DIAGNOSIS OF ROTAVIRUS INFECTION BY CELL CULTURE

A. S. BRYDEN, HEATHER A. DAVIES*, M. E. THOULESS AND T. H. FLEWETT

Regional Virus Laboratory, East Birmingham Hospital, Birmingham, B9 5ST

PLATE VIII

ROTAVIRUS infection in man and animals has been diagnosed mainly by electronmicroscopy (EM) of faeces after differential centrifugation. However, Woode *et al.* (1974) were able to diagnose infection in calves by inoculating faeces on to calf-kidney (CK) monolayer cultures followed by immunofluorescent staining (FA) to demonstrate growth of the virus. Many workers (personal communications), including ourselves, have been unsuccessful in applying this technique to the diagnosis of human rotavirus infection. Wyatt *et al.* (1974, 1976) succeeded in propagating the human virus in organ cultures of human embryonic small bowel, and could then passage the virus 14 times on human embryo-kidney (HEK) monolayer cultures, but they were unable to repeat this result from the original sample.

Recently, Banatvala *et al.* (1975), also using FA, found that the human virus could be made to infect monolayer cultures of a pig-kidney cell line by centrifuging the specimen on to the cells. We now describe the application of this method to detect rotavirus in faeces, with cell cultures of HEK cells, CK cells and a line of monkey cells (LLC-MK2); the three cell cultures were compared for sensitivity in isolating virus.

MATERIALS AND METHODS

Specimens. Samples of faeces were collected from children with acute diarrhoea. Approximately 20% suspensions were made in phosphate-buffered saline (PBS), pH 7.3, and centrifuged at 3000 r.p.m. for 15 min. The supernates were examined, as described below, immediately, or after storage either at 4°C for not more than 48 h or at -35°C for longer periods.

Electronmicroscopy. Supernates from faecal specimens were concentrated by high-speed centrifugation and, marked by day-book numbers only, were passed to the electronmicroscopist (H. A. D.) for examination by a method previously described (Flewett, Bryden and Davies, 1974).

Virus-isolation technique. Monolayer cultures of LLC-MK2 cells (obtained from Flow Laboratories Ltd), primary or secondary HEK cells, and primary CK cells were grown on 13-mm round coverslips in flat-bottomed screw-capped blood-collection tubes (Pathlab Supplies Ltd). Each tube was seeded with either $3-5 \times 10^5$ cells, for primary HEK and CK cultures, or $1-2 \times 10^5$ cells, for LLC-MK2 and secondary HEK cultures, in 1 ml of Eagle's MEM containing 10% (v/v) of foetal calf serum (FCS), 1% of non-essential amino acids (Flow Laboratories Ltd), gentamicin (40 µg per ml) and Fungizone (10 µg per ml). When confluent monolayers had formed they were re-fed with 1 ml of Eagle's MEM, containing 2% (v/v) of FCS and buffered with HEPES (20 mM) instead of sodium bicarbonate (16 mM), and infected with 0.1-ml amounts of faecal supernates. The tubes were centrifuged at 1200 g for 70-75 min. and then incubated at 37°C for 18 h. The coverslips were removed with a barbed hypodermic needle and forceps, washed in PBS, fixed in acetone for 1-2 min. at room temperature and allowed to dry. The infected monolayers were then treated with a

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* Present address: Division of Electron Microscopy, Clinical Research Centre, Watford Road, Harrow HA1 3UJ.

Requests for reprints should be sent to T. H. F.

1 in 10 dilution of convalescent serum from a gnotobiotic calf infected with calf rotavirus (Woode *et al.*, 1976) for 45 min. at 37°C, washed in PBS, stained with fluorescein-conjugated rabbit antiovine serum, washed again, counterstained with 1 in 150 000 Evans Blue and mounted in buffered glycerol, pH 8.4. They were examined in a microscope fitted with a vertical illuminator and designed for blue-light immunofluorescence. Fluorescent cells were easily detected with the low-power objective ($\times 10$), but doubtful objects were inspected with a $\times 20$ objective. To avoid bias on the part of the observer (A. S. B. or T. H. F.) the coverslips were coded, and only afterwards were the FA and EM findings compared.

For examining large numbers of specimens and for serum-neutralisation tests, Microtiter plates (Flow Laboratories Ltd), with flat-bottomed wells 6.5 mm in diameter, were used. The wells were seeded with 5×10^4 LLC-MK2 cells in 0.2 ml of growth medium. When the cells were confluent they were re-fed with maintenance medium, and 0.05 ml of faecal supernate was added to each well; larger amounts were sometimes toxic. The plates were sealed with Sellotape, centrifuged at 1200 *g* for 1 h in an MSE Magnum centrifuge on swing-out platforms and then incubated for 18 h at 37°C. The medium was then discarded and the plates were rinsed three times with PBS, immersed in methanol at -70°C for 1-2 min., stained by the FA method described above, rinsed in water and allowed to dry.

The cell sheets were observed through the floor of the wells of the inverted plates with the vertical illuminator. Although the thicker areas of the plastic plate appeared pale green under the fluorescence microscope, when the stained monolayers were viewed through the thin floors the observational conditions were similar to those of the glass coverslips.

Neutralisation tests. These were done with human rotavirus, prepared by filtering infected faeces through a 0.45- μ gradocol membrane, and with a tissue-culture-adapted strain of bovine rotavirus, by the Microtiter-plate method described above. Filtrate or tissue-culture supernatant fluid, respectively, were diluted so that 0.025 ml would give 50-100 fluorescent cells in each well. The viral suspensions were mixed with equal volumes of doubling dilutions of either convalescent gnotobiotic calf serum or two sera from convalescent children, and the mixtures were incubated at 37°C for 90 min. and then inoculated in 0.05-ml amounts on to LLC-MK2 cells in wells. The antibody titre of each serum was expressed as the highest dilution that gave a 50% reduction in the number of fluorescent cells.

RESULTS

Of 84 specimens of faeces examined for rotavirus, 31 were positive by both FA and EM and four were positive only by EM; no specimen was positive by FA only (table). Fluorescence was always cytoplasmic (fig. 1), without nuclear involvement. Occasionally the fluorescence was concentrated in granules, which may have been inclusion bodies; in other instances, there were also what appeared to be fluorescent fibrils in the cytoplasm.

The four cell cultures were comparable in their sensitivity for detection of virus. However, because the same specimens could not always be examined in all four types of cell, statistical comparisons could not be made. Moreover, some specimens were already known to be positive in LLC-MK2 cultures before testing in primary and secondary HEK cells, and, although the coverslips were coded, observer bias could not be entirely eliminated.

The highest proportion of infected cells was obtained with LLC-MK2 and secondary HEK cultures, more than a quarter of the cells fluorescing in some preparations; primary HEK and primary CK cultures showed fewer infected cells. When one strongly positive sample was titrated in LLC-MK2 and secondary HEK cells by the micromethod, the titre was found to be 10 times higher in LLC-MK2 cells. The effect of any differences among the virus strains tested or the various kidneys used for cell-culture preparation is not yet known and will require further investigation. Infected multinucleate cells were sometimes seen in the LLC-MK2 (fig. 2) and secondary HEK cultures, but this was not a cytopathic effect of the virus since similar non-fluorescing multinucleate cells were also present in uninfected control cultures. Faecal samples rich in virus particles often produced fluorescence in a few cells, even without the aid of centrifugation, but when the specimen was centrifuged on to the cultures the number of infected cells increased more than a thousand-fold.

TABLE

Sensitivity of cell-culture—fluorescent-antibody technique for detecting rotavirus in faeces, compared with electronmicroscopy

Cell culture	Number of faecal specimens examined	Number positive by fluorescent-antibody staining (and number of faeces positive by direct electronmicroscopy)
LLC-MK2 cell line	72	26* (30*)
Primary human embryo kidney	16	10 (11)
Secondary human embryo kidney	9	4 (5)
Primary calf kidney	12	5 (5)
Total	84	31* (35*)

* One specimen each was at first negative by FA or EM, but positive on re-examination.

Four of the five specimens positive by EM but negative by FA contained fewer than 10^6 particles per ml; one proved positive on retesting in LLC-MK2 cells, and another contained mostly distorted and damaged virus particles that might not have been viable. The fifth specimen contained 10^9 – 10^{10} particles per ml, but despite several attempts cell cultures could not be infected; the particles, however, lacked their outer capsid layer and this may have made them less infectious. Tissue-culture-adapted calf virus without the outer capsid layer has been found to be a thousand times less infectious than complete virus (Bridger and Woode, 1976; M. M. Elias, 1976, *personal communication*).

Twenty-four specimens were also tested by the micromethod. One, positive by EM, was toxic for the cells even in a dose of only 0.025 ml; it was also very toxic for coverslip cultures, but enough cells survived for a positive result to be read by this method. Eleven of the other 23 specimens were positive by EM and 10 of these were positive by FA on both coverslips and Microtiter plates. There were no obvious differences between the two FA systems, either in the proportion of cells fluorescing or in the quality of fluorescence. The macro- and micromethods were equally sensitive when three specimens were titrated for virus activity, in parallel, by both methods; any differences were within acceptable limits of experimental error.

In the microneutralisation tests the serum from the infected calf and the two human convalescent sera neutralised only the homologous virus; the respective titres were 160 and, for both human sera, 80.

DISCUSSION

Our results confirm the findings of Banatvala *et al.* (1975) that FA staining of tissue-culture cells infected by centrifugation, although not quite as sensitive as EM, is very useful for routine diagnosis of rotavirus infection, especially when many specimens have to be examined or when an electron microscope is not available. Possibly, concentration of the specimens before inoculation will further increase the sensitivity of the test, as it does for EM (Flewett, Bryden and Davies, 1974). An advantage of EM is that it can often detect other viruses, but the tissue-culture technique should also be able to do this, provided the cells are susceptible, either by showing cytopathic effects or by FA staining. The LLC-MK2 cell line is very easy to grow and to handle and appears to be the cell of choice for rotaviruses, although we have not yet been able to compare it with the IB RS2 cells used by Banatvala *et al.* (1975).

The micromethod should prove particularly useful for large numbers of specimens, e.g., in surveys, or when many replicate cultures are required. Toxicity of the specimen was the greatest problem, but this did not occur often and could be dealt with by further dilution of the specimen. Care is needed to avoid cross-contamination of wells by droplets or aerosol. We used a Finnpiptette (Jencons (Scientific) Ltd, Hemel Hempstead, England), allowing the inoculum to run down the side of the well.

During the course of these studies, a piece of small intestine from a fatal case of gastroenteritis in a 1-year-old boy was submitted for investigation. It was emulsified and examined for rotavirus in the same ways as faeces; positive results were obtained by EM but not by FA. The virus particles, however, looked as if they were coated with immunoglobulin. This was apparently confirmed when, after treating the emulsified specimen with an equal volume of glycine-HCl buffer, pH 2.8, for 1 h at room temperature, it gave positive FA staining in LLC-MK2 cells. Naturally occurring antibody in faeces that may prevent virus from infecting cell cultures must, therefore, be regarded as a potential cause of false-negative results when testing by the FA technique. The close correlation that we get between EM and FA staining in examinations of faeces indicates that this is not a common problem, at least with faeces.

Our results with the neutralisation test suggest that it is species specific, unlike the complement-fixation and immunofluorescence tests which are group specific (Woode *et al.*, 1976). However, neutralising antibodies in adult sera and in sera from hyperimmunised animals may be more group reactive than those in sera from calves and children (unpublished observations). Because the immunofluorescence test is group specific for all known rotaviruses (Woode *et al.* 1976), any rotavirus antiserum can be used for the cell culture-FA technique. We used an antiserum to bovine rotavirus, prepared in a gnotobiotic calf, in the present study as it was considered to be the one least likely to give false-positive reactions, but pooled, random bovine or rabbit sera also gave good antibody titres in the tests.

SUMMARY

Rotaviruses were detected by electronmicroscopy in 35 of 84 specimens of faeces from infants with diarrhoea, and in 31 by fluorescent staining of tissue cultures infected with the help of centrifugation. LLC-MK2 cells were found to be the most sensitive, although primary and secondary human-embryo-kidney and primary calf-kidney cells could also be used.

A micromodification of the tissue-culture method provides a relatively simple technique for the diagnosis of rotavirus infection, for the titration of virus infectivity and for estimating neutralising antibodies in serum.

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