JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1984, p. 347–350
0095-1137/84/090347-04$02.00/0

Vol. 20, No. 3

Radioimmunofocus Assay for Detection and Quantitation of Human Rotavirus

SHIQING LIU,[1] CHRIS BIRCH,[2] ANTHONY COULEPIS,[2] AND IAN GUST[2]*

Institute of Medical Biology, Chinese Academy of Medical Sciences, Kunming, People's Republic of China,[1] and Virology Department, Fairfield Hospital for Communicable Diseases, Fairfield, Victoria 3078, Australia[2]

Received 16 April 1984/Accepted 25 May 1984

A radioimmunofocus assay suitable for quantitation of cell culture-adapted human rotavirus was developed. The method was reproducible, more sensitive than plaque assay, and useful to detect and quantify strains of rotavirus which do not produce plaques. Preliminary results also suggested that the technique will be a useful means of serotyping cell culture-adapted strains of the virus.

Rotaviruses are important causes of infantile gastroenteritis in both humans and animals. Direct isolation of rotaviruses from fecal specimens from patients with diarrhea (1, 3, 5, 8, 10) and rescue of noncultivable human strains by genetic reassortment with cultivable bovine rotavirus (2) have been achieved, and at least four serotypes can now be detected by means of conventional plaque reduction neutralization assays (12).

A radioimmunofocus assay (RIFA) has been recently described by Lemon et al. (6) for the detection and quantitation of hepatitis A virus. The present report describes the modification of this technique to detect and quantify cell culture-derived rotaviruses and compares its sensitivity to that of plaque assays (PA).

MATERIALS AND METHODS

Cells. CV-1 cells, a continuous line of African green monkey kidney cells, were grown in acetone-resistant plastic petri dishes (diameter, 60 mm; Lux Permanox; Miles Scientific, Div. of Miles Laboratories, Inc., Naperville, Ill.). The growth medium was Eagle minimal essential medium with Earle salts, L-glutamine, nonessential amino acids (GIBCO Laboratories, Grand Island, N.Y.) and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid supplemented with 10^5 U of penicillin-streptomycin per ml, and 10% (vol/vol) fetal bovine serum. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and were used when confluent.

Viruses. Three rotaviruses, considered to be representative of three distinct serotypes, were used to establish the optimal conditions for the assay. FH77, a long electropherotype human strain, Hu7, a short electropherotype human strain, and a local strain of simian agent, SA11, were chosen. Serotyping of strain FH77 with reference antisera has not been carried out; however, this virus is thought to be either serotype 1 or 4. Hu7 is a serotype 2 rotavirus (R. Bishop, personal communication), and SA11 has been shown to be antigenically similar to human serotype 3 (11).

Antisera. Hyperimmune sera against FH77 and Hu7 were prepared in guinea pigs. Virus was grown in roller bottles as previously described (1) and then concentrated and partially purified by centrifugation at 200,000 *g* for 90 min through 45% (wt/vol) sucrose with an SW41 rotor in a Beckman L8 ultracentrifuge. Three sequential doses of virus in Freund complete adjuvant were administered intramuscularly at 3-week intervals. Hyperimmune serum against SA11 was prepared in a rabbit by a similar procedure, except that virus was administered subcutaneously.

Iodination of hyperimmune anti-rotavirus globulin. Saturated $(NH_4)_2SO_4$ (1 ml) was added dropwise to 1 ml of hyperimmune SA11 serum every 10 to 15 s at 4°C with constant stirring. The resultant precipitate was deposited by centrifugation at 1,400 × *g* for 15 min, and the pellet was suspended in 1 ml of 50% saturated $(NH_4)_2SO_4$ at 4°C. After centrifugation as above, the pellet was suspended in 1 ml of 0.0003 M phosphate buffer (pH 8.0) and dialyzed against the same buffer for 3 days. Iodination was carried out by a modification of the method of Salacinski et al. (7). Briefly, 20 μl of antibody was added to 0.1 μg of Iodogen (Pierce Chemical Co., Rockford, Ill.) together with 1 mCi of ^{125}I (Amersham Radio-Chemicals, Australia) in 10 μl of distilled water. The reaction was allowed to proceed for 10 min at room temperature and was stopped by the addition of 200 μl of 0.1% NaN_3 in phosphate-buffered saline (PBS), pH 7.2. The reaction mixture was allowed to stand for 15 min after which unreacted ^{125}I was separated from iodinated antibody by filtration through a Sephadex G-25 column (Pharmacia Fine Chemicals, Uppsala, Sweden) which had been equilibrated in PBS. The labeled antibody was then diluted in 5% (vol/vol) fetal calf serum in PBS until an activity of 35,000 counts/10 μl per 10 s (2.1×10^7 cpm/ml) was obtained.

RIFA for rotavirus. The RIFA was carried out by a modification of the method of Lemon et al. (6). Briefly, confluent monolayers of CV-1 cells in petri dishes were washed three times with a total of 15 ml of maintenance medium (minimal essential medium supplemented with 10^8 U of penicillin-streptomycin, 376 mg of L-glutamine, 35 ml of 5% $NaHCO_3$, and 1.25 mg of trypsin per liter). The dishes were inoculated with 0.5 ml of virus diluted in maintenance medium. Virus was allowed to absorb for 1 h at 37°C in an atmosphere of 5% CO_2. The inoculum was then removed, and the cells were overlaid with 5 ml of a 2× concentrate of maintenance medium containing an equal volume of 1% (wt/vol) Bacto-Agar (Difco Laboratories, Detroit, Mich.) at 45°C. After the agar had solidified, the dishes were incubated at 37°C in an atmosphere of 5% CO_2. To detect viral antigen after the required incubation time, we gently removed the overlay and washed the monolayer once with 5 ml of prewarmed (35°C) Hanks balanced salt solution. The dishes were air dried, and the cells were fixed with 2 ml of cold acetone at 4°C for 2 min. The dishes were again air dried, and 2 ml of ^{125}I-anti-SA11 (2.1×10^7 cpm/ml) was added, followed by incubation at 37°C for 4 h. The radiolabel was then aspirated, and the cells were washed 10 times with a

* Corresponding author.

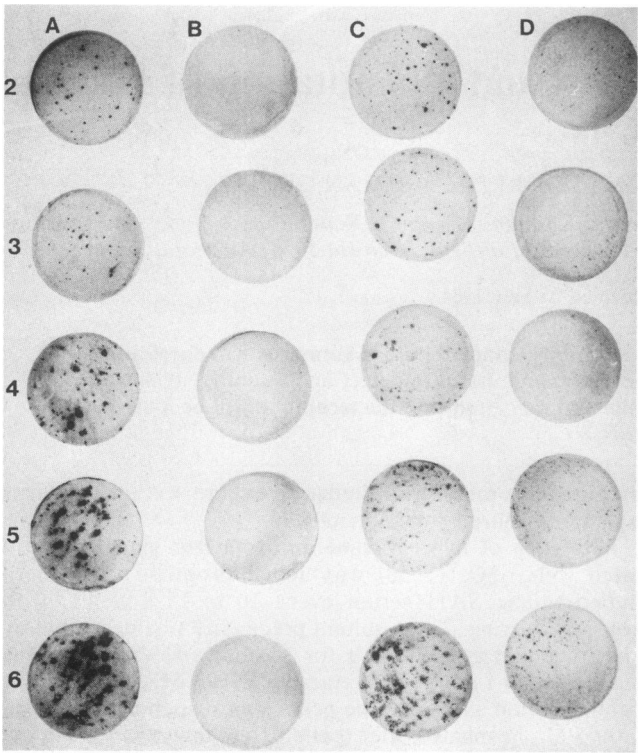

FIG. 1. RIFA autoradiograms prepared 2, 3, 4, 5, and 6 days after the inoculation of cultures with 60 RFU of (A) SA11, (B) uninfected control, (C) Hu7, and (D) FH77.

total of 20 ml of PBS. When dry, the sides of each dish were cut away with scissors. Autoradiography was carried out by placing the dishes in a Kodak X-Omatic cassette containing regular intensifying screens and Cronex 4 medical X-ray film (E. I. du Pont de Nemours & Co., Inc., Sydney, Australia) for 3 to 5 days at −20°C. The films were then processed through a Dupont Automatic Processor, and the autoradiograms were examined for foci of developed grains. The titer of virus in the stock preparations was then expressed in terms of radioimmunofocus-forming units (RFU) per milliliter (6).

Specificity of RIFA procedure. To demonstrate the specificity of the RIFA procedure, we carried out a neutralization assay by using a standard concentration of rotavirus (240 RFU per ml) with a fixed concentration (1:100 in PBS) of acute (preinfection) and convalescent anti-FH77, anti-Hu7, and anti-SA11. Equal volumes (0.25 ml) of virus and antiserum were mixed and incubated at 37°C for 1 h before being added to washed CV-1 petri dishes. Plates were then treated and autoradiographed as above, and the percentage of reduction in the number of RFU was calculated by comparing the number of plaques obtained after preincubation with acute serum with that obtained with convalescent serum.

PA. For PA, petri dishes containing confluent CV-1 cells were washed, inoculated with virus, overlaid, and incubated in a manner identical to that for RIFA. Cells were fixed in acetone for 2 min at 4°C, and plaques were visualized by flooding the petri dishes with 0.2% crystal violet for 2 min at room temperature and then washing each dish under running tap water.

Statistical analysis. Confidence limits were estimated by the method of Kleinbaum and Kupper (4).

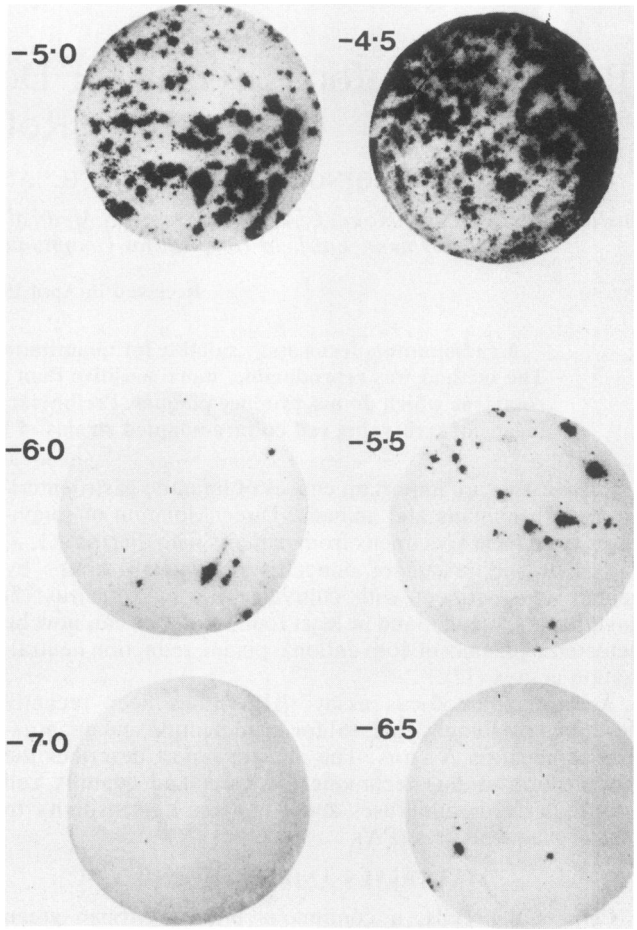

FIG. 2. RIFA autoradiograms at 6 days postinfection of cultures inoculated with various dilutions of SA11.

RESULTS

Rate of appearance of radioimmunofoci. After 24 h of incubation, all three viruses produced foci, but these were not clear, especially for FH77. However, by 48 h postinfection, all three produced clearly visible foci although for FH77 they were only pinpoint sized. However, this size proved to be characteristic of the foci produced by this virus. The development of foci between days 2 and 6 for each of the viruses is shown in Fig. 1. Generally the number of foci did not change with time but did increase in size. The number of visible radioimmunofoci became less clear with increasing

TABLE 1. Sensitivity of RIFA compared with PA for quantitation of rotavirus at 6 days postinfection[a]

Strain	RFU/ml	PFU/ml
SA11	2.9×10^7	7.9×10^6
	2.8×10^7	7.8×10^6
Hu7	1.1×10^5	7.7×10^3
	1.2×10^5	7.2×10^3
FH77	3.5×10^4	0[b]
	3.6×10^4	0

[a] Autoradiography was for 6 days.
[b] FH77 did not produce plaques by a conventional plaque assay system.

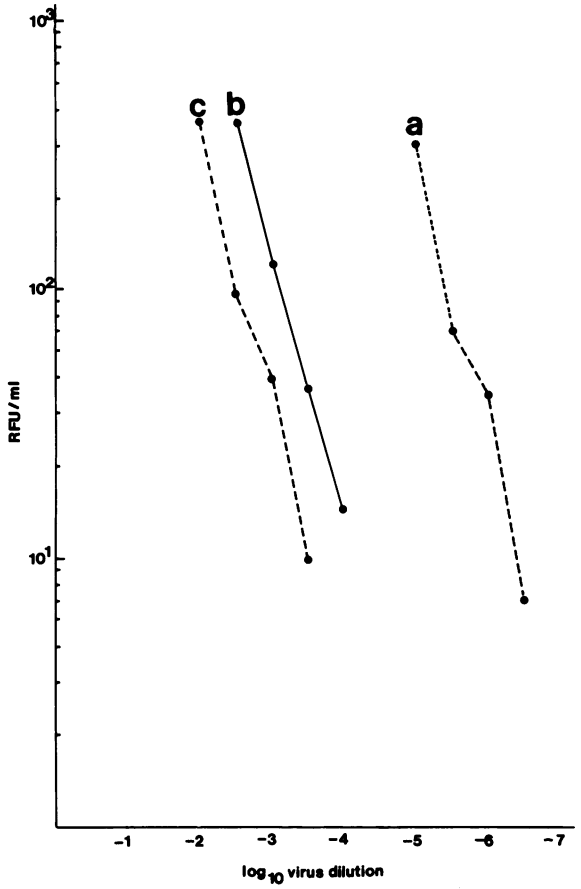

FIG. 3. Results of a typical experiment showing the number of RFU visible for SA11 (a), Hu7 (b), and FH77 (c) at 6 days postinfection.

time because, as the size of each focus increased, fogging of the X-ray film occurred. Repeated experiments showed that by day 6 each of the viruses had produced foci of characteristic diameter, those of SA11 ranging up to 8 mm, those of Hu7 ranging up to 7 mm, and those of FH77 not increasing beyond 1 to 2 mm. Autoradiography for 6 days appeared to be optimal for visualization of all foci, as use of shorter periods occasionally resulted in failure to reveal a small number of plaques later visible at 6 days.

Effect of titration on the number of RFU. Foci of SA11 were confluent at a dilution of $10^{-3.5}$, and the entire area of cells was heavily exposed, but at higher dilutions individual foci became increasingly discernible (Fig. 2). Of the three viruses, SA11 had the highest titer, with FH77 and Hu7 having titers that were similar but lower than that of SA11, probably reflecting the degree of adaption to cell culture of the three strains (Table 1). There was a linear relationship between the number of RFU per milliliter and the dilution for each of the viruses tested (Fig. 3). Titration of virus stocks in separate experiments revealed a minimal variation in the number of RFU detectable at each dilution. In each case the results obtained were within the range predicted by the coefficient of variation (4).

Specificity of RIFA procedure. With each virus, addition of homologous antiserum completely blocked the subsequent appearance of radioimmunofoci, whereas incubation of virus with preimmunization serum did not result in any reduction. Incubation of heterologous antiserum with each of the viruses before inoculation into cells resulted in a reduction in, but not complete elimination of, the number of plaques. A similar reduction rate was seen when the same procedure was applied to the PA test (Table 2).

Sensitivity of RIFA compared with PA. Table 1 shows the comparative sensitivities of RIFA with PA. With a standard inoculum of 60 RFU in each experiment, the RIFA procedure was only marginally (fourfold) more sensitive than PA for SA11 but considerably more sensitive (15-fold) for Hu7. Comparison of RIFA versus PA for FH77 was not possible because FH77 did not produce plaques within 6 days (nor did it regularly produce plaques in more than 6 days).

DISCUSSION

The radioimmunofocus described here was originally developed for the quantitation of hepatitis A virus by Lemon et al. (6) and is especially useful in the quantitation of noncytopathic viruses, nonplaquing viruses, or both. It can also be easily modified to permit simple neutralization tests to be performed and has potential for assessing the susceptibility of viruses to antiviral drugs and chemicals. The method was sensitive, more so than was PA, and this feature outweighs the rather long period of autoradiography required to achieve optimal results. Although we used 6 days for autoradiography, this period can, in most cases, be shortened to 3 to 4 days. In our experiments the increased sensitivity of RIFA over PA was only minimal with SA11, probably reflecting the extent and nature of the cell culture adaption of SA11, which has been passaged many times in different cell lines in this laboratory and does not have an absolute requirement for trypsin, rather than an inherent property of the RIFA. On the other hand RIFA was more than 10-fold more sensitive than PA for one human isolate (Hu7) and reproducibly detected plaques for another isolate (FH77) which did not produce plaques by a conventional plaque assay system.

Given that not all strains of human rotavirus adapted to growth in cell culture produce plaques (10, 12) and that the appearance of cytopathic effects is variable (1), the major advantage of RIFA is the provision of an alternative method for serotyping strains of rotavirus. Wyatt et al. (12) have reported that up to 34% of their isolates do not produce plaques and that an identical proportion produce only small, faint plaques. We have noted similar results in our laboratory. Under these circumstances a large proportion of cell culture-adapted rotavirus will be untypable by PA. Preliminary results obtained from plaque neutralization experi-

TABLE 2. Percentages of reduction in plaque numbers obtained when viruses were incubated with single dilutions of homologous and heterologous sera[a]

Strain	Test	% Reduction with antiserum:		
		Anti-SA11	Anti-FH77	Anti-Hu7
SA11	PA	100	84	84
	RIFA	100	83	85
FH77	PA	No plaques	No plaques	No plaques
	RIFA	80	100	89
Hu7	PA	92	63	100
	RIFA	93	65	100

[a] 60 RFU of virus was used in each neutralization experiment; results represent the average of two separate experiments.

ments with homologous and heterologous antisera to establish the specificity of the RIFA procedure suggest that the method could be modified to serotype all rotavirus strains adapted to growth in cell culture.

ACKNOWLEDGMENTS

These studies were assisted by grants from the National Health and Medical Research Council and the Australian Development Assistance Bureau.

We thank Margaret Veale and Poppy Kargas for their excellent technical assistance and John Albert (Royal Children's Hospital, Melbourne) for kindly providing us with an isolate of Hu7 virus.

LITERATURE CITED

1. **Birch, C. J., S. M. Rodger, J. A. Marshall, and I. D. Gust.** 1983. Replication of human rotavirus in cell culture. J. Med. Virol. **11**:241–250.
2. **Greenberg, H. A., R. G. Wyatt, A. Z. Kapikian, A. R. Kalica, J. Flores, and R. Jones.** 1982. Rescue and serotypic characterization of noncultivable human rotavirus by gene reassortment. Infect. Immun. **37**:104–109.
3. **Hasegawa, A., S. Matsuno, S. Inouye, R. Kono, Y. Tsurukubo, A. Mukoyama, and Y. Saito.** 1982. Isolation of human rotaviruses in primary cultures of monkey kidney cells. J. Clin. Microbiol. **16**:387–390.
4. **Kleinbaum, D. J., and L. L. Kupper.** 1978. Applied regression analysis and other multivariable methods, p. 56–58. PWS Publishers, Boston, Mass.
5. **Kutsuzawa, T., T. Konno, H. Suzuki, A. Z. Kapikian, T. Ebina, and N. Ishida.** 1982. Isolation of human rotavirus subgroups 1 and 2 in cell culture. J. Clin. Microbiol. **16**:727–730.
6. **Lemon, S. M., L. N. Binn, and R. H. Marchwicki.** 1983. Radioimmunofocus assay for quantitation of hepatitis A virus in cell cultures. J. Clin. Microbiol. **17**:834–839.
7. **Salacinski, P. R. P., C. McLean, J. E. C. Sykes, V. V. Clement-Jones, and P. V. Lowry.** 1981. Iodination of proteins, glycoproteins and peptides using a solid-phase oxidising agent 1,3,4,6-tetrachloro-3α-6α-diphenyl-glycouril (Iodogen). Anal. Biochem. **117**:136–146.
8. **Sato, K., Y. Inaba, T. Shinozaki, R. Fujii, and M. Matumoto.** 1981. Isolation of human rotavirus in cell cultures. Arch. Virol. **69**:155–160.
9. **Urasawa, S., T. Urasawa, and K. Taniguchi.** 1982. Three human rotavirus serotypes demonstrated by plaque neutralization of isolated strains. Infect. Immun. **38**:781–784.
10. **Urasawa, T., S. Urasawa, and K. Taniguchi.** 1981. Sequential passages of human rotavirus in MA-104 cells. Microbiol. Immunol. **25**:1025–1035.
11. **Wyatt, R. G., H. B. Greenberg, W. D. James, A. L. Pittman, A. R. Kalica, J. Flores, R. M. Chanock, and A. Z. Kapikian.** 1982. Definition of human rotavirus serotypes by plaque reduction assay. Infect. Immun. **37**:110–115.
12. **Wyatt, R. G., H. D. James, A. L. Pittman, Y. Hoshino, H. B. Greenberg, A. R. Kalica, J. Flores, and A. Z. Kapikian.** 1983. Direct isolation in cell culture of human rotaviruses and their characterization into four serotypes. J. Clin. Microbiol. **18**:310–317.