridger: I think you do see infected single cells, but the fluorescence with the B/NIRD-1 virus isn't clearly in discrete columnar cells, as it is for the limited number of group A or group C rotaviruses that I have studied. The most obvious effect seems to be syncytia formation.

Bourne: You implied, Dr Hall, that there are two possible mechanisms in the pathogenesis of the calicivirus lesions: first, viral damage of tissue and, second, a degenerative process, possibly associated with increased levels of gut hormone (enteroglucagon). Similar lesions can be associated with immune responses, in graft-versus-host reactions in the mouse, and in association with hypersensitivity to dietary antigens (Stokes et al 1986). Might this play a part in the pathogenesis of lesions in these viral infections?

Hall: It is possible. I am not suggesting that the increase in gut hormone that we saw is the only mechanism. One could envisage immune reactions in the region of the crypts releasing chemicals which stimulate crypt cells to divide either more slowly, or more rapidly. There must be local mechanisms that control the crypt cell production rate, as well as the more general (hormonal) mechanism for which we have evidence.

Woode: Like you, we have never seen calicivirus ultrastructurally in the infected cells. That seems to need some explanation.

Blacklow: In regard to that, I was struck that of all the viruses that you discussed, the calici-like virus pathological changes seemed indistinguishable from what we have noted with Norwalk virus.

Hall: I agree. There are only two clear differences; in Norwalk infection you reported vacuolation, and the vacuoles didn't contain lipid; and you noticed a neutrophil and round cell infiltration of the lamina propria, which I have not seen in Newbury agent infection in calves. But these are not major differences.

Woode: Do you think that pathological effects on the epithelial cell may not be a response to viral replication, but perhaps are due to a toxin or a hormone?

Hall: We can certainly immunostain viral antigen in mature villus enterocytes by the immunoperoxidase method, so viral replication presumably occurs in these cells. We saw some immunofluorescence as well, in the calves infected with Newbury agent, but it was faint.

Bridger: If the particles are not clustered and they are present in the low numbers that would be expected from the numbers in the gut contents and in the faeces, their small size would make it difficult to see scattered individual particles. You would need some immunostaining of the thin sections, to visualize them.

Hall: We plan to develop an immuno-gold staining method for the detection of viral antigen. We are starting with rotaviruses, where you can at least see the target; if successful there, we would try to link colloidal gold particles to antibody to Newbury agents and identify areas in the enterocytes where we think viral antigen may be formed.

Saif: On the astroviruses, I wasn't sure of your interpretation of the viruses seen in the M cells. Is virus being taken up just like normal antigens into M cells in the domes of Peyer's patches, or are the virus particles infecting the M cells? And are any of them infecting the underlying lymphoid tissue?

Hall: Both types of uptake seem possible. It could be that there is more virus in the M cells because it is their job to take up antigen. One wonders also whether, if a virus is not very virulent, it may be unable to get into the absorptive enterocytes very easily but enters more readily into M cells, whose job is to take up particles. We haven't seen astrovirus in the lymphoid tissue, but we haven't really looked yet.

Saif: So you are not sure yet whether astrovirus replicates in M cells?

Hall: No.

Woode: We thought that Breda virus was taken up passively, by M cells; it looked as though it was encased in vesicles, as if it was being phagocytosed. Whereas the astrovirus was distributed diffusely in the cytoplasm and appeared to have replicated.

Hall: Yes; in your studies, astrovirus appeared to be causing lesions in the cytoplasm of M cells.

Kurtz: In tissue culture (human embryo kidney cells) and in human enterocytes taken at duodenal biopsy, where viruses are multiplying, the arrays of astroviruses look very similar to those in the M cells. That would be evidence for their replication there.

Wadell: We heard earlier about the situation with Norwalk virus where

antibody-positive individuals are not protected. With astrovirus infection in calves, is it possible that the infected calves have antibodies, which bind to the virus, and then home the immune complex on to the M cells via Fc receptors? Have you looked at the astrovirus-specific antibody level?

Bridger: No. These calves were all gnotobiotic and were antibody negative.

Saif: Concerning parvoviruses, I don't understand how the virus, when given orally, would get across the absorptive epithelial cell and into the blood and from there home to the crypt. Another mechanism might be more likely, namely the one postulated for reovirus 1, whereby virus is taken up via the M cells into the Peyer's patches and then infects crypt cells, where a receptor is postulated on the basolateral membrane (Rubin et al 1986). Has anyone looked for that type of receptor for parvovirus on crypt cells, or is there a receptor for parvoviruses on the absorptive epithelial cells?

Hall: I can't answer that. I haven't worked on this myself, but in experimental infections the virus was assumed to have entered the body through the alimentary tract, although the exact site was not identified (Macartney et al 1984).

Bourne: On the general question of the regeneration of villous tissue and the return to normal physiological function, you studied villus height in calves up to 10 days after Newbury agent infection (Fig. 6, p 199). You said that by that time, normal villous architecture had been restored. Do you know if full physiological function was obtained by then?

Bridger: No, because we looked at xylose absorption only at the time of expected clinical disease, or rather the day after (namely four days after infection); so I can't tell you about recovery of function.

Woode: We did show full recovery after group A rotavirus infection, at 10 days, in our early work (Woode et al 1978). However, D-xylose absorption is virtually a passive absorption and does not reflect the physiological activity of a cell; it probably just requires some physical integrity, and then D-xylose will enter the system.

Bourne: We have studied this in the pig in the post-hypersensitivity state after oral presentation of soya. Two days later we see hyperplasia of crypt cells as the initial lesion. That is followed by villous atrophy, which is associated with maldigestion and malabsorption, with the associated reduction in enzyme levels on the enterocytes. Recovery takes place by Day 10–12 after the initial insult. At that time there is a full return of digestive and absorptive capacity. Villous architecture remains stunted and the villi do not return to their finger-like appearance seen before weaning or before antigenic challenge, but the gut retains full physiological function.

Hall: We measured xylose absorption on the day before necropsy and found that xylose absorption recovered sooner than lactase activity.

Bourne: In a weaned animal you would expect that, as a result of milk withdrawal.

Hall: In fact, the calves were being fed evaporated milk!

Greenberg: What is the evidence that the enteric adenoviruses infect the basolateral side of the epithelial cell, as opposed to the luminal side?

Hall: The only evidence is that viraemia is found, with virus being isolated from the blood before the enteric disease process, so there is the possibility of infection in this way.

Woode: Dr J.P. Orr studied an enteric adenovirus infection in a calf. There was extensive cytopathology of the dome epithelium in addition to vasculitis, thrombosis and necrotizing enterocyte lesions. This implied that the virus might have reached the gut via the vascular system (Orr 1984).

Kapikian: Can you speculate, Dr Hall, on the mechanism of the diarrhoea caused by the novel rotaviruses?

Hall: One can only assume that diarrhoea results from the loss of digestive and absorptive function. The novel rotavirus infection that we studied was in pigs. Our experience with calves is that they don't get diarrhoea when infected with atypical rotaviruses.

Kapikian: Do you think that enough functional epithelium is removed as a result of infection with these viruses?

Hall: Yes; also, you may end up with undigested sugars in the large intestine which may act osmotically to aggravate the situation (Argenzio 1978). We didn't measure absorptive or digestive function in those two infected piglets.

Kapikian: Do you then think that an orally administered vaccine would be better than a systemically administered one, in order to achieve protection at the site of infection?

Hall: I would say that antibody in the gut lumen at the time that virus enters the gut would be the most effective prophylaxis. Several approaches could be used to get that antibody there. It could be given passively, with the objective of keeping the right level of antibody there for the whole period that the animal is likely to be exposed to infection. The alternative approach would be to encourage local secretion of antibody onto the gut surface.

Bourne: I agree with this, but I also think that it is too much to expect humoral antibody, secretory IgA, on the mucosal surface to prevent viral penetration of enterocytes. IgA has a mitigating effect on infection and certainly reduces mucosal contamination, but does not always prevent penetration, although passive antibody can be highly protective in young animals. Other mechanisms are also probably involved in gut protection, but we don't know how to stimulate them using local presentation of a non-replicating antigen.

Saif: Several studies have demonstrated that passive protection against transmissible gastroenteritis (TGE) virus of swine can be accomplished by artificially feeding antiserum, sIgA, IgG or IgM antibodies to piglets (Haelterman 1963, Stone et al 1977). In the TGE virus model system we showed a poor correlation between virus antibody titres associated with IgG (which were usually present only in low levels) in the milk of infected or vaccinated sows and protection

when we challenged their nursing piglets with TGE virus. In contrast, infected sows that had high sIgA TGE virus antibody titres in their milk provided essentially complete protection against TGE virus challenge to their nursing piglets (Saif & Bohl 1979, 1981). Purified porcine sIgA antibodies have been shown to neutralize TGE virus or rotavirus *in vitro* (Saif & Bohl 1979, 1981) and a similar neutralization may occur *in vivo*, thereby preventing or retarding viral penetration of intestinal epithelial cells and hence infection.

This question about local versus systemic protection is very pertinent. One reason that it is so important to define the mechanisms of pathogenesis is because of the implications for immunity. There are situations where crypt gut cells may be infected secondarily via a viraemia, by way of the 'back door' into the intestinal tract (O'Sullivan et al 1984, Meunier et al 1985). In such cases, one can speculate that systemic immunity could be protective against gut infections. This has been the case with canine parvovirus, where high titres of actively produced or passively administered circulating antibodies provided protection against parvovirus challenge in dogs (Carmichael et al 1983, Meunier et al 1985). On the other hand, with a localized infection whereby virus arrives at the site of replication by way of the luminal surface, one would speculate that IgA antibodies are important, and cell-mediated immunity mechanisms as well, in terminating the infection.

Greenberg: With the parvoviruses, is it known if the vaccine works because it stops viral spread back from bloodstream to the intestine, or because it stops the spread to other systemic organs such as the heart? Spread to most organ systems would be prevented by systemic immunization, presumably.

Saif: I am not sure if that is known; but knowing the mechanism of pathogenesis, one can predict that a great deal of virus could be neutralized before it reaches the crypt cells (Meunier et al 1985). But there are indeed complications in parvovirus infection, such as myocarditis (O'Sullivan et al 1984), and presumably circulating antibodies would also stop that aspect of the illness.

*Hall:*It is generally thought that high levels of circulating antibody are likely to reduce the severity of the viraemic stage, so that fewer of the cells that one would expect to be infected during the viraemia are actually infected. Whether myocytes or enterocytes are susceptible to infection, the number of cells infected is considerably reduced, or infection is prevented, by circulating antibody.

Kurtz: Does anybody understand why any of these viral gastrointestinal infections stop as quickly as they do? There are vast numbers of cells in the gut but relatively few are infected in these diseases, which tend to last for only 24 or 48 hours.

Saif: A key question here—in terms of rotavirus infections, at least—is whether the cells migrating up from the crypts are susceptible or resistant to the infection. I am not sure of the answer.

Snodgrass: One should not underestimate the number of cells that are infected. In experimental infections with lamb group A rotavirus, almost all mature enterocytes in the ileum are infected during the incubation period, before there is any clinical illness. By the onset of clinical disease, many infected cells have already been sloughed, and after two days only scattered individual cells are infected (Snodgrass et al 1977a).

Kurtz: That answers one question: the diarrhoea comes on quite a time after the virus infection is maximal.

Woode: An excellent study on TGE virus by Dr Pensaert is relevant here. He did timed killing of pigs (Pensaert et al 1970). We repeated that with porcine rotavirus (group A) (Crouch & Woode 1978). It is quite clear that even when infectious virus surrounds the recovering villi, some of the cells are refractory to infection. There is a minor wave of immunofluorescence during this late phase, but the majority of cells do not become infected, despite high titres of infectious virus around them. Whether that is blocking by local IgA, or the effect of interferon, or whether the cell is refractory because it is immature, has been debated.

Bourne: Crypt hyperplasia is likely to play a part in pathogenesis. The fact that you get immature enterocytes on the villus will lead to increased secretion into the gut, which contributes to the diarrhoea. It could be that the immaturity of these cells makes them resistant to viral infection. But I would question, on the basis of gut physiology, whether there is clinical cure in two days. The diarrhoea might stop, but the water content of the faeces is likely to remain high for a number of days more.

Flewett: I don't know if it has been shown in humans, because they go home from hospital so soon, but in some pigs after rotavirus infection, if you follow them through, the virus is present in quantity in the faeces during the diarrhoea and then disappears; then a small second wave of viral replication follows, without much diarrhoea. This suggests that when the new enterocytes are mature, there may still be enough virus present to infect them, but by that time there is so much IgA present that no more epithelial cells are infected.

Horzinek: On this question of what stops the infection: in a study by Hubert Laude, Thiverval-Grignon, France (personal communication) in the TGE system in piglets, significant amounts of interferon were found in the lumen of the small intestine.

Holmes: On the question of whether immature epithelial cells are refractory to viral infection, in our laboratory Dr G. Raghu was trying to characterize receptors for group A rotaviruses on brush border membranes of piglets, by extracting membrane proteins and glycoproteins, running protein gels, transferring the protein bands as Western blots to nitrocellulose and looking for virus binding to particular proteins (Raghu 1985). He was able to separate out the cells from the crypts, from higher up the villi, and from the tips of the villi, giving a gradient of the cells in terms of maturity. The rotavirus was not

adsorbed to any glycoproteins on cells from crypts or the base of the villus, but it bound to two or three high molecular weight glycoproteins from brush borders from the more mature enterocytes. We took that as evidence that the receptors developed as the cells matured. We think it is something to do with the carbohydrate structure of the brush border glycoproteins. We can't be certain that this is specific receptor activity.

Hung: Are there any indications of allergic reactions being involved in the pathogenesis of viral diarrhoea? Do mast cells play any roles in the allergic reactions?

Bourne: Professor E.H. Bohl recognized a cell-mediated immunity (CMI) response in pigs infected with TGE virus which coincided with villous damage, by which time (3–4 days later) the virus had disappeared. He suggested that an allergic, type IV hypersensitivity response might be involved in the development of the villous lesion (Frederick & Bohl 1976).

Woode: That appears to be in contradiction to Dr Pensaert's study, showing recovery while TGE virus was still there in the gut lumen (Pensaert et al 1970).

Kapikian: On the role of local antibody, we have been proceeding on the concept that high levels of antibody passively acquired from the mother have a protective effect against neonatal or infantile rotavirus gastroenteritis when that antibody reaches the surface of the gut. Does systemic (humoral) antibody get through to the mucosal surface of the small intestine and, if so, how does it do that? Perhaps these compartments are not as separate as we used to think?

Bourne: There is evidence in both the pig and the calf that passively administered IgA antibody absorbed from the intestinal tract into the serum is re-secreted on to mucosal surfaces, such as those of the respiratory tract and the urinary tract. The half-life of this antibody is not known.

Kapikian: I was thinking of a human infant who acquires antibody (IgG) transplacentally, rather than passively acquired local IgA antibody via breast milk.

Bourne: There is actually very little IgA absorption from the intestinal tract in the human infant. IgG certainly transudes back to the gut surface in all species, by a transudation mechanism rather than by active transport. Evidence for this is the mitigating influence of serum IgG1 on intestinal disease in the calf (Bourne et al 1978).

Snodgrass: Rotavirus infection and diarrhoea in neonatal animals and human infants can be prevented by feeding rotavirus antibody from colostrum, post-colostral milk, serum, or even IgG (Snodgrass et al 1977b, Snodgrass & Wells 1978a, Fahey et al 1981, Barnes et al 1982). After intraperitoneal injection of hyperimmune rotavirus antiserum to neonatal lambs, rotavirus excretion was reduced and diarrhoea prevented (Snodgrass & Wells 1978b), presumably due to selective transfer of immunoglobulin into the gut.

Woode: How did your intraperitoneal injection compare with the natural absorption of gammaglobulin through colostrum?

Snodgrass: The serum IgG concentrations after intraperitoneal inoculation were 6–8 mg/ml, considerably less than a well-suckled animal would receive (Snodgrass & Wells 1978b).

Woode: So if the normally suckled animal received enough colostrum, you would expect some protection?

Snodgrass: Yes; it is not complete protection but better than that in the colostrum-deprived calf.

Saif: Our work in calves has shown something similar (Saif et al 1983, Saif & Smith 1985). After feeding 'immune' colostrum for five days we find extremely high IgG1 rotavirus antibody titres. There is a period thereafter, for up to seven days after the termination of colostrum feeding, when the calf is still protected, before it becomes susceptible to rotavirus diarrhoea. The most likely mechanism for this protection is that IgG1 antibodies from the serum are transuded back into the intestine and provide a transient protection. But it is not as complete a protection as when local antibodies are present almost continuously in the gut.

Flewett: One ought not to extrapolate too far between species, and maybe not from the calf or pig to the human, because certainly in the human with poliomyelitis, when killed poliovirus vaccine was first used, wild-type strains virtually disappeared from well-immunized human populations. Everybody said that this wouldn't happen, because nobody believed that serum IgG would prevent infection of the gut lining. Maybe it is the same for enteric infections due to diarrhoea viruses.

Bridger: When you speak about passive antibody preventing rotavirus infection, do you really mean that, or do you mean the prevention of rotavirus disease? You can show an active immune response when animals are protected from rotavirus challenge by passive antibody. Do you suggest that rotavirus infection is prevented by passive antibody, or actual disease?

Saif: We have seen both situations. We have seen calves that were totally protected, in that we detected no shedding of rotavirus and no diarrhoea for the entire period for which they received colostrum from rotavirus-immunized cows (Saif et al 1983). We have also had instances where, say, one in eight of these calves, when the colostrum feeding was terminated, was susceptible to re-challenge, which means that it did not develop an active immune response. Two other calves were protected against challenge, so they must have developed subclinical infections followed by active immunity. We have seen the whole gamut of protective effects after colostrum feeding (Saif & Smith 1985).

Snodgrass: The result is dose dependent. In an experimental situation, depending on the relative concentrations of virus and ingested antibody, the result can vary from complete protection against infection, through mild subclinical infection, to no effect on virus excretion or diarrhoea. In a real-life immunization you want the animal to have a subclinical infection under cover of the passive immunization, which enables it to mount its own active immunity, and generally speaking that is what happens.

Bourne: My comment earlier was in reference to infection. Mucosal surfaces that are normally sterile do not rely on IgA for this, but on some other immune mechanism. The immune response is primarily a non-invasive, non-inflammatory response in the gut, or at any surface that has a normal bacterial flora. At a sterile surface, such as the lower respiratory tract, inflammatory sterilizing responses occur.

Kapikian: Why is IgA not as effective as IgG at the mucosal surface for inducing 'sterility' of the surface mucosa?

Bourne: IgA acts in concert with the innate immune mechanisms to allow these to cope in a way that doesn't lead to tissue damage. These innate mechanisms in the gut are primarily peristalsis and mucus production, and the IgA is anti-adhesive; it may also neutralize viruses.

*Holmes:*IgA will neutralize viruses just as well as IgG, surely.

*Greenberg:*Most virus-neutralizing antibodies do not appear to act by blocking binding of the virus to the cell surface. Dr Paul Offit purified anti-rotavirus IgA and IgG and showed that IgA was more efficient per microgram than IgG at protecting mice from rotavirus challenge. Both immunoglobulin types were effective, however.

Saif: Although cellular immunity may play a role in some of these enteric viral infections, athymic nude mice can clear a rotavirus infection, which suggests that antibody could be an important factor, in the absence of CMI (Eiden et al 1986, Riepenhoff-Talty et al 1986).

Greenberg: The only problem with those results is that mice, unlike many other species, become resistant to rotavirus infection at around 15 days of age in the absence of any immune mechanisms. It is hard, therefore, to separate cellular and humoral immunity in a mouse rotavirus model. A 15-day-old mouse with no immune system can still clear a rotavirus infection. Humans with combined immunodeficiency syndrome, on the other hand, do not always clear rotavirus infections; some of them go on to a chronic infection. Something is evidently missing, and passive transfer of antibody doesn't necessarily eradicate the infection in humans.

Hung: I wonder whether there is any viraemia in viral diarrhoea, since this may be related to the question of immunity?

McNulty: Lizbeth Kraft had some evidence for that with the mouse group A rotavirus (EDIM virus).

Flewett: She found small amounts of infective EDIM virus in the liver and in other organs as well in infected baby mice, so showing there must have been viraemia.

Bishop: Are any of these 'enteric' viruses found in other organs?

Flewett: In some earlier rotavirus work in animals, fluorescing mononuclear cells were found in the lamina propria, which thus contained antigen, at least, and probably the virus particles, no doubt getting into the circulation; so it would not be surprising if infectious virus were reaching the circulation from the small intestine. Presumably some antigen must get into the bloodstream, or

you wouldn't have the good rapid IgG and IgM responses that are found.

Hall: Infected macrophages, or rather macrophages containing antigen, have been detected in the lamina propria, for coronavirus and Breda virus and for group A rotavirus.

Horzinek: In cat coronaviruses, the switch from replication in an enterocyte towards replication in the macrophage may cause quite a different disease picture; it causes fatal feline infectious peritonitis when the virus replicates in the macrophage, but only a mild diarrhoea when it replicates in the enterocytes.

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Clinical trials of rotavirus vaccines

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Abstract. The clinical efficacy of candidate rotavirus vaccines has been tested in Tampere, Finland, over four winter and spring rotavirus epidemic seasons in 1983–1986. Testing against natural challenge has demonstrated that heterologous oral rotavirus vaccines induce cross-protection to human rotavirus diarrhoea. The trials have also given insight into mechanisms of protection in human rotavirus diarrhoea.

After the oral vaccination of infants aged six to 12 months the highly attenuated bovine rotavirus strain RIT 4237, titre 10^8 per dose, probably 'takes' in most vaccinees, producing a symptomless intestinal infection with a low virus excretion rate and an antibody response in over 80% of the initially seronegative subjects. Upon natural challenge such vaccination gives no protection against human rotavirus infection but gives 50–60% protection against any clinically detectable rotavirus-associated illness and 80–90% protection against severe rotavirus diarrhoea, regardless of the infecting human rotavirus serotype.

The less attenuated rhesus monkey rotavirus RRV-1, titre 10^5–10^6 per dose, is more infectious in humans, and virus multiplication in the intestine results in excretion of vaccine virus in the stools and some clinical symptoms, mainly fever, 3–4 days after vaccination. The degree of protection against human rotavirus diarrhoea appears similar to that induced by bovine rotavirus vaccine.

1987 Novel diarrhoea viruses. Wiley, Chichester (Ciba Foundation Symposium 128) p 218–237

Soon after the discovery of human rotaviruses it was found that human and many animal rotaviruses showed serological cross-reactivity at the level of a common inner capsid group antigen (Kapikian et al 1976). Wyatt and co-workers demonstrated that infection of fetal lambs *in utero* by a bovine rotavirus made them resistant to challenge by a human rotavirus after birth: hence the original idea of using a bovine rotavirus as a human vaccine (Wyatt et al 1979). Subsequently the NCDV (Nebraska calf diarrhoea virus) strain of bovine rotavirus was attenuated by 147 tissue culture passages, and the new strain was designated RIT 4237 (Delem et al 1984). This strain was again shown to induce cross-resistance against human rotavirus infection in a piglet model (Zissis et al 1983).

It was apparent at this point that the potential value of the attenuated bovine rotavirus as a human vaccine could only be tested in man, and specifically in

young children. Preliminary testing of the RIT 4237 strain in adults and children in Finland in 1982 indicated that the virus did not produce any appreciable symptoms in the recipients and was not usually excreted in the stools, but about 70% of the recipient children developed an antibody response to it (Vesikari et al 1983a). In order to test the protective efficacy of such immunity we decided to take advantage of the known and predictable epidemiological pattern of rotavirus diarrhoea in Finland (Vesikari et al 1981, 1983a) by vaccinating infants at greatest risk (age six to 12 months) before the rotavirus epidemic season and testing the vaccine-induced immunity against natural challenge during the rotavirus season in winter and spring. We have studied the clinical efficacy of RIT 4237 rotavirus vaccine in infants during two winter seasons according to such a plan, and we are currently studying the efficacy of neonatal RIT 4237 vaccination against natural challenge.

Rhesus monkey rotavirus strain RRV-1 has been introduced as another candidate heterologous rotavirus vaccine for man (Kapikian et al 1985). We have tested its efficacy under similar epidemiological conditions as used for the trials of RIT 4237. At the same time as the clinical efficacy of the candidate rotavirus vaccines has been established, we have collected information on various factors that may influence the success of rotavirus vaccination. Altogether our experience contributes to understanding the mechanism of protection against rotavirus diarrhoea after rotavirus vaccination and also after natural infection.

Materials and methods

The vaccines, virological and serological methods, and the performance of the clinical studies have been described in detail in earlier publications, and only a brief outline is given below.

The vaccines

The RIT 4237 vaccine was derived from the NCDV strain of bovine rotavirus, attenuated by passaging 147 times in primary bovine kidney cells, and produced at 154th passage level in primary monkey kidney cells (Delem et al 1984). Safety testing of the vaccine was carried out according to the WHO guidelines (WHO 1982). Although different lots of the vaccine were used in the clinical trials, most lots had titres of between $10^{8.1}$ and $10^{8.3}$ tissue culture infective doses per 0.5 ml, which was the volume administered orally. The vaccine was always supplied freeze-dried from Smith Kline-RIT, stored at $-20\,°C$, and reconstituted shortly before administration.

The RRV-1 strain of rhesus monkey rotavirus (originally designated MMU 18006) was passaged a total of 34 times in tissue culture and produced for testing in primary monkey kidney cells (Kapikian et al 1986). The vaccine bulk was

shipped on dry ice and stored at −70 °C; dilutions of 1:10 and 1:100 of the bulk, with titres of approximately 10^6 and 10^5 per ml, respectively, were prepared before use in Tampere. The dose administered orally was 1 ml.

Laboratory tests

Rotavirus serology in studies of the RIT 4237 vaccine was done in Tampere or at Smith Kline-RIT, Rixensart, Belgium. The method used for the rotavirus enzyme-linked immunosorbent assay (ELISA) IgG test has been described (Vesikari et al 1984b), and the ELISA IgM test and microneutralization test will be reported separately (A. Delem & T. Vesikari, submitted). Serological testing of the RRV-1 vaccinees was done also at the Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, USA. The method for the plaque reduction neutralization test has been described (Hoshino et al 1985).

Virus isolation studies were done at the Division of Virology, Infectious Disease Unit, St. Pierre Hospital, Brussels (Dr G. Zissis), at the Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, USA (Dr A.Z. Kapikian), and at the WHO Collaborating Centre for Reference and Research on Rotaviruses, Birmingham, UK (Dr T.H. Flewett). The methods for serotyping (Thouless et al 1982) and subgrouping (Lambert et al 1984) of the rotavirus isolates have been described.

Clinical setting

All the vaccinees were healthy infants born at the Tampere University Central Hospital and living in the city or the surrounding area. The infants were vaccinated at the paediatric outpatient clinic of the hospital, where clinical cases were also seen by the investigators. The centralized follow-up system made it possible to record and investigate clinically practically all the cases of diarrhoea in the study groups. Thus the clinical investigators evaluated the condition of each child with suspected diarrhoea, and, when necessary, gave instructions for treatment.

Results and discussion

RIT 4237 bovine rotavirus vaccine

Clinical symptoms and virus excretion. About 700 infants between the ages of four and 12 months have received the RIT 4237 vaccine in Tampere, and clinical reactions attributable to the vaccine have never been observed. Specifically, there has been no increased incidence of fever or diarrhoea by comparison with control infants given uninfected tissue culture fluid, within a week after

vaccination (Vesikari et al 1983b, 1984a, 1985b). In neonates, a slight increase in the frequency of watery stools, but no significant diarrhoea or illness, was seen in about 15% of the vaccine recipients on the first day after vaccination (T. Vesikari, T. Ruuska, A. Delem & F.E. André, unpublished work).

In the most closely monitored study the RIT 4237 vaccine virus was isolated in the stools of five (21%) of the 24 vaccine recipients; the virus isolation studies were done by laboratories using sensitive techniques (Vesikari et al 1986b). In one of the first trials the vaccine virus antigen was detected in the faecal specimens of three of the 26 vaccinees (Vesikari et al 1983b).

The absence of clinical symptoms in close home monitoring of young infants seronegative for rotavirus suggests that the RIT 4237 virus is highly attenuated for man and probably does not multiply effectively in the human host. This is supported by the low degree of excretion of infectious virus. It is possible that many intestinal epithelial cells are infected, but the virus multiplication remains incomplete and only a small amount of new infectious virus is produced. The fact that the mild clinical reactions observed in neonates occurred the next day after vaccination may be due to a high degree of initial infection of intestinal epithelial cells. Since little new infectious virus is produced, the secondary spread of this virus may be limited and not sufficient to give clinical symptoms. The initial infection of the epithelial cells by the RIT 4237 vaccine virus may also form the main stimulus for an immune response.

Serological response: effect of gastric acid, pre-existing antibody and breast-feeding. The serum antibody response to rotavirus in the RIT 4237 vaccine recipients has been used as an indicator of the vaccine virus 'take'—that is, intestinal infection. There is evidence that virus multiplication, even if incomplete, plays a role in the antibody response: administration of the same amount of RIT 4237 virus after fasting or after a milk meal resulted in a poorer seroconversion rate in the fasting subjects, probably as a result of inactivation of the vaccine virus by gastric acid (Vesikari et al 1984b, 1985c). Thus a higher dose of infectious virus resulted in a better serological response. A comparison of different vaccine dosages (10^8, 10^7 and 10^6 per dose) indicated that the lower the dose, the poorer the serological response (Vesikari et al 1985c). This can be interpreted to mean that the amount of infectious virus in the original vaccine, rather than the amount of virus resulting from virus multiplication in the host, is critical for the induction of an immune response to RIT 4237 vaccine.

In all studies the measurable serum antibody response to RIT 4237 rotavirus vaccine has shown a correlation with the prevaccination level of serum rotavirus antibody; that is, the serological response rate in seronegative vaccinees has always been better than that in seropositive ones (Vesikari et al 1984a, 1985c, 1986c). Table 1 shows the serological responses of six- to 12-month-old children and newborn infants to the standard dose (10^8) of RIT 4237 vaccine. Of the 6–12-month-old infants, the majority were seronegative (ELISA titre less than

TABLE 1 Rotavirus ELISA IgM antibody response to RIT 4237 rotavirus vaccine (titre $10^{8.3}$ per dose) in 6–12-month-old and newborn infants in relation to their prevaccination serum rotavirus antibody status

Prevaccination serum rotavirus ELISA antibody	*No. with response/no. tested*	
	6–12 month-olds[a]	*Neonates*[b]
Seronegative	58/69 (84%)	3/3 (100%)
Seropositive	25/37 (68%)	36/116 (31%)

[a] Data from Vesikari et al 1986c.
[b] T. Vesikari, T. Ruuska, A. Delem & F.E. André, unpublished work.

1:200) and 81% of them developed a detectable serum antibody response, compared to 69% of those with pre-existing serum rotavirus antibody (Vesikari et al 1986c). In neonates the overall response rate was much lower, as most of the neonates had a high titre of transplacentally acquired rotavirus antibody. Still, the few neonates with no measurable serum antibody responded well (Table 1), and of the seropositive ones the 31% who responded tended to have a low titre of serum antibody before vaccination (T. Vesikari, T. Ruuska, A. Delem & F.E. André, unpublished work).

The lower serological response rate in the seropositive vaccinees can be explained in two ways: (1) either the pre-existing antibodies prevent infection by the vaccine virus, or (2) the antibodies do not prevent the initial infection but only suppress an active antibody response to the infection. We tend to favour the latter explanation.

According to this view the vaccine virus would 'take' in virtually all recipients, and even those vaccinees who do not develop an active antibody response would benefit from the immunization, possibly by developing cell-mediated local immunity. This is supported by our findings in the clinical protection studies: protection against diarrhoea was found to be better than the serological response after vaccination (Vesikari et al 1984a, 1985b). Nevertheless, the vaccinees who did develop an antibody response were better protected than those who did not (Vesikari et al 1984a, 1985b).

Since secretory IgA antibodies appear to have an important role in protection against rotavirus diarrhoea in the mouse model (Riepenhoff-Talty et al 1986) we have intensively studied the effect of breast-feeding on the 'take' of RIT 4237 vaccine (Vesikari et al 1985c, 1986c). In our experience, breast-feeding does not significantly interfere with oral RIT 4237 vaccination (Table 2). This finding is of great practical significance, since the simplest and best available antacid before rotavirus vaccination would in most circumstances be breast milk. On the other side, the absence of an inhibitory effect of breast milk suggests that passively administered secretory IgA antibody does not effectively prevent infection by a

TABLE 2 Serological response of breast-fed and non-breast-fed infants to RIT 4237 rotavirus vaccine

	No. with response/no. tested	
Feeding	*6–12 month-olds*[a]	*Neonates*[b]
Breast milk	26/32 (81%)	39/119 (33%)
Cow's milk or infant formula	32/37 (86%)	
Glucose		40/119 (34%)

[a] Data from Vesikari et al 1986c.
[b] T. Vesikari, T. Ruuska, A. Dalem & F.E. André, unpublished work.

TABLE 3 Protection against rotavirus infection and diarrhoea in two placebo-controlled trials of RIT 4237 rotavirus vaccine in 6–12-month-old infants

	Vaccine protection rate	
Clinical description	*Study 1*[a] (*n* = *178*)	Study 2[b] (*n* = *328*)
Rotavirus infection	None	None
Any rotavirus-associated gastrointestinal illness	50%	58%
Moderate or severe rotavirus diarrhoea	88%	82%

[a] Vesikari et al 1984a.
[b] Vesikari et al 1985b.
n, vaccine and placebo recipients combined.

heterologous rotavirus. To be effective the local IgA antibodies should perhaps be serotype specific and virus neutralizing: hence a heterologous bovine rotavirus might more easily break through the antibodies present in human breast milk, whereas human rotaviruses might be neutralized.

Protection against rotavirus diarrhoea and infection. Table 3 summarizes the results of two placebo-controlled clinical protection studies of one epidemic season's duration with the RIT 4237 vaccine, in 6–12-month-old infants. In the first trial (Vesikari et al 1984a) the follow-up lasted from February to May 1983. Rotavirus infections were common, as determined by rotavirus antibody responses during the season. In the vaccinated group 45% of the children and in the placebo group 40% of the children had a significant rotavirus ELISA antibody increase from February to May. Some of the 'excess' serological responses in the vaccinated group may represent late vaccine-induced sero-conversions, but in any case it can be concluded that the RIT 4237 vaccination did

not prevent rotavirus infections but that these occurred equally often in the vaccinated and placebo-treated recipients. Very similar results were obtained in the second study (Vesikari et al 1985b). Therefore it appears that the RIT 4237 vaccine-induced protective mechanism does not operate at the first line of defence (secretory antibodies), and rotavirus infection is enabled to take place.

In contrast to the lack of protection against rotavirus infection, there was a clear vaccine-induced protection against rotavirus diarrhoea, which was even more evident when only clinically significant cases requiring therapeutic intervention were taken into the analysis (Table 3). It can therefore be stated that prior vaccination with RIT 4237 vaccine reduces the clinical severity of gastrointestinal symptoms associated with subsequent human rotavirus infection. It is possible that all the vaccinees, regardless of antibody response, derive some benefit: even in the 'breakthrough' cases of clinically significant rotavirus diarrhoea in the vaccinees, the duration of diarrhoea was shorter (mean 2.8 days) than in the placebo recipients (mean 4.4 days) (Vesikari et al 1985b).

In both studies presented in Table 3 the protection against rotavirus diarrhoea was, however, better among those vaccinees who responded serologically to vaccination. Also, in the follow-up during the second winter season, rotavirus diarrhoea occurred almost exclusively in those placebo recipients who remained rotavirus seronegative after the first season (Vesikari et al 1986a). Both study groups were followed over two years (Vesikari et al 1985a, 1986a), but clinical rotavirus diarrhoea was much less frequent in the second season than in the first. The total vaccine-induced protection for two years was as follows: eight cases of (moderate to severe) rotavirus diarrhoea in the 246 vaccinated subjects, compared to 52 cases in the 260 placebo recipients, which equals an 84% protection rate.

Evidence accumulated so far suggests that the RIT 4237 vaccine-induced protection against rotavirus diarrhoea is not limited to a particular rotavirus serotype, but rather covers a whole range of human rotaviruses. In the second protection study (Vesikari et al 1985b) the rotavirus isolates were serotyped by T.H. Flewett (Birmingham, UK). Serotypes 1, 2, and 3 were discovered, and there appeared to be protection against diarrhoea associated with each of these serotypes. In the first protection trial (Vesikari et al 1984a) the rotaviruses were not serotyped but were subgrouped by G. Zissis (Brussels); the majority of the viruses found in that season were of subgroup 2, whereas the RIT 4237 vaccine virus falls in subgroup 1. Thus the spectrum of clinical protection appears to be wide and not restricted by serotype or subgroup specificities. Consequently, it seems that neutralizing antibodies against the epitopes that determine the serotype specificities are not essential for protection against rotavirus diarrhoea.

We are currently investigating whether neonatal rotavirus vaccination, despite the poor antibody response in the presence of cord blood antibody, will induce clinical protection against rotavirus diarrhoea. The analogy would come from neonatal rotavirus infection with a nursery strain: in the follow-up study of

Bishop et al (1983) it was found that a natural neonatal infection gave partial protection against rotavirus diarrhoea, but no protection against rotavirus infection, in the next three years. In our first study group of 239 neonates there were 14 cases of rotavirus-associated gastrointestinal illness in the 120 vaccinees and 10 cases in the 119 placebo recipients during a 16 months' follow-up, and hence no protection. However, of the 10 cases of diarrhoea in the placebo-treated group, seven were severe and three moderately severe, whereas in the vaccine group there was only one severe episode, with four episodes of moderately severe rotavirus diarrhoea (T. Vesikari, T. Ruuska, A. Delem & F.E. André, unpublished work). Therefore RIT 4237 vaccination in the neonatal period seems to induce significant protection against rotavirus diarrhoea. Consequently, rotavirus vaccinations of newborns and/or any age group up to six months, when passively acquired rotavirus antibodies are commonly present, remain as alternatives for future rotavirus vaccination programmes, even though the primary antibody responses in these age groups may appear disappointingly low.

RRV-1 rhesus monkey rotavirus vaccine

The rhesus monkey rotavirus vaccine RRV-1 was first given to adults and older children in the United States, and in these trials the virus induced good serological responses with no clinical reactions (Kapikian et al 1986). Subsequently the vaccine has been tested in young infants in the USA, Venezuela, Sweden and Finland (Kapikian et al 1986). The only comparative trial so far of the RRV-1 and RIT 4237 vaccines for immunogenicity and safety was carried out in Tampere in 6–8-month-old infants (Vesikari et al 1986b). A clinical protection study is under way in Tampere and only preliminary data on clinical efficacy are available.

Clinical symptoms and virus excretion. A 1:10 dilution (approximate titre 10^6) of the RRV-1 vaccine induced significant morbidity in the 6–8-month-old infants: more than half the children had fever over 38 °C with concomitant general irritability on Days 3 and/or 4 after vaccination, and about 20% had diarrhoea on Days 4 and/or 5 (Vesikari et al 1986b). Similar fever reactions on Days 3 and 4 were also reported by Losonsky et al (1986) in a study in Maryland, and by L. Gothefors in Sweden (Kapikian et al 1986).

Altogether the symptoms observed are consistent with the timing of peak virus multiplication in the host. In RRV-1 virus infection much more infectious virus is produced than in RIT 4237 infection: 84% of the RRV-1 vaccinees excreted detectable infectious virus, and most of them had virus in several successive stools (Vesikari et al 1986b).

It was thought that the RRV-1 vaccine might cause fewer reactions if the

vaccine dose was reduced or, alternatively, if the vaccine was given to infants younger than six months of age, who would be protected by maternally acquired antibody. In a second study in Finland the RRV-1 vaccine was diluted 1:100 (approximately 10^5 per dose) and given to 100 infants between two and five months of age. In this trial 25% of the infants had fever over 38 °C on Days 3 and/or 4, and the clinical illness was milder than in those who had received the higher dose (T. Vesikari, T. Rautanen & A.Z. Kapikian, unpublished work). Evidently a reduction of the vaccine virus dose decreases clinical reactions but does not completely abolish them. Fever reactions were seen in both seronegative and seropositive vaccine recipients. On the other hand, the same dose of RRV-1 vaccine has been given to neonates in Venezuela with no significant clinical reactions (J. Flores, personal communication). The reason for the different experience is not clear, but one possibility is that neonates in Venezuela possess a greater amount of transplacentally acquired rotavirus antibody than infants in Finland. Altogether the reactogenicity of RRV-1 vaccine remains an unsettled and controversial issue. It is clear that the reactions associated with the high-dose vaccine make it unsuitable for use in the susceptible population of 6–8-month-old children in countries like Finland and Sweden. A lower dose might be acceptable in terms of side-reactions, but a reduction in dose may compromise immunogenicity and clinical protection.

Serological response. Vaccination with the RRV-1 vaccine results in a stronger antibody response than that following RIT 4237: in the comparative trial in Tampere, 88% of the RRV-1 vaccinees and 75% of the RIT 4237 vaccinees produced a detectable antibody response, but the titres were generally higher in the RRV-1 vaccinees (Vesikari et al 1986b). However, a few vaccinees did not respond serologically to RRV-1, nor did they excrete the vaccine virus; these may have been primary vaccine failures (Vesikari et al 1986b). The full serological results of the vaccination of 2–5-month-old infants in Finland are not yet available, but it is probable that the responses will be lower than those in older infants because of the suppressive effect of maternal antibody (like that observed in RIT 4237 vaccinees).

Protection against rotavirus diarrhoea. So far there is only limited information on the clinical protection induced by the RRV-1 vaccine. The results of the first placebo-controlled clinical protection study in Umeå, Sweden, will be available shortly (L. Gothefors, personal communication). In Tampere, a placebo-controlled trial of the '1:100' vaccine in 200 infants aged 2–5 months was started in December 1985, and preliminary results as of 15 May 1986 have been made available by breaking the code for the observed eight cases of rotavirus diarrhoea (Table 4). These preliminary data suggest that the protection may be similar to that induced by RIT 4237 in a comparable setting.

TABLE 4 Rotavirus diarrhoea in the follow-up period from 1 January to 15 May 1986 in infants who received the RRV-1 vaccine (1:100) or placebo at the age of two to 5 months

	Number of episodes	
Clinical description	*RRV-1 vaccine (n = 100)*	*Placebo (n = 100)*
Moderate diarrhoea	2	3
Severe diarrhoea	0	3
Total	2	6

Protection rate, 75%.
T. Vesikari, T. Rautanen & A.Z. Kapikian, unpublished work.

Summary and conclusions

The clinical studies of candidate rotavirus vaccines in Finland have, above all, demonstrated that heterologous rotaviruses induce significant cross-protection against human rotavirus diarrhoea and could therefore be used as human vaccines. The initial success in the prevention of rotavirus diarrhoea in our studies does not mean that either of the candidate vaccines, RIT 4237 or RRV-1, is 'ready' for use in general rotavirus vaccination, even in Finland, and much less in developing countries where the need for a rotavirus vaccine is greatest. The two vaccines may in fact represent extreme approaches in terms of attenuation (Table 5): the RIT 4237 may be overattenuated and RRV-1 too little attenuated for use in man. We have preliminary evidence that an earlier passage level (20th) of the NCDV bovine rotavirus may be more immunogenic in man than the RIT 4237 vaccine (T. Vesikari, T. Rautanen, A. Delem and F.E. André, unpublished work). On the other hand it might be possible to develop a further-attenuated rhesus monkey rotavirus to reduce reactogenicity and possibly to improve vaccine virus yield in tissue culture.

The experience in Finland clearly indicates that heterologous rotavirus vaccines will not prevent rotavirus infections and therefore will not have much effect on rotavirus epidemiology. However, diarrhoea and dehydration can be prevented by a heterologous rotavirus vaccine, and this should be sufficient for all practical purposes. The RIT 4237 vaccine induces clinical protection against rotavirus diarrhoea up to the age of three years, which can be regarded as meeting the main requirements for a rotavirus vaccine (Vesikari et al 1985a, 1986a, Vesikari 1985).

A rotavirus vaccination programme in Finland and comparable countries with a similar rotavirus epidemiological picture could follow the pattern of our clinical trials, with vaccination of children at the age of highest susceptibility before their first rotavirus season. Alternatively it might be possible to give

TABLE 5 Comparison of RIT 4237 bovine and RRV-1 rhesus monkey rotavirus vaccines and vaccination in infants

Property	*Bovine rotavirus vaccine* (*RIT 4237*)	*Rhesus monkey rotavirus vaccine* (*RRV-1*)
Serotype specificity	Serotype 6 Not cross-reactive with human rotavirus serotypes 1–4	Serotype 3 Identical with human rotavirus serotype 3
Degree of attenuation	High 154 tissue culture passages	Low 34 tissue culture passages
Vaccine dose	High ($10^{8.3}$ per dose)	Low (10^6 to 10^5 per dose)
Virus multiplication in human host	Probably low: ≈20% of vaccinees excrete infectious virus	High: over 80% of vaccinees excrete infectious virus
Vaccine virus-associated illness	Very low Mild gastrointestinal symptoms in neonates on Day 1 after vaccination	Moderately high Fever reactions on Days 3 and 4 Diarrhoea on Days 4 and 5 after vaccination
Vaccine-induced immunity	Homologous antibody response in over 80% of seronegative recipients, less in seropositive ones 'Take' of vaccine better than serological response? Cell-mediated immunity?	Close to 100% homologous antibody response in seronegative recipients, less in seropositive ones
Vaccine-induced protection	No protection against rotavirus infection 80–90% protection against moderate to severe rotavirus diarrhoea Duration of protection ⩾2 years Spectrum of protection not restricted by human rotavirus serotype	Preliminary evidence of cross-protection against human rotavirus diarrhoea obtained

rotavirus vaccination together with other immunizations any time between birth and six months of age, even though such vaccination would not always elicit a detectable antibody response. In this regard an unresolved problem is the value of repeated oral rotavirus vaccinations: it is not clear whether repeated vaccinations will produce better immunity and protection against rotavirus diarrhoea than a single vaccination. The primary vaccination might theoretically induce homotypic immunity which could interfere with repeated vaccinations by the same virus. If this were so, a secondary infection by natural rotavirus or another vaccine strain might be more effective in boosting immunity.

Altogether, it will probably be necessary to find ways to stimulate rotavirus vaccine-induced immunity from the level seen in studies in Finland before rotavirus vaccination will be clinically effective in developing countries. There is already preliminary evidence that RIT 4237 vaccine has been less efficacious in trials in Ruanda and in Peru than in Finland (F.E. André, personal communication). In developing countries the primary 'take' of the vaccine may be prevented by interference by other enteric viruses present in the gut. Furthermore, the vaccine-induced immunity may be weaker in poorly nourished children and therefore protection against challenge less effective. Finally, the infectious dose in conditions with poor hygiene may be greater than in Finland, and a high dose of wild rotavirus may more readily surpass vaccine-induced immunity.

All these possibilities foretell difficulties for candidate rotavirus vaccines in developing countries. Ways of overcoming the problems may include stimulation of the heterologous vaccine-induced immunity by repeated vaccinations with the same or different vaccine, including possibly an inactivated injectable vaccine. Another approach would be to prevent the rotavirus infections, and not only diarrhoea, by means of human rotavirus vaccines or recombinants of human and animal rotaviruses which induce neutralizing antibodies against various human rotavirus serotypes (Kapikian et al 1986).

While the ultimate test of all such approaches will be in developing countries, it will be important to continue studies of new rotavirus candidate vaccines in developed countries where the testing can be done in 'cleaner' conditions with rotavirus as the main causative agent of childhood diarrhoea. Finally, a future rotavirus vaccination programme should preferably be started from developed countries, to pave the way for rotavirus vaccination in the Third World.

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DISCUSSION

Woode: In the veterinary field, the attenuated bovine rotavirus vaccine has not given protection against some rotavirus diarrhoeas in calves. We are also finding that cross-protection doesn't correlate well with serotype. If I understand your results correctly, with a different serotype you might not have seen much protection? You get good protection to serotype 1, but if most of your cases had been serotype 3, then it might not have been so protective?

Vesikari: There is no definite answer to that question, but all the available evidence suggests that protection is not restricted by serotype. For example, serotype 1, which is the most common human rotavirus causing disease in this age group, is different by neutralization specificity from NCDV (serotype 6). With the RIT 4237 vaccine we can achieve protection against this serotype, which is first of all important and useful as such, because serotype 1 is clinically so significant; secondly, it suggests that we could predict protection against other human serotypes as well.

Woode: The results you obtained did not show protection against serotype 3?

Vesikari: The results are inconclusive, because we have so little evidence on this point. This is why I am looking forward to serotyping the rotavirus isolates from our ongoing protection study of RIT 4237 vaccine given to neonates.

Bridger: We have experimental evidence in calves, with one pair of group A rotaviruses, that there is cross-protection when viruses are unrelated by serotype. We used a bovine rotavirus (17/4) as our 'vaccine' strain and we challenged 21 days later with a virulent rotavirus (CP-1). Five calves were used; four were protected and one was unprotected. Three shed virus and all five seroconverted. We measured neutralizing antibody levels at challenge to the cloned challenge virus, CP-1; in the serum of the protected calves, the means titre was less than 19, whereas the mean homologous titre was 313. The unprotected calf had no detectable neutralizing activity to the CP-1 virus in its serum at a 1:10

dilution but had seroconverted to the 'vaccine' virus. In the faeces we also found very low levels of neutralizing activity to the challenge virus (CP-1) on the day of challenge: <1:50 in the protected calves and 1:29 in the unprotected calf. So we have protection in four of five calves against clinical disease with very little neutralizing activity, or no detectable levels. This conflicts somewhat with published data on cross-protection between serotypes.

Bishop: What virus serotypes were you using?

Bridger: I haven't related them to the human serotypes, but the difference in neutralization titres between the 17/4 and CP-1 rotaviruses is >219-fold in one direction and more than 24-fold in the other direction. By the '20-fold' serotypic criterion they are different serotypes of bovine rotaviruses, but I can't tell you how they are related to serotypes from other species.

McCrae: These studies were done in gnotobiotic calves over a relatively short period. Have you been able to take the animals out of the isolator, without giving them a challenge, and keep them for a while, and then give them a heterologous challenge? Is this feasible?

Bridger: It is feasible for us to do that; we try to use a standard experimental set-up with not too many variables, so we haven't done it, but we could if we thought it worth while.

Greenberg: Have you done a similar protection study in a passive way? That is, have you immunized the mothers and then seen whether immune milk also protects calves heterologously?

Bridger: No, we haven't at Compton.

Snodgrass: Passive protection also appears to be serotype specific. We used two distinct calf rotavirus serotypes, and hyperimmune rabbit antisera to each. In gnotobiotic animals fed serum and infected with virus, good homotypic but not heterotypic protection was achieved (Snodgrass et al 1984). The serotype specificity of passive protection has also been confirmed in mice (Offit & Clark 1985). In passive immunization through maternal vaccination, however, the response to vaccination of the dam who has experienced multiple rotavirus infection is probably heterotypic, and protective neutralizing antibody against a range of serotypes is produced in milk (Snodgrass et al 1984).

Greenberg: I am wondering whether, if you had serum with exactly that heterotypic neutralization specificity and you fed that, it would protect against heterotypic challenge.

Bridger: I don't know, but the mechanisms of active and passive protection are probably quite different.

Saif: Have you had any trouble in reproducing a diarrhoeal syndrome and viral shedding in 21-day-old calves? We found much inconsistency at that age in reproducing diarrhoea.

Bridger: Not with the rotavirus strain we used. Our latest paper, on variation in virulence of bovine rotaviruses, may help to explain the discrepancies between laboratories using different rotaviruses (Bridger & Pocock 1986). Not

unexpectedly, there is a range of virulence in group A rotaviruses which is not well recognized. We have different strains which produce different effects in calves of different ages. In the experiment I described, all four challenge controls infected with the CP-1 rotavirus developed diarrhoea.

Saif: We have done some of the passive immunization experiments about which Harry Greenberg was asking. We have hyperimmunized cows with the NCDV strain of bovine rotavirus (Saif et al 1984). They had colostral rotavirus antibody titres of 360000 against NCDV rotavirus by virus neutralization. We fed that colostrum to unsuckled specific pathogen free (SPF) calves and challenged with an unrelated second serotype of bovine rotavirus. We found only partial protection, as opposed to complete protection after challenge with the homologous strain.

We did a similar study in conventional naturally suckled pigs whose dams were vaccinated with OSU porcine rotavirus (Saif 1985). In this study we relied on natural challenge with rotavirus to evaluate rotavirus-associated diarrhoea and shedding among litters of vaccinated and control sows. Maternal vaccination did not prevent natural infection with rotavirus in nursing piglets, but it did delay the onset and shorten the duration of both rotavirus infection and diarrhoea. Thus partial protection was also seen in this vaccination study.

Greenberg: This is basically what Dr Vesikari has found.

Saif: Yes; but we don't know the serotype(s) of the strains that infected the pigs.

Kapikian: Dr Bridger, we know from Dr Vesikari's studies in humans that the RIT 4237 vaccine did not protect significantly against mild diarrhoeal illnesses but did give significant protection against clinically significant diarrhoea. Did you grade the illnesses you observed in your animal studies?

Bridger: In four out of five calves it was total protection against illness, which we measure quantitatively, as well as by subjective assessment.

Kapikian: Is it a mild illness at that age?

Bridger: In the challenge controls which we infected with the CP-1 virus at about four weeks of age, it is an illness with faecal colour change for four days, increased faecal ouptut for four days with peak levels about six-fold higher than normal, and a variable degree of anorexia between calves, but it's not fatal, at 21 days of age. It is not the most severe rotavirus disease that we are working with and I would describe its severity as mild to moderate.

Svensson: We have analysed by a radioimmunoprecipitation assay (RIPA) the immune response in children to different polypeptides of human rotavirus after vaccination with RIT 4237 and RRV-1 vaccines. Antibody responses were directed against VP2, VP4 and VP6. We could not find any activity to VP7, which is surprising. This might be due to the fact that sera collected on Day 28 preferentially contain antibodies of IgM type that are not recognized in our RIPA. We have also noted that many of the pre-vaccination sera were positive for rotavirus antibodies. If these antibodies are due to a previous rotavirus

infection and are not maternally acquired, the vaccine-induced response might be regarded as a booster. The relatively low level immune response as seen by RIPA after vaccination, compared to that after a natural infection, rather suggests that the pre-existing antibodies are of maternal origin.

Chiba: There is no doubt about the need for an effective rotavirus vaccine, but there are a number of questions about homotypic as well as heterotypic immunity which must be answered before an effective strategy for immunization can be developed. In the course of our longitudinal observations of gastroenteritis in infants living in an orphanage, which I mentioned earlier, we experienced three consecutive outbreaks due to serotype 3 rotavirus. Taking advantage of the serotype determination involved in those outbreaks, and also of the availability of pre- and post-outbreak sera, we studied the relationship between pre-existing homotypic and heterotypic antibody levels and resistance to infection, or clinical disease due to type 3 rotavirus (Chiba et al 1986). As you can see from Table 1, in the group of seronegative infants or infants with antibody titres of 1:32 or below, almost all of the 26 infants were infected and became ill, except for two cases. But in the group of 23 infants with neutralizing antibody titres of 1:128 or greater, all but one of them escaped illness, although many of them became infected. Thus the correlation of pre-existing antibody levels with protection against clinical disease was clear cut. We couldn't find such a correlation with heterotypic antibody titres. From these results, the immune protection against a rotavirus gastroenteritis in humans was found to be serotype specific and the protective titre of antibody was estimated as 1:128. This level of antibody could be a useful index for monitoring the immune resistance to rotavirus gastroenteritis.

We also investigated the possibility of inducing heterotypic immunity. The majority of infants infected with serotype 3 rotavirus showed concomitant antibody responses against serotypes 1 and 4 rotavirus, but only a minority of infants had a concomitant increase in antibodies against serotype 2 virus. Fig. 1 shows the neutralizing antibody response to type 1 virus in infants after exposure to the outbreaks of type 3 rotavirus gastroenteritis: 80% of the infants infected with type 3 virus showed a significant antibody response against type 1 virus. I would like to emphasize that all but one of the seronegative infants, presumably having no previous infection with serotype 1 virus, were seroconverted to type 1 virus and acquired heterotypic antibody. These results may suggest the possibility of inducing heterotypic immunity by a rotavirus vaccine.

Bishop: Has heterotypic protection been observed in animal experiments and, if so, what is the basis for it?

Snodgrass: It is a confusing area; we have got used to the idea of using hyperimmune antisera prepared in a guinea-pig or rabbit, and that is how we have divided up the serotypes. If you take an animal with no rotavirus experience and infect it with a single-serotype rotavirus, you get a different situation; you do not get a solely monotypic antiserum. For example, we have infected

TABLE 1 ***(Chiba)*** **Relations between pre-existing neutralizing antibody titres to type 3 rotavirus and infection or clinical illness during three outbreaks of type 3 rotavirus gastroenteritis**

Antibody titre	*Number tested*	*Number infected*	*Number of patients*
<1:16	7	7	6
1:16	13	13	12
1:32	6	6	6
1:64	12	9	8
1:128	6	5	1
1:256	8	2	0
≥1:512	9	2	0

From Chiba et al 1986.

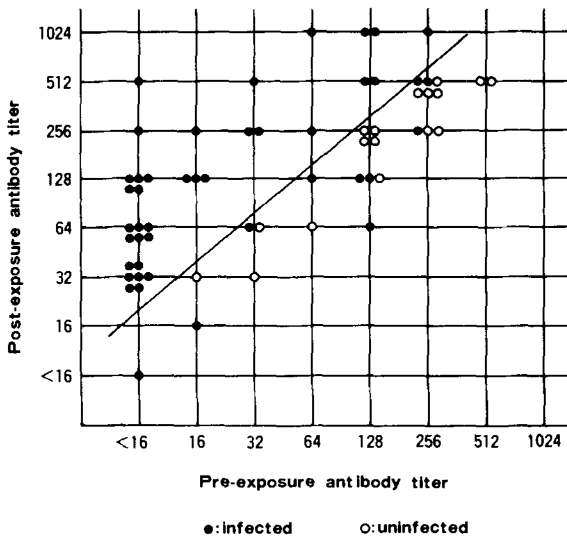

FIG. 1 (*Chiba*). Neutralizing antibody titres to type 1 virus before and after exposure to outbreaks of type 3 rotavirus gastroenteritis.

gnotobiotic lambs and calves with rotavirus serotype 6 (on Hoshino's classification: Hoshino et al 1984). We get a good neutralizing antibody response to serotype6, and also neutralizing responses to some other rotavirus serotypes, and we are not clear about the basis of that: is it a response to protein VP3 or is there some other partial relationship with VP7? Antibody response is not as clear cut after infection as it is after immunization, and this underlies some of the confusion.

Greenberg: Dr Chiba, do you know whether the heterotypic response is due to cross-reactivity to VP7 or to VP3, in the sera of those children?

Chiba: I haven't looked at that yet. Your question is very helpful, and I will try to clarify that point.

Woode: I agree with David Snodgrass's experience. We find that heterotypic cross-protection is not really predictable. It happens with some strains of bovine rotavirus (group A) and not with others. Also, some strains induce a broad serum response to different serotypes and others induce very specific responses. Our interpretation is that cross-protection does not directly correlate with serotyping by neutralization, because we can have neutralizing antibody in the faeces and blood, but the animal is not protected, depending on the combination of viruses you have used.

Flewett: If you simply do serological comparisons by hyperimmune sera and plaque reduction titration you will perhaps get a misleading result. If you use hyperimmune sera, and do serotyping by fluorescent focus neutralization using raw virus from raw faeces, you still demonstrate the serotype difference but, usually, titre ratios are smaller. And if you look at convalescent sera—this certainly applies to the infant and to our collaborative work with Gerald Woode (Thouless et al 1977)—there is a lot of heterotypic cross-neutralization; you get a titre difference between rotaviruses from different species of 1:16- or 1:32-fold by fluorescent focus neutralization using convalescent sera. David Snodgrass helped us with sera for that investigation. It depends how you do the titration, and the results you get by plaque neutralization with hyperimmune sera certainly distinguish well between different strains of rotavirus, but may give a misleading impression of how different in terms of cross-immunity the different virus strains are.

Kapikian: Dr Chiba's elegant studies demonstrate quite clearly one of the problems in rotavirus vaccine development, by showing that with naturally occurring rotavirus infections, low levels of pre-existing serum neutralizing antibody did not protect against infection or illness with the homotypic rotavirus. Dr Vesikari and his colleagues have shown that RIT 4237 vaccine does not prevent subsequent natural rotavirus infection, and Dr Bishop and her coworkers showed that rotavirus-infected neonates could also be reinfected naturally within the next three years of life (Bishop et al 1983, Vesikari et al 1984, 1985). However, in spite of this, the vaccine-induced or naturally induced infection protected against clinically significant (but not milder) diarrhoea during reinfection. This is reminiscent of, although not exactly analogous to, an immunization strategy for influenza described by Dr Kilbourne, in which infection was a necessary event after immunization with an influenza neuraminidase which does not participate in virus neutralization but is protective because of its damping effect on multicycle infection. Thus, a modified and immunizing infection would occur when the wild virus infected the host, post-vaccination (Kilbourne 1985). He called the strategy 'infection-permissive

vaccines for the modulation of infection'. A rotavirus vaccine probably operates in a somewhat similar although not identical manner. Since natural rotavirus infection does not appear to give effective protection against reinfection but, as Dr Chiba showed, a certain level of serum antibody will induce protection, it is not likely that vaccination would be more effective than naturally occurring infection. But, rather, vaccination should also modulate the subsequent naturally occurring rotavirus infection such that severe illness will be prevented, and mild illness may or may not be prevented.

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The diagnostic gap in diarrhoeal aetiology

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Abstract. It is well established that rotaviruses of group A are the most important cause of severe diarrhoea in children. The causes of most cases of infectious diarrhoea still remain unidentified, however, and there must be other viruses to be found. 'Novel' rotaviruses have recently been discovered, mainly in animals (serogroups B and C in pigs and humans, D in birds, and one or more further groups in sheep and other mammals). Except for the group B virus which has caused widespread outbreaks of quite severe diarrhoea in adults in China (still not reported from outside China) these novel rotaviruses are rarities in the human and probably represent uncommon zoonotic infections. We speculate that the Chinese virus might have arisen by reassortment of genetic segments of animal group B viruses or perhaps by mutation, and so became infectious for man.

The problem of identifying and determining the importance of small round viruses is reviewed. It seems likely that the group of small, round structured viruses, including Norwalk and the viruses of plainly calicivirus morphology, are all representatives of a whole group of enteric caliciviruses. Until they can be more easily cultivated it will be difficult to make diagnostic reagents available to all. Astroviruses and genuine parvoviruses have been found by many people in many countries. Astroviruses are probably more important as pathogens in lambs than in children or calves; parvoviruses can only be established as significant in epidemics. The coronavirus-like particles, first found in Vellore and Bristol, are still enigmatic and their role in diarrhoea is uncertain. Toroviridae, recently discovered as causes of diarrhoea in ungulates, do not seem to be at all important as causes of diarrhoea in humans.

Possibly fruitful approaches to future searches are outlined: firstly to make more extensive use of immuno-electron microscopy; and secondly to try to improve existing tissue culture systems to make them more sensitive to enteric viruses.

1987 Novel diarrhoea viruses. Wiley, Chichester (Ciba Foundation Symposium 128) p 238–249

We have heard a lot in this symposium about many different viruses and their entrancing behaviour in the laboratory, but only in the last part have we heard about the unpleasant things that these viruses do to children and to animals. Diarrhoea kills 4–5 million children each year throughout the world. Children

get it in the UK; but if a child is brought into hospital in time it should not die from the diarrhoea. It is very different in the shanty towns of the Third World where gross faecal–oral transmission of infective agents is the rule in children already infected with heavy loads of intestinal parasites. As Dr Mata showed at a previous Ciba Foundation Symposium (Mata et al 1976), even if a child with only moderate malnutrition recovers from successive attacks of diarrhoea it will never attain the size to which it should have grown, but will be permanently stunted. High mortalities in the first year of life up to 240 per thousand live births have, even recently, been reported from some parts of the world. Such infant mortality rates were found in London and other Western cities 200 years ago. Even in England 90 years ago, when provision of sanitation was already fairly well developed, diarrhoeal diseases killed over 30/1000 children in their first year in England and Wales. We don't have earlier figures for diarrhoeal disease deaths, but the mortality in the 18th century must have been much higher (Wilson & Miles 1953). The rising standards of living should make an improvement, provided that population growth is kept under control (McKeown 1965).

It has by now been very well established that rotaviruses of group A are the most important cause of severe diarrhoea among children. Nevertheless, numerous studies, some published, many unpublished, based on epidemiological work funded by the World Health Organization, revealed that we are still looking for an aetiology for many, usually most cases of infectious diarrhoea (Brandt et al 1983, Black et al 1980). Other agents are important; but studies from Bangladesh indicated that although infection by Norwalk or Norwalk-like viruses was widespread in Bangladeshi children in the early years of life, this did not appear to contribute greatly to morbidity (Greenberg et al 1979). Enterotoxigenic *Escherichia coli* has been implicated in only a comparatively small proportion of early childhood diarrhoea (Black et al 1980). Clearly, other viruses remain to be found.

The novel rotaviruses

With the notable exception of the pioneering work of Horace Hodes (Light & Hodes 1943, 1949), hardly repeated by anyone, perhaps because the authors didn't give enough details of exactly how they carried out their experiments and how they isolated their calves, we have had to rely upon the vets for the identification of new diarrhoea viruses. They have the enormous advantage that they can carry out transmission experiments and kill their patients at an interesting stage of the disease to see what is happening; and of course they can keep their patients gnotobiotic. Many medicals ignorantly suppose that the vets have a lot to learn from them, whereas in reality it is often the other way round. Your Chairman and I would have found rotaviruses in children long before 1973 if we had taken note of what Chuck Mebus was doing in Nebraska in 1969 and 1970! (Mebus et al 1969, 1971.)

Novel rotaviruses have indeed been discovered, mainly in animals (group B and C viruses in pigs, the group D viruses in birds and one or two more groups in sheep and other mammals) (Pedley et al 1986). All the indications are that with the notable exception of the Chinese virus, which *still* has not been reported from outside China, these novel rotaviruses, although very important in animal infections, are rarities in the human, and I would guess represent occasional zoonotic infections, because nobody so far has reported clustering of cases. Antibody surveys confirm that. These infections are not so easy to diagnose by electron microscopy as are infections by group A rotaviruses because group B and C viruses seem to disintegrate rapidly in phosphotungstate; nevertheless, enough people have been using RNA profile analysis as a diagnostic tool (Espejo et al 1980, Rodger et al 1981, Pereira et al 1983) to indicate clearly that, had these infections been common, they would have been detected.

Where could the Chinese rotavirus have come from? The high adult attack rate is what one would expect from a completely new variety of virus, by analogy with, for example, influenza. One would suspect that this virus might have arisen as a genetic reassortment of human and animal group B viruses in pigs or rats, or perhaps a group B infection in birds; this might be a route of entry, through handling of chicken carcasses. Alternatively, point mutations may have occurred within some segment or segments of an animal group B virus rendering it pathogenic for man. Its epidemiology—more in villages than in cities, failure to cross the Chinese border—suggests that a high dose is required to infect human beings; this would be consistent with an animal virus which had only recently adapted to man. When a lot more genomic segments have been sequenced by the molecular biologists we shall probably know the answer. Our own observations, reported here (p 54) indicate that this is also very much the case for group B as well as group C rotavirus infections, at least in England.

Other viruses

Small round viruses

We must therefore look for something other than rotaviruses to explain much human diarrhoea, because I cannot accept the excuse that used to be made before rotaviruses were discovered that 'oh well, it's probably just a feeding problem'. What about small round viruses? Diagnosis of these is difficult because one small round virus looks very much like another in the electron microscope and it is only when one has an outbreak associated with a particular food, as Hazel Appleton and others have shown, that these can be convincingly implicated (Appleton & Pereira 1977, Christopher et al 1978). Nevertheless, they must be circulating in the community to get via sewage into shellfish or other food in the first place, and we still know very little about them.

Interpretation is even more difficult because small round viruses from plants, such as tomato bushy stunt virus in tomatoes, can appear in human faeces

(Tomlinson et al 1982) and may cause confusion. Small round viruses in faeces may even be bacteriophages; small round phages do exist; there are even parvovirus bacteriophages, such as ϕX174. Without reference antisera to agglutinate them, they cannot be distinguished.

Caliciviruses

It now seems likely that the Norwalk viruses are but one serotype of a whole group of caliciviruses. We have been in great difficulty in the investigation of caliciviruses in general as causes of diarrhoea because, with the exception of David Cubitt's limited success in cultivating them in dolphin cell lines (W.D. Cubitt, personal communication 1985), we can't get enough virus antigen to work with. If we had plenty of good really specific sera we could try antibody capture methods to get enough virus. If we had plenty of virus we could make plenty of good specific serum—and until you have a really good detecting method, you can't make monoclonal antibodies because you don't know whether the monoclonal antibodies that you have are specific for the virus or for some other component of faeces. We have been very much in this 'chicken and egg' situation ourselves in trying to raise monoclonal antibodies against the Chinese rotavirus. Fortunately at last we do have, as you heard, a specific ELISA test, and we may now be lucky (see my discussion of group B rotaviruses and of toroviruses, p 54 and p 183).

Parvoviruses

Genuine parvoviruses do perhaps cause diarrhoea (Clarke et al 1972, Appleton & Higgins 1975), although we (Flewett et al 1974a, b) found them as often, in small numbers, in faeces of children who did not have diarrhoea. Parvoviruses, probably adeno-associated viruses (AAVs), were found in huge numbers in some specimens from Scotland that we looked at over ten years ago (Flewett 1977). 10^{13} particles/g faeces were counted in one sample, but we haven't seen them since. Where can they have gone? We couldn't be missing them. Of course, viruses do appear and disappear (human immunodeficiency virus, HIV; swine vesicular disease virus; canine parvovirus; and whatever caused sleeping sickness).

We talked about the small round 'hairy' viruses. They may perhaps belong to the calicivirus group, though their taxonomy is very uncertain. We, like the Toronto group (Middleton et al 1975), who called them mini-rotaviruses, have occasionally seen them in paediatric ward outbreaks of mild diarrhoea.

Astroviruses

Astroviruses have now been well established as animal pathogens by Dr Snodgrass's group and others (Snodgrass & Gray 1977, Kurtz et al 1977).

Undoubtedly they can cause human infections, but how much actual disease they cause still remains uncertain; probably not very much.

Coronaviruses cause diarrhoea in piglets and calves and respiratory infection in humans (Tyrrell et al 1978); can we be missing them in human faeces? They can, I am told, be very difficult to find in calf faeces by direct electron microscopy (G.N. Woode, personal communication 1985). And we have the coronavirus-like particles independently discovered in Bristol and Vellore in 1975, frequently found in faeces of quite healthy people in the tropics (Mathan et al 1975, Caul et al 1975). In England we see them rarely and usually in diarrhoea, for instance in the faeces of someone who has recently returned from India. I do believe that they are viruses—but are they pathogens? One recent study (Sitbon 1985) gave the embarrassing result that they were more frequently found in children who did *not* have diarrhoea than in children who did. One could still argue a case for them as a cause of diarrhoea, but unconvincingly without more evidence.

The Toroviridae

It has been the general rule that when a pathogen has been discovered in several mammalian species, sooner or later it will turn up in man. Coronaviruses, caliciviruses, parvoviruses and leukaemia viruses are all examples of this rule. The discovery of the Berne virus and later the Breda viruses and of the relationship between them therefore made us very excited and, of course, we started looking for similar viruses in human faeces and had some results pointing to the possible existence of similar agents in children (Beards et al 1984). Faeces from one child certainly appeared to contain particles closely related to or identical with Breda virus type 2; enough virus was present to agglutinate rat cells, so we could do haemagglutination inhibition tests. Whether this virus had anything to do with the child's diarrhoea was doubtful, because this sample also contained many rotaviruses. Until very recently the only evidence of relationship that we had was by immuno-electron microscopy. I confess I am very nervous about interpreting results by this method, firstly because it is so easy for a microscopist to see what he wishes to see—though one can get over that by having coded specimens examined independently by two different observers—and also because experience with monoclonal antibodies has shown that similar epitopes may appear in sites which are, on the face of it, quite unrelated to each other. I think one human infection at least was 'genuine' because we were able to go back repeatedly to the original specimen and get the same result. After some difficulties we developed an ELISA test for Breda virus, as I described (p 183). But, so far, the toroviruses don't appear to be important human pathogens.

Vaccines

We have heard about the problems of vaccines from Dr Vesikari and Dr

Kapikian. Previous experience, with other viruses, has always shown better protection after immunization with the homologous virus serotype than with less closely related viruses. We have also heard about rotaviruses in gnotobiotic piglets. In Third World villages, piglets are far from gnotobiotic; they wander in and out of the houses (or shacks) and defaecate at will where the children are crawling on the earthen floor—a great opportunity for the transmission of infection. Do we therefore need to protect children in such countries against animal strains of rotaviruses? Are animal strains ever important human pathogens? We simply do not know; no good investigation of animal-to-children transmission has yet been done.

Future searches

I am asked to go on to 'where do we go from here?' I shall be very surprised if there are not more diarrhoea viruses waiting to be discovered. How could we detect a virus in faeces having the morphology of a featureless blob unless it was cytopathic in tissue culture or unless we had an antiserum which would specifically agglutinate it to clearly defined aggregates? We might look first for epidemics or clustering of cases. In general, for all kinds of virus, virological investigation of sporadic cases has been much less rewarding than the investigation of outbreaks. Then, try animal inoculation. But except for the piglet and the mouse with the rotavirus serotype 3 and, of course, Horace Hodes' original experiments with calves (Light & Hodes 1943, 1949), animal inoculation hasn't contributed a great deal to human rotavirus infection. If it won't 'go' in animals, human volunteers might be worth a try, but most adults unfortunately are likely to be immune already. Two approaches seem to me to be possibly fruitful. The first is to persevere with immuno-electron microscopy using convalescent sera, reacting them with faecal extracts fractionated in different ways. After all, the hepatitis B antigen was discovered by reacting an unknown antibody with an undiscovered virus (Blumberg et al 1965). Of course, Blumberg wasn't a virologist—no virologist would have tried such a thing. If you find a virus but can't purify it in sufficient quantity to raise antisera and make an ELISA test, and if you can't grow it, then clone it, and you can use a dot-blot test for epidemiological purposes. If you can persuade your clone to produce antigen, that could be used either to develop an ELISA test or to make a vaccine.

Another approach is to look for better tissue culture systems. All diarrhoea virus work has been bedevilled by the reluctance of all the diarrhoea viruses to grow in tissue cultures of any kind, and most still won't. Work would advance so much more rapidly if some bright young man or woman could discover how to make cells in tissue culture as susceptible to diarrhoea viruses as are the enterocytes of the small intestine. The same discovery might perhaps be applied to the isolation of human papillomaviruses—there might even be a Nobel Prize in it!

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DISCUSSION

Woode: Are there epidemiological and/or pathophysiological markers that would predict a virus involvement in gastroenteritis? In my experience, in animals, where we have applied certain criteria, such as the pattern of spread between animals, and have searched for long enough (as with Breda virus, which took two years to find), we usually end up by finding a virus. Perhaps our medical colleagues, who do more epidemiological studies and probably more pathophysiological investigations, can say whether there are markers that are reasonably predictive of viral causation, as against bacterial diarrhoea or toxic diarrhoea.

Flewett: If there are abundant leucocytes in the faeces, combined with a strong polymorphonuclear leucocyte response, you probably have a bacterial diarrhoea.

Vesikari: One characteristic of viral infections in general is leucopenia; certainly in rotavirus infections we see a significant reduction in the number of blood leucocytes (Mäki 1981). I don't know how well this applies to other viruses that cause diarrhoea.

Wadell: In Dr Uhnoo's study, the rotavirus-infected children did not show a rise in blood leucocytes but 33% of children infected with enteric adenoviruses did show this (Uhnoo et al 1986).

Flewett: Faecal leucocyte numbers tend to be low in pure virus infections,

and high in bacterial infections. But you very commonly have a double infection, or bacterial invasion potentiated by viral infection, and that can cause confusion.

Vesikari: Another general feature of rotavirus diarrhoea is the relatively low sodium concentration of the stools, as compared to the toxigenic diarrhoea induced by bacteria (Sack et al 1982).

Chiba: In addition, there is no tenesmus in a viral diarrhoea, because the viral invasion occurs only in the small intestine.

Bishop: Dr Woode asked also about the epidemiological characteristics of viral diarrhoea. Most clinicians would say that a child aged less than three years who presents with severe watery diarrhoea during the winter months in temperate climates is probably infected with group A rotavirus. They are not always right, but almost always!

Flewett: I would say that it is probably a virus infection, and in the UK it is probably a rotavirus, but Minnie Mathan looking at children in Vellore might say it's more likely not to be a rotavirus—because you don't find rotavirus in most of your diarrhoeic children, do you?

Mathan: We find fewer rotaviruses. Faecal leucocytes are also found in children with rotavirus diarrhoea in our patient group, even though quantitatively they are more frequent in bacterial diarrhoea. Tenesmus is restricted to children with dysentery.

Flewett: This is extremely interesting, because your ill babies largely come from an area with a high risk of faecal–oral contamination; I suspect that if enterocytes are being destroyed in patches by rotavirus, this must assist a coliform bacterium coming in afterwards. I recall that some early work at Compton showed that if you added any coliform to a rotavirus infection in a gnotobiotic calf, the diarrhoea was much worse.

Woode: We are publishing a study on this (Runnels et al 1986). There appears to be a synergistic effect of mixed infections of rotavirus and enterotoxigenic *E. coli* in calves, resulting in more severe lesions and fatalities. There is the problem that there may be competition between the virus and *E. coli* for the same epithelial cells. We have field observations on invasive salmonellosis following enteric virus infection. As most enteric salmonella infections in calves are confined to the gut, it is interesting that we have observed severe salmonellosis with rotavirus or coronavirus infections.

Hall: The effects of dual infection with enterotoxigenic *E. coli* and rotavirus in calves have been studied by several groups (Gouet et al 1978, Runnels et al 1980, Tzipori et al 1981a,b, Hess et al 1984, Torres-Medina 1984). An important point to note, however, is that these two organisms rarely occur together in field outbreaks. In calves, enterotoxigenic *E. coli* cause disease in the first 48 hours of life and the peak age for rotavirus-induced diarrhoea is 11 days, so an artificial combination has been studied. At Compton we tried to look at combined rotavirus and salmonella infections in germ-free calves. Unrealisti-

cally large inocula are required to set up experimental salmonella infections in conventionally reared calves, and we hoped that rotavirus infection might enable us to produce salmonellosis with a much smaller inoculum of salmonellas. Our attempts to test this hypothesis with germ-free calves failed, because when small numbers of salmonellas were given orally to gnotobiotic calves, the salmonellas grew very rapidly, and there was no problem with the inoculating dose.

One other point: we recently surveyed the causes of enteritis in calves over two winters (Reynolds et al 1986). During the survey 21 calves were purchased in a moribund state and came to necropsy (G.A. Hall, unpublished work). Of the 21, only two had uncomplicated infections, one with rotavirus and the other with coronavirus. All the other calves were infected with more than one enteropathogen. In thirteen of the 21 calves bacteria were adherent to the surface of the large bowel, which may have contributed to the disease process. In some of the cases the adherent bacteria were enteropathogenic *E. coli* rather than enterotoxigenic *E. coli*, but they were not all of the same serological type. Some of the adherent bacteria may have been other bacterial species and not *E. coli*. In my experience, in these conditions it's more likely that one will see surface infections with bacteria, rather than invasive bacterial infections. Nevertheless, on the gut surface they could be contributing to disease by modifying function in the large intestine.

Flewett: Or even in the small intestine?

Hall: Yes; but we have seen them in the large.

Mathan: We have just studied an epidemic of Echovirus type 11, as I mentioned earlier (p 159). Following the echovirus infection there was a shigella infection (*S. flexneri*) as a second wave in the same village. Patients who were infected by the echovirus had a more severe infection with a longer duration in the second wave. This suggests that prior virus infection may increase the possibility of subsequent bacterial infection, in the human.

Snodgrass: I agree with Graham Hall about rotavirus and enterotoxigenic *E. coli* (ETEC) not occurring together naturally, but it has been a model studied in calves, so it may be relevant to Dr Flewett's point, and to human medicine. The consensus seems to be that rotavirus infection enables ETEC to establish infection in circumstances where for reasons of age of animal or titre of inoculum it would not otherwise do so (Gouet et al 1978, Tzipori et al 1981a, Hess et al 1984, Snodgrass et al 1982). So in this experimental situation there is interaction between the two agents.

On the same theme, in our field observations, the commonest pair of organisms was rotavirus and *Cryptosporidium*, found together in 11% of diarrhoeal calves (Snodgrass et al 1986). No clinical significance has been found for their interaction, however (Tzipori et al 1981b).

Bishop: It puzzles me that the most sensitive indication of rotavirus infection in children given the rhesus monkey rotavirus vaccine strain seems to be fever,

rather than symptoms obviously related to the intestinal tract. What is the mechanism underlying the occurrence of fever? Have veterinary workers found fever in experimental infection with avirulent rotavirus strains?

Woode: We always take the body temperatures of our rotavirus-infected calves, but we haven't seen fever (more than 1 °C rise) as a response to infection.

Bridger: In gnotobiotic calves given a virulent strain of bovine rotavirus there was a mean elevation of 0.4 °C (Hall et al 1985). We are now looking at colostrum-deprived calves born by Caesarian operation (still nowhere near the conventional situation) using the same virus. In five calves temperatures were 0.1°C to 1.5°C (mean 0.5°C) above 39.2°C. So we do see fever, but these calves had no serum antibody.

Saif: We have seen that response too in SPF (specific pathogen free) colostrum-deprived calves given bovine rotavirus, but never more than a degree above average normal temperature. Fever usually peaks just before the onset of diarrhoea.

Vesikari: Fever is part of the clinical picture of natural rotavirus infection and diarrhoea in humans. When we see the full-blown typical case, it is often associated with high fever (39–40 °C). This is useful in the clinical distinction of a typical rotavirus case from other cases of acute diarrhoea in childhood (Mäki 1981). So I am not surprised that we see some degree of fever response in association with a rhesus monkey rotavirus vaccine, which also multiplies in the human host. The magnitude of the fever responses following rhesus rotavirus vaccination were clearly dependent on the dose of vaccine virus, and probably on the level of pre-existing rotavirus antibody.

The other point, which was brought up earlier in connection with some of the other diarrhoea viruses, is that we are really looking at diarrhoea as the indicator of all these viruses, so we are starting with diarrhoea, whereas many of the viruses discussed here may cause quite a lot of other symptoms, and not only diarrhoea. Because we start with diarrhoea, we see only what occurs in addition to that. If we were looking for 'fever viruses', for example, we might have a totally different distribution of agents.

Kurtz: Astroviruses, certainly in volunteers, produce fever rather than diarrhoea (see our Fig. 4, p 97). I suspect that the fever, or the degree of fever, is related to the degree of insult to the gut. Mild fever would indicate a mild assault on the small intestine, whereas with a severe rotavirus infection the temperature would be higher. I don't think the mechanism of the fever is known.

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Final general discussion

Criteria for non-group A rotaviruses

Bishop: Earlier in the symposium (p 47) we asked a subgroup of participants to try to reach a consensus on the novel rotaviruses—on how to find them, and what to do when they are found. This group has now come to some agreement, and Malcolm McCrae will present this for discussion.

McCrae: Drs Bridger, Holmes, McNulty and Saif and I have reached a straightforward consensus on one point, and not quite a consensus on another. Concerning whether one is dealing with a virus that is 'non-group A', there is little disagreement that to say that one has a non-group A virus one should be able to show (1) a complete lack of serological cross-reaction in the standard group A detection assays, and (2) some gross perturbation of the genome profile. These two criteria should ideally go together, but may not always. I think this proviso is necessary to reflect the fact that a number of people only do genome profile analyses. Reliance on a single experimental criterion is dangerous; we have already seen examples of isolates appearing that have quite a different genome profile from the standard rotavirus genome profile; but they are clearly group A serologically. The avian viruses of Stewart McNulty are a good example of this. I hope most people will feel comfortable with those two criteria. What I like is that they are independent criteria.

Cubitt: Which assay are you suggesting we use for group A? An ELISA?

McCrae: Yes, because this is the assay that most people are doing; but there are several other assays that will give the same answer.

Bishop: May we say, then, that any serological assay which estimates the rotavirus group A *group* antigen is acceptable.

McCrae: I agree. Then comes the more difficult question of the definition of new groups of non-A viruses beyond those already defined. Dr Bridger and I have always been careful to use two completely independent criteria to assign a virus to a new group, one serologically based and the other based on nucleic acid studies. I take the pragmatic view that in future the main procedure for defining a new group of non-group A viruses has to be a serologically based assay. If a lack of serological cross-reaction can be shown in a two-way cross with the previously isolated groups, A–E, this should be sufficient to say tentatively that one is dealing with a new virus group. Having decided that a new group exists on the basis of the serological assay, I would hope that investigators would be able to show a gross sequence divergence, across all genomic segments, from previously isolated groups of atypical rotaviruses. My

feeling is that up to the present the different groups are almost certainly genetically isolated. In the longer term, if these non-A rotaviruses become medically important, one will have to consider vaccine strategies. The concept of genetic isolation from the point of view of vaccine production is an important one. I personally would be sad to see the sole criterion being serological, although it has to be the major one for pragmatic reasons.

Greenberg: Were you to use that genetic criterion only, the avian group A rotaviruses would not be classified with the other group A viruses. By hybridization analysis they are as different from other group A viruses as the group B rotaviruses are. They do, however, share several cross-reactive antigens.

McCrae: This is why both criteria are necessary. We have the possibility of two independent ways of looking at the virus which, at least at the operational level, do not relate to one another. Scientifically, this seems a good idea.

*Kapikian:*The serological assay is in fact a rather complex test to do. Where are investigators to obtain the necessary reagents for groups A–E?

Bishop: These could be obtained from the laboratories of the participants in this symposium who have experience with novel rotaviruses.

Enteric adenoviruses

Bishop: If we can now turn to the enteric adenoviruses, are there any additional points that should be made?

Chiba: It is obvious that enteric adenoviruses are the second most frequently detectable viruses in association with diarrhoea everywhere, and I feel that they are certainly an interesting subject for molecular epidemiology, just like the respiratory adenoviruses. Actually, in collaboration with Professor K. Fujinaga's group, we have found a new genomic variant of type 40 adenovirus (M. Demura et al, unpublished paper, 34th Annual Meeting of the Society of Japanese Virologists, Fukuoka, October 1986).

Wadell: We still know very little about the distribution of enteric adenoviruses throughout the world. One large study was done by Alistair Kidd in Soweto in 616 children. He found that 13.8% shed rotaviruses and 6.5% shed enteric adenoviruses, during a seven-month period. It could be totally different in other areas, but that is something which has to be studied. Then we need to know if the enteric adenoviruses induce cross-protecting immunity. They cross-neutralize to a large extent. When this information is available, one could start to discuss means of prophylaxis. There are already three effective vaccines against adenoviruses being used in the USA on 15 million military recruits (serotypes 4, 7 and 21), but they have not been evaluated in children. In the industrialized world there seems to be no need for a vaccine against diarrhoea caused by enteric adenoviruses, unless they are shown to be of importance in gluten intolerance, which is possible and has still to be studied.

Small round viruses

Bishop: We had a long discussion on the small viruses and reached a reasonable consensus that they are beginning to be categorized, but that further advances require their culture and biochemical study.

Snodgrass: The human parvovirus studies left me in some confusion. The work by Dr Appleton seemed to indicate some involvement of parvoviruses in gastroenteritis, but Dr Kapikian disagreed with these observations. Is there any agreed position on that?

Appleton: The situation is that there is a lot of disagreement about these viruses at present, and we do not know what their role is in gastroenteritis or how important they are. Serological tests are difficult. We can show seroresponses to some of the human parvovirus agents and have identified at least three different serological groups. IgM antibody can be seen on virus particles mixed with convalescent serum, but you don't get the striking antibody responses obtained to some other enteric viruses. As far as some of the shellfish-associated outbreaks are concerned, we see these small viruses among many other kinds of viruses in the patients, but we have also managed to see small numbers of similar-looking viruses in incriminated shellfish samples themselves. I have no idea what that means.

Flewett: The parvovirus particles that you have found in faeces are extremely difficult to interpret. If you find them appearing with diarrhoea and disappearing when the diarrhoea ceases in a number of patients in an outbreak, that is fair presumptive evidence that they have something to do with it, but there are parvovirus-shaped bacteriophages (φX174, for example), and unless you have an extremely clean preparation, which you won't get from faeces, you will not see the dimers on the corners of the particle which enable you to recognize it as φX174. Moreover, roughly one person in 20 in the UK population has serum antibodies to φX174 which will agglutinate the phage, and simply finding agglutinating antibodies, unless you can show a change in titre with disease, doesn't prove an association. If you can show a correlation with the presence of parvoviruses in the faeces—if they appear with the diarrhoea and disappear when it goes and if they do that in a significant proportion of patients in a group—that would be a criterion suggesting that parvoviruses are important.

Appleton: All the parvoviruses we have reported have been examined after density gradient purification, when we would expect to differentiate φX174.

Human coronaviruses

Bishop: One area we have not discussed sufficiently is the fringed viruses. We considered Berne and Breda virus at length, of course, but there are other fringed viruses, in particular the coronaviruses, which are an important cause of disease in cattle. We have not dealt with their potential for disease in humans. Owen Caul probably has more experience than anyone else here of enteric coronavirus infection in man.

Caul: In fact, much higher incidences of coronavirus or coronavirus-like particles have been reported in other parts of the world than have been reported in the UK; however, I do want to raise the topic of necrotizing enterocolitis in babies. There are now two independent reports of human enteric coronaviruses associated with colitis in neonates, one from France (Chany et al 1982) and Dr Resta's report from Dallas (Resta et al 1985). In the French work, coronavirus-like particles were detected in the intestinal lumen by negative-staining techniques, as well as in mucosal cells of the small intestine by thin sectioning, and the virus was grown in cell culture. It now seems agreed by most workers that the virus propagated by the French group was a bovine coronavirus. At that time it was reasonably easy to identify the particles as a bovine coronavirus by the double fringe of projections, because no other coronavirus has a double fringe that I am aware of.

In the Dallas outbreak a coronavirus was propagated in intestinal organ culture. It failed to replicate in any other cell culture. A 50K protein has been identified in purified coronavirus preparations which appears to be unique to coronaviruses. Western blotting, ELISAs and other serological tests were done with paired sera from the affected babies and seroconversions were demonstrated against the homologous isolate.

Kapikian: The data of Dr Resta and her colleagues on human coronaviruses are very convincing, and the identification of the agent of the Dallas outbreak of necrotizing enterocolitis as a coronavirus seems quite firm. No further information has appeared since the first report (Resta et al 1985).

Caul: Did the Dallas virus, which you have discussed with Dr Resta, have a double fringe of projections?

Kapikian: I don't think so.

Woode: It seems fairly convincing that there is a coronavirus in humans that is serologically and morphologically similar to the bovine one, which is reminiscent of the relationship between human and bovine respiratory syncytial viruses.

Caul: The Dallas group's human enteric coronavirus has no antigenic relationship to any of the known coronaviruses that they tested, which is an interesting observation.

Horzinek: I have asked Sylvia Resta for sera to compare it with the 777 virus isolate of Maurice Pensaert (Ghent, Belgium), which is another non-cultivable enteric virus in pigs. We have identified the protein profile of the 777 virus, and it is a coronavirus, but unrelated to any known antigenic clusters. The human isolate did not react in any test with the porcine strain (H.F. Egbering & M.C. Horzinek, unpublished results).

Kapikian: The work published by Dr Gerna and co-workers on human enteric coronaviruses also appears to be quite convincing. I examined an electron microscope grid from their study with Dr Passarani, one of the co-authors, when she was a guest researcher in our lab., and the particles appeared to be coronavirus-like (Gerna et al 1985).

Woode: The human coronavirus (OC43) cross-reacts serologically with the

bovine enteric coronavirus. That creates the problem that one might be studying convalescent sera to a respiratory OC43 infection, which is antigenically related to the bovine coronavirus.

Kapikian: Gerna et al (1985) associated their coronavirus with acute gastroenteritis in infants and young children and showed that this enteric coronavirus was related antigenically to respiratory coronavirus OC43.

Caul: The Dallas group have not shown any cross-reaction with their human enteric coronavirus to the bovine coronavirus or to OC43, so it would appear that their isolate is not a laboratory contaminant.

*Bishop:*There are also coronavirus-like particles, that Professor Mathan and others have identified in abundance in faeces.

Mathan: These particles are frequent in our population in all age groups, with increasing prevalence with increasing age. The highest prevalence is found in epidemics of tropical sprue in adults. During acute diarrhoeal illness, the prevalence actually falls, compared to controls of the same age group. We have seen serological responses to CVLP using immuno-electron microscopy but we have not tried ELISA. There was antigenic variation among these particles, both with patients' serum, and with hyperimmune sera produced against CVLP in rabbits.

Caul: Coronavirus-like particles in many preparations show a considerable degree of pleomorphism. This worried us initially. During the course of our studies we have looked at nasopharyngeal aspirates from children with upper and lower respiratory tract infections, and detected parainfluenza viruses and respiratory syncytial virus (RSV), which were subsequently isolated in cell culture. In the clinical specimens, these enveloped RNA viruses can be extremely pleomorphic with sizes up to about 1 μm with RSV. So the pleomorphism of the coronavirus-like particles worries us less now; any enveloped virus budding from the endoplasmic reticulum may well be pleomorphic.

Horzinek: At Cornell Veterinary School, Cheryl Stoddart has found particles in a cat colony which are virtually indistinguishable from Dr Mathan's. These particles apparently could be grown in MA104 cells for two or three passages. We are now labelling these structures *in vitro* and using radioimmune precipitations to determine their protein profile.

Mathan: In small intestinal biopsies from adults with tropical sprue we have shown morphologically similar vesicles to those shown with coronaviruses.

Caul: We have done purification work on coronavirus-like particles. In the purified preparations we see sheets of particles, the majority appearing completely collapsed. Within that population there are good solid particles—more respectable coronaviruses, perhaps! In thin section work with purified coronaviruses and after Nonidet treatment followed by negative staining, we have evidence for an internal component in about one in 10^5 particles. This internal component is about 8 nm in diameter, which corresponds well with the nucleocapsid-like structures that we described in organ cultures infected with the

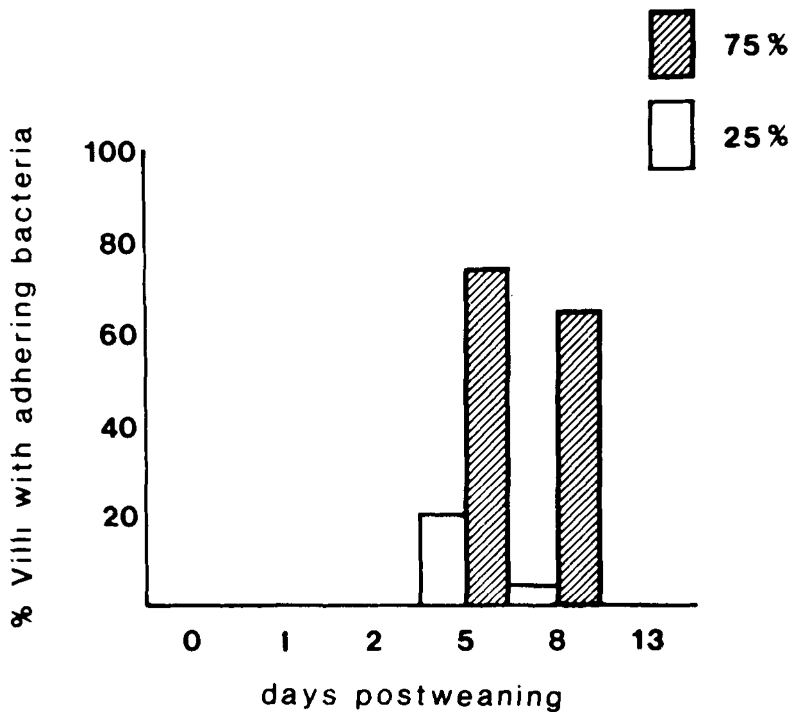

FIG. 1 (*Bourne*). Adherence of *Escherichia coli* to the small intestine in the postweaned pig after stimulation of crypt cell hyperplasia by dietary change. Each histogram represents the mean of five pigs. Each pig was examined by scanning electron microscopy at two sites, 25% and 75% along the small intestine. Ten villi at each site were examined for adherent bacteria.

human enteric coronavirus; it is also compatible with the internal component of coronaviruses.

Comparative pathology of diarrhoeal infections

Bishop: We had considerable discussion of Graham Hall's paper on the comparative pathology of novel viral enteric diseases. He indicated an interest in physiological studies designed to try to elucidate underlying mechanisms of disease.

Mathan: Dr Hall mentioned the hypothesis that gut hormones play a part in crypt cell hyperplasia. There is evidence for this. It has been reported that in patients with tropical sprue and small intestinal lesions there is an increase in enteroglucagon in the serum. This is a trophic hormone for crypt cell hyperplasia. Also, our work on colonic mucosal changes in tropical sprue has shown a marked increase in the number of endocrine cells in the colon, when there is

severe damage to the small intestine. In acute gastroenteritis, also, the numbers of these endocrine cells in the colon are increased.

Bourne: A small point in relation to gut pathology: in the postweaned pig we stimulate crypt cell hyperplasia with dietary change. The hyperplasia starts on Day 2 after weaning. *E. coli* adherence occurs on Day 4 or 5, and the organisms persist for the next four to five days, after which they disappear (Fig. 1). This correlates with the appearance of immature enterocytes on the villus. Also, these same enterocytes have a greatly increased susceptibility to the enterotoxin. Similar mechanisms would be expected to occur in viral infection, and in this way viral infections could have an influence on secondary bacterial growth.

Hall: Is the implication that the immature gut epithelial cell has better receptors for enterotoxigenic *E. coli* adherence than the mature cell?

Bourne: We have no evidence for that in the pig, but there is evidence of this in the rabbit (Cheney & Boedeker 1984).

Hall: Your study might relate to what David Snodgrass said earlier about the ability to set up dual infections with enterotoxigenic *E. coli* and rotavirus in older calves (p 247). It looks as though it is easier to get your bacteria to stick to immature cells.

Bourne: There has to be some insult which leads to crypt hyperplasia, although a number of factors are likely to be involved in *E. coli* growth—the removal of milk antibody, the presence of undigested food in the lumen, and so on.

Vaccination against rotavirus diarrhoea

Bishop: Turning to the final topic of vaccination against group A rotaviruses, Dr Mathan has some information relating to problems that may restrict their use in a developing country.

Mathan: The experience with live oral polio vaccine in India suggests that there are likely to be problems with other live oral vaccines. This is probably related to competition between viruses, since we can isolate enteroviruses from about 40–50% of faecal samples from children. Furthermore, one wonders whether the high antibody titres to rotavirus in our population would interfere with the take of the vaccine strain.

Since we have some evidence to suggest that a prior virus 'infection' may enhance susceptibility to bacterial enteric pathogens, and bacteria account for 60–70% of pathogens isolated from children with diarrhoea, the effect of 'infection' by the vaccine strain will have to be studied carefully. The frequency of rotavirus as a causal agent of diarrhoea is different in different parts of India and the overall maximum contribution may be only around 20%. The question is whether a rotavirus vaccine would significantly reduce the incidence of diarrhoea in children in India.

Holmes: Is there any information from the trials of the RIT (bovine) rotavir-

us vaccines on interference between rotavirus and oral poliovirus vaccine?

Vesikari: A study has been done in Yugoslavia and another in The Gambia. In Yugoslavia there was one-way interference; the poliovirus vaccine interfered with the rotavirus vaccination, but not vice versa (I. Vodopija, personal communication).

Kapikian: If the 'Jennerian' approach is not effective in inducing protection against heterotypic rotaviruses (Kapikian et al 1986a), another approach is the use of rotavirus reassortants produced by co-infection of cell cultures under selective pressure of antibody. Thus, rhesus rotavirus could be used as a donor of attenuating genes that can be transferred, in co-infection of cell cultures with a human rotavirus serotype, to form a reassortant that possesses only the major neutralization protein (VP7) of a human rotavirus serotype donor and the remaining genes from the rhesus rotavirus donor. Dr Midthun and colleagues in our lab. have now prepared such single-gene substitution reassortants for each of the four human rotavirus serotypes with the rhesus and/or bovine (UK) strains as the donor of the remaining 10 genes (Midthun et al 1985, 1986).

We have recently begun phase I studies with two of these reassortants. The first is a D (human serotype 1) × rhesus rotavirus strain which has 10 genes from rhesus rotavirus and a single gene from the human rotavirus which encodes VP7, the major neutralization protein. Thus this reassortant is classified as human rotavirus type 1 according to its VP7. Studies with the D × rhesus rotavirus reassortant were recently carried out at Johns Hopkins University in collaboration with Drs Clements, Sears and Black, in adult volunteers. No associated illness was observed. Following this, studies in older children, 3–10 years of age, have just been carried out with this reassortant by Dr Wright and colleagues at Vanderbilt University. Since no associated reactions were observed, progressively younger children will be tested in phase I trials with the orally administered vaccine candidate.

Another reassortant under study is a DS-1 (human serotype 2) × rhesus rotavirus strain which has 10 genes from rhesus rotavirus and a single gene from the human rotavirus which encodes VP7. The reassortant is classified as human rotavirus type 2 according to its VP7. Studies with this reassortant have recently been carried out at Johns Hopkins University with the collaborators already mentioned. No associated illness was observed and therefore studies in older children are planned.

Perhaps when all phase I trials are completed successfully we can initiate phase II trials with each of the reassortants and possibly with a trivalent vaccine containing the serotype 1 reassortant, the serotype 2 reassortant and rhesus rotavirus, which is a serotype 3 rotavirus. Such a combined vaccine might yield the desired broad coverage against these three serotypes at least, if the 'Jennerian' approach is not successful (Kapikian et al 1986a). As was said earlier, we are not expecting to prevent infection or mild illness with a rotavirus vaccine, because, as Dr Chiba showed, individuals with pre-existing antibody under

natural conditions to a specific rotavirus serotype can be reinfected and can develop illness with the same serotype if their serum antibody titre is not high enough. We cannot therefore expect an 'all-or-none effect' on the prevention of illness by rotavirus antibody, as characteristically described following live measles virus vaccination. We hope however to prevent clinically significant diarrhoea caused by prevalent serotypes with an antigenic vaccine.

Significant reactions to rhesus rotavirus vaccine (RRV-1) were first recognized conclusively by Dr Vesikari and co-workers in Finnish children and later in other studies (Vesikari et al 1986, Losonsky et al 1986, Anderson et al 1986, Kapikian et al 1986a,b, Wright et al 1986, Rennels et al 1986). In collaborative studies, Ms Perez-Schael, Dr Flores and colleagues evaluated the reactogenicity and antigenicity of lowered doses of the RRV vaccine in 4–10-month-old children in Venezuela and found it to be non-reactogenic when compared to a control, and quite antigenic at the 10^4 PFU/ml dose (Perez-Schael et al 1986). The reason for the absence of significant reactions in this study and in the initial phases of USA studies was unclear, but when levels of pre-existing serum neutralizing antibodies to RRV were compared among 6–8-month-old children in Finland, and 4–12-month-old children in Venezuela and the USA, the Finnish children were found to have significantly lower antibody levels (Kapikian et al 1986b). For example, 37% of them lacked neutralizing antibody to RRV, whereas 2% of the Venezuelan children and none of the USA children lacked such antibody. This suggested that the antibody may have modified the clinical response to the vaccine in Venezuela and possibly in early phases of certain USA studies. This prompted us to consider vaccination of infants of a lower age group where the potential protective effect of maternal antibody acquired transplacentally might modify the reactogenicity of the vaccine and, in addition, enable evaluation of the vaccine in an age group where the need for vaccination was greatest in the developing countries. Also, although less attenuated than the RIT 4237 vaccine, the RRV vaccine might break through the maternal antibody barrier and evoke satisfactory antibody responses in infants in the first few months of life. In 1–4-month-old children, the vaccine did not cause significant reactions, and it induced antibody in approximately 75% of them (Perez-Schael et al 1986). Field trials of a 10^4 PFU/ml dose of RRV are currently under way in infants less than five months of age.

Snodgrass: I wonder whether our studies on passive rotavirus immunization in cattle have any relevance to the human situation? Although we and others have shown that passive protection is serotype specific in the experimental situation, that may not be so in real life, where one is vaccinating dams with a wide range of previous exposure to rotavirus. These animals seem to respond after single-serotype vaccination by producing neutralizing antibodies against all the serotypes to which they have been exposed. This may overcome the need for multivalent vaccines; the single-serotype vaccine may protect against all the rotaviruses in an environment. I appreciate that there are problems with the

vaccination of pregnant women, but perhaps such vaccination could be postponed until after birth. In the Third World the major incidence of rotavirus diarrhoea is not in the immediate neonatal period, and breast-feeding continues for a considerable time after birth. The possibility of passive immunization of post-partum mothers should not be ignored, in case the current major research input into active immunization is not as fruitful as is hoped.

Kapikian: Such an approach may be effective for a limited period of time when the antibody levels are sufficiently high to afford protection. However, as you say, the neonatal period is a time of lower risk for rotavirus diarrhoea in humans and it is during this very period that the maternal antibodies acquired passively would be highest. But protection against rotavirus diarrhoea is needed for at least the first two years of life and active immunization of the child would be essential to achieve this goal. Of course, the approach you suggest might elevate the maternal antibodies to levels that might increase the duration of such protection but this would be important for a limited time only and might be difficult to implement as a programme, unless it were a supplement to the active rotavirus immunization of infants.

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Chairman's closing remarks

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1987 Novel diarrhoea viruses. Wiley, Chichester (Ciba Foundation Symposium 128) p 261–263

The symposium has ranged widely over viruses that are or may be implicated in the aetiology of acute diarrhoea in animals and man. All have in common the fact that they were seen initially by electron microscopy of diarrhoeal faeces. There are many intriguing questions still to be answered about a number of these viruses.

Novel rotaviruses can be defined as having the typical morphology of rotaviruses but differing from group A rotaviruses in at least two respects: they show no serological cross-reactions in tests that detect the group antigen of group A rotaviruses, and they show a gross alteration in the arrangement of the eleven bands of their genome profiles when compared with group A rotaviruses. Novel rotaviruses are at present classified as groups B–E. If new groups are proposed they should be first shown to have no serological relationship to the existing five groups in two-way cross neutralization assays using sera specific for each of these groups. Novel rotaviruses infect man and other animals, but appear to be less virulent than group A rotaviruses, since outbreaks associated with their excretion are rare. The widespread epidemics due to group B rotaviruses in adults (and children) in China may represent zoonoses, perhaps caused by viruses from pigs or rats. There is a need for further studies, particularly serological surveys of the extent of present-day infection. These surveys should include attempts to detect infection with novel rotaviruses before 1970. Cross-protection studies between groups A–E should be continued.

Adenovirus infection by serotypes 40 and 41 is relatively common and has been identified in 7–10% of young children admitted to hospital in developed countries. When specific assays are developed, such as ELISA using monoclonal antibodies, it should be possible to study the epidemiology of enteric adenovirus infection in children in developing countries. The association of these adenovirus serotypes with chronic infection, and with gluten intolerance, also requires further investigation. Other (cultivable) adenoviruses may be excreted by children with acute diarrhoea. It is still necessary to demonstrate

aetiological involvement in disease. Shedding should be temporally related to disease in longitudinal studies, and should be shown to be accompanied by seroconversion.

A variety of small viruses (20–40 nm) are often shed in faeces during acute diarrhoea. These can be classified in a preliminary fashion according to their morphology in negatively stained faecal preparations examined by electron microscopy. Many may lack the characteristic morphology as a result of degenerative changes that occur during the storage of specimens, or because their surface is obscured by antibody. Astroviruses are recognized by their star appearance, which is usually evident on 5–10% of particles. Caliciviruses are also recognizable. Some particles lacking calicivirus morphology may eventually be classified as such. Norwalk virus can be identified by radioimmunoassay, although specific antisera are in short supply. This limits the number of studies that can be done at present. Accurate classification of many small viruses will not be possible until sufficient antigen is available for genome and protein profiles to be characterized.

Many small viruses are associated with food-borne epidemics of diarrhoea in adults, particularly involving the ingestion of shellfish contaminated by sewage. Other small viruses may be spread predominantly from person to person by the faecal–oral or respiratory routes. There is still no convincing evidence that parvoviruses cause diarrhoea in humans. Their frequent appearance in diarrhoeal stools in 'mixed infections' may result from the increased production of particles in crypt cells stimulated to replicate after damage due to another agent.

Large fringed particles are not uncommon in faeces. The only group characterized so far is the Berne–Breda group of viruses. These are cultivable and have been proposed as a new family, to be called Toroviridae. These viruses have been identified mainly in animals and appear to be widespread in horses and cattle, although they are seldom associated with disease. Occasional human infections have been detected and may be rare zoonoses. Coronaviruses, unrelated to respiratory tract infections, have been described and may be implicated in acute diarrhoea in adults in crowded living conditions and in newborn babies who develop necrotizing enterocolitis. Fringed particles that appear different from both Berne–Breda viruses and coronaviruses are common in some settings, including Indian villages and among non-urban Australian Aboriginals. Although present in faeces in large numbers, they may not be implicated in disease. Further proof of their infectious nature is required. When large fringed particles are seen in faeces they should be carefully compared with the published micrographs of Berne–Breda viruses and coronaviruses before any conclusions about their identity are drawn.

Histopathological changes produced in experimental animals by all these categories of novel diarrhoea viruses have been studied. Most of them infect the mature epithelial cell of the small intestine. Coronaviruses, and the parvo-

viruses responsible for enteritis in dogs, infect crypt cells. The cell infected by many 'small viruses' has not yet been identified, although histological changes can be detected in duodenal mucosa. Further studies of experimental infections in animals may yield information about physiological changes and reparative processes. Such studies are rare, as yet, and could prove fruitful. Further animal studies are also required to elucidate mechanisms of immunity and the extent of cross-protection. Studies on the duration of protection require longitudinal follow-up after experimental or natural infection.

Trials of candidate group A rotavirus vaccines are in progress. Prophylaxis in farm animals will focus on parenteral vaccines aimed at producing high levels of maternal immunity which will passively protect young animals from birth. Human vaccination has focused on the ingestion of live rotavirus strains that include heterologous animal strains (bovine and simian). A balance between attenuation and efficacy may be hard to achieve. The existence of cross-protection between different serotypes of animal and human rotaviruses is still controversial and will not be fully understood until the components of the immune response to different neutralizing proteins on rotaviruses can be defined.

A major problem hampering progress in the further study of all novel diarrhoea viruses is our inability to adapt *any* representative strains to cell culture. Repeated attempts based on cells and techniques suited to the cultivation of group A rotavirus strains have not succeeded. It is necessary to persevere, duplicating as far as is possible the natural conditions favouring growth of these viruses in the human and animal intestine. The starting point for successful culture may be basic studies of cell replenishment and maturation *in vivo*.

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