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Radioimmunofocus Assay for Quantitation of Hepatitis A Virus in Cell Cultures

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A new method is described for the quantitation of hepatitis A virus in cell cultures, based on the immune autoradiographic detection of foci of infected cells (radioimmunofoci) developing beneath an agarose overlay 14 days after the inoculation of petri dish cultures of continuous African green monkey kidney cells (BS-C-1). The number of foci developing in each culture was linearly related to the dose of hepatitis A virus (either HM-175 or PA-21 strain) inoculated. Focus development was prevented by prior incubation of virus with specific antisera, and the specificity of the radiolabeled antibody reaction was confirmed in competitive blocking experiments. This new assay method retains many of the advantages of conventional plaque assays for virus. Compared with existing end-dilution methods for the quantitation of hepatitis A virus, the radioimmunofocus assay offers greatly improved accuracy and comparable sensitivity, yet is relatively rapid and highly conservative of reagents.

Hepatitis A virus (HAV) has been propagated successfully in several different types of cell cultures (2, 6, 12), and efforts at vaccine development have resulted in substantial progress (11). However, because HAV is not cytopathic in any cell culture studied thus far, detection of viral replication is dependent upon the demonstration of HAV antigen by an immunological method such as immunofluoresence or solid-phase radioimmunoassay (2, 12). In addition, optimum development of HAV antigen in infected cell cultures requires 3 to 4 weeks, even with cell culture-adapted virus. These factors have made the titration of virus and the measurement of neutralizing antibody (12) difficult, lengthy, and laborious tasks. In this paper, we describe a new method for the quantitation of HAV. This method, which is based on the autoradiographic demonstration of foci of HAV-infected cells developing beneath an agarose overlay, is sensitive and extremely accurate, yet relatively rapid, and thus offers substantial advantages over previous assays. Variables affecting the assay are described, as are experiments with two different strains of HAV.

MATERIALS AND METHODS

Cells. Continuous African green monkey kidney cells (BS-C-1) were obtained from H. Hopps, Bethesda, Md., and were used for these studies between the 79th and 105th passage levels (7). Cells were grown at 35°C in Eagle minimal essential medium with Earle salts (HEM Research Inc., Rockville, Md.), supplemented with 2 mM glutamine (M. A. Bioproducts, Walkersville, Md.), 10% fetal bovine serum (Rehatuin; Armour Pharmaceutical Company, Tarrytown, N.Y.), 100 μg of streptomycin per ml, and 100 U of penicillin per ml. Cells were fed weekly with maintenance medium containing 2% fetal bovine serum or were split 1:2 after treatment with trypsin or trypsin-EDTA (GIBCO Laboratories, Grand Island, N.Y.).

Virus. The initial isolation of HAV was accomplished in primary African green monkey kidney cells (Lederle Laboratories, Wayne, N.J.) or BS-C-1 cells (L. N. Binn and S. M. Lemon, unpublished data). The PA-21 strain of HAV was isolated in BS-C-1 cells from the liver of a naturally infected Panamanian owl monkey (10). PA-21 virus seed was prepared at the sixth passage level by repeated freeze-thawing of the mechanically removed cell monolayer, followed by sonication and clarification by centrifugation at 7,000 × *g* for 30 min. The HM-175 strain of HAV was originally recovered from the feces of a naturally infected human in Australia (2) and was obtained as sixth marmoset-passage liver from S. M. Feinstone, Bethesda, Md. HM-175 was initially isolated and passed 10 times in primary African green monkey kidney cells, followed by one passage in BS-C-1 cells. The HM-175 seed was prepared from the supernatant medium of BS-C-1 cells 21 days after inoculation with virus and was clarified by centrifugation as described above. Virus seed preparations were kept at −70°C until use.

Sera. Reference preinfection and postinfection serum specimens were collected from a colony-bred chimpanzee which had been inoculated intravenously with the MS-1 strain of HAV (S. M. Lemon and W. H. Bancroft, unpublished data). These sera were negative and positive, respectively, for antibody to HAV (anti-HAV) when tested by radioimmunoassay (Havab; Abbott Laboratories, North Chicago, Ill.). Paired preinfection and postinfection sera from a chimpanzee experimentally infected with HM-175 virus were the gift of S. M. Feinstone. Preinfection and postinfection

sera were also collected from an owl monkey inoculated intravenously with the PA-33 strain of HAV (J. W. LeDuc et al., Infect. Immun., in press). The PA-33 and PA-21 isolates of HAV are epidemiologically related, both having been obtained from owl monkeys involved in the same colony-centered outbreak of HAV infection (10), and almost certainly represent the same original virus strain. Paired preinfection and postinfection human sera were collected during the investigation of a focal outbreak of hepatitis A among American soldiers at Grafenwohr, Federal Republic of Germany, during July 1982.

^{125}I-labeled anti-HAV. The immunoglobulin G fraction of a human convalescent serum sample (anti-HAV titer, 1:16,000 by immune adherence hemagglutination) was purified by a combination of ammonium sulfate precipitation and anion exchange chromatography and radioiodinated as previously described (9). The specific activity of ^{125}I-labeled anti-HAV preparations was approximately 10 μCi/μg of immunoglobulin G. Radiolabeled antibody was diluted in 0.25 M phosphate buffer, pH 7.2, containing 1 mg of KI per ml and 10% fetal bovine serum, and was passed through a 0.22-μm filter before use.

Radioimmunofocus assay (RIFA) for HAV. BS-C-1 cells grown in a 490-cm^2 plastic roller flask were split as described above and seeded into a total of 40 acetone-resistant plastic petri dishes (60-mm diameter; Lux Permanox; Lab-Tek Products, Div. Miles Laboratories Inc., Naperville, Ill.) and placed at 35°C under a 5% CO_2 environment. Cell cultures were inoculated with virus 3 to 5 days later when they were confluent. Virus dilutions were prepared in Hanks balanced salt solution (HBSS) (HEM Research Inc.) containing 100 μg of streptomycin per ml, 100 U of penicillin per ml, and 5% fetal bovine serum. Growth medium was aspirated from individual petri dishes, and each culture was inoculated with 0.25 ml of virus. Virus was allowed to adsorb for 90 to 120 min, and, without removal of the inoculum, the culture was overlaid with 4.5 ml of maintenance medium containing 0.5% agarose ME (Seakem; Marine Colloids Inc., Rockland, Maine) at 45°C. After the agar had solidified, the cultures were incubated at 35°C under 5% CO_2. Infected cultures were generally held for 14 days without feeding or, if held for longer periods of time, received a second agarose overlay at day 14.

Testing for viral antigen was accomplished by gently dislodging and removing the agarose from the culture and washing the cell monolayer once with 5 ml of HBSS prewarmed to 35°C. After being thoroughly air dried, the cells were fixed with 2 ml of acetone at room temperature for 2 min. To each dish we then added 2 ml of ^{125}I-labeled anti-HAV (500,000 cpm/ml), followed by incubation at 35°C for 4 h. The radiolabeled antibody was then aspirated from the dishes, and the treated cells were washed five times with a total of 10 ml of phosphate-buffered saline, pH 7.2. After air drying, the bottom of each petri dish was cut out and affixed with tape to a cardboard sheet. Autoradiography was carried out by placing the mounted cultures with Kodak X-AR5 film (Eastman Kodak, Rochester, N.Y.) in a Kodak X-Omatic cassette with regular intensification screens for 4 to 6 days at −70°C. The film was then processed through a Kodak RP Automatic Processor, and completed autoradiograms of individual petri dishes were examined for foci of developed grains. From the number of such foci, the amount of virus in the inoculum could be estimated in terms of radioimmunofocus-forming units (RFU) per milliliter.

End-dilution titration of HAV. Titration of HAV by the end-dilution method was carried out in borosilicate glass tubes (12 by 75 mm) containing confluent cultures of BS-C-1 cells. Six to eight replicate cultures were inoculated with 0.1 ml of each dilution of virus in a 10-fold dilution series. After adsorption at 35°C for 2 h, cultures received 0.5 ml of maintenance medium and were then incubated at 35°C under 5% CO_2 for 21 to 28 days with weekly feeding. Viral replication was detected by an in situ radioimmunoassay similar to that described previously by Kojima et al. (8). Cell sheets were washed twice with HBSS and fixed with acetone for 2 min at room temperature. After drying, 0.2 ml (200,000 cpm) of ^{125}I-labeled anti-HAV was placed in each tube, and the tubes were reincubated at 35°C for 4 h. The labeled antibody was then aspirated from each tube, and each tube was washed five times with 2 ml of phosphate-buffered saline per wash. Tubes were counted for residual radioactivity in a Rackgamma-II automatic gamma counter (LKB-Wallac, Turku, Finland). A positive result in an individual culture tube was indicated by a counts per minute value greater than or equal to 2.1 times that of uninfected control tubes. This method was determined to be approximately as sensitive as direct immunofluorescence for the detection of HAV antigen in infected cultures (L. N. Binn et al., unpublished data). Tissue culture infectious doses ($TCID_{50}$) were calculated by the method of Reed and Muench (13), with 95% confidence intervals estimated as described previously by Dulbecco and Ginsberg (4).

Solid-phase radioimmunoassay for HAV antigen. HAV antigen was detected by a microtiter solid-phase radioimmunoassay method described previously (10). Results are presented as P/N ratios (P/N = counts per minute of test sample divided by the mean counts per minute of HAV-negative control samples); samples yielding P/N ratios equal to or greater than 2.1 were considered positive.

RESULTS

RIFA for HAV. The HM-175 virus seed preparation was diluted in $10^{0.5}$ increments from 10^{-1} to 10^{-6} and inoculated into petri dish cultures of BS-C-1 cells as described above. Cytopathic effect did not develop under the agarose overlay during a 14-day period of observation. The completed RIFA autoradiograms, prepared 14 days after inoculation of the cultures, are shown in Fig. 1. At the $10^{-1.0}$ virus dilution, foci of viral antigen were confluent, and the entire area of the film under the cells was heavily exposed. Individual foci of antigen, represented by developed grains on the autoradiogram, were discernable at the $10^{-2.0}$ dilution but were still too numerous to count. At the $10^{-2.5}$ and greater virus dilutions, discrete foci were present and could be readily counted. These radioimmunofoci were heterogeneous in size, ranging from 1 to 6 mm. At progressively higher dilutions of

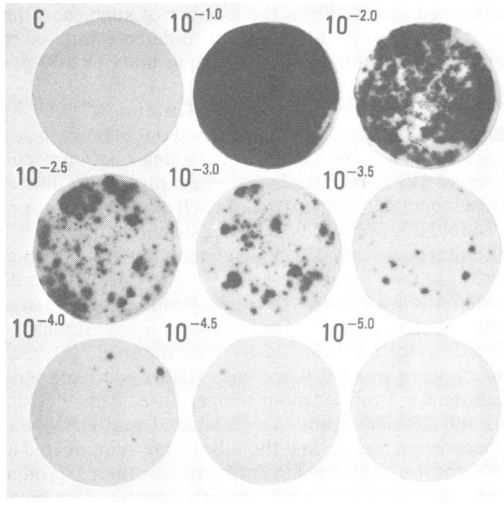

FIG. 1. RIFA autoradiograms of petri dish cultures prepared 14 days after inoculation with either HBSS (C) or various dilutions of HM-175 virus seed preparation.

virus, there were fewer radioimmunofoci per dish. Uninoculated control dishes showed no foci.

Figure 2 presents the estimated virus content (RFU per milliliter) of each of the HM-175 dilutions, determined in four replicate cultures inoculated with each dilution. The estimated viral content of each dilution (proportionate to the number of radioimmunofoci observed per petri dish culture) followed a linear relationship with the dilution of virus between the $10^{-3.0}$ and $10^{-4.5}$ dilutions. A similar experiment was carried out with the PA-21 strain of HAV (Fig. 3). As with the HM-175 strain, the number of foci observed in each petri dish culture was linearly related to the dilution of PA-21 inoculated.

Specificity of RIFA procedure. To demonstrate the immunological specificity of the RIFA procedure for HAV, two different experiments were conducted (Fig. 4). First, petri dishes held for 28 days after inoculation with HM-175 virus were washed and fixed as usual. Replicate cultures were then stained with the ^{125}I-labeled anti-HAV preparation to which had been added a 1:10 dilution of either preinfection or postinfection reference chimpanzee serum (Fig. 4A and B). The addition of postinfection serum almost totally blocked the appearance of radioimmunofoci, whereas the preinfection serum was without effect.

To further demonstrate the specificity of the test procedure, we incubated approximately 80 RFU of HM-175 virus with a 1:40 dilution of either preinfection or postinfection, heat-inactivated human hepatitis A serum for 1 h at 35°C. The virus-serum mixtures were then inoculated onto petri dishes containing BS-C-1 cells (Fig. 4C and D). Incubation with postinfection serum resulted in a >90% reduction in radioimmunofoci developing by 14 days after inoculation, whereas no reduction was observed when virus was incubated with the same dilution of homolo-

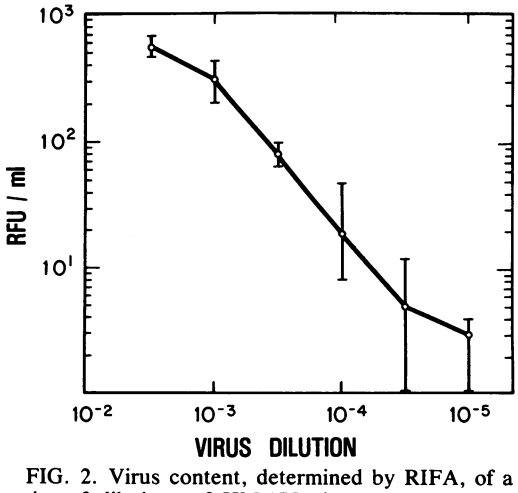

FIG. 2. Virus content, determined by RIFA, of a series of dilutions of HM-175 virus. Four separate petri dish cultures were inoculated with each dilution of virus: O, mean values; bars, range of values for replicate cultures. Cultures were incubated for 14 days.

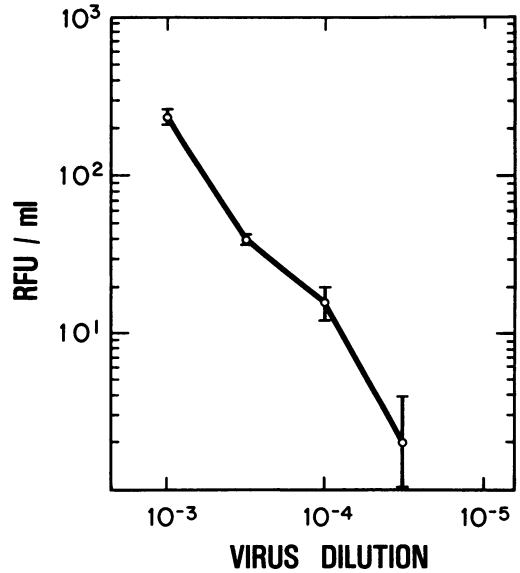

FIG. 3. Virus content, determined by RIFA, of a series of dilutions of PA-21 virus. Two petri dish cultures were inoculated with each dilution of virus: O, mean values; bars, range of values for replicate cultures. Cultures were incubated for 14 days.

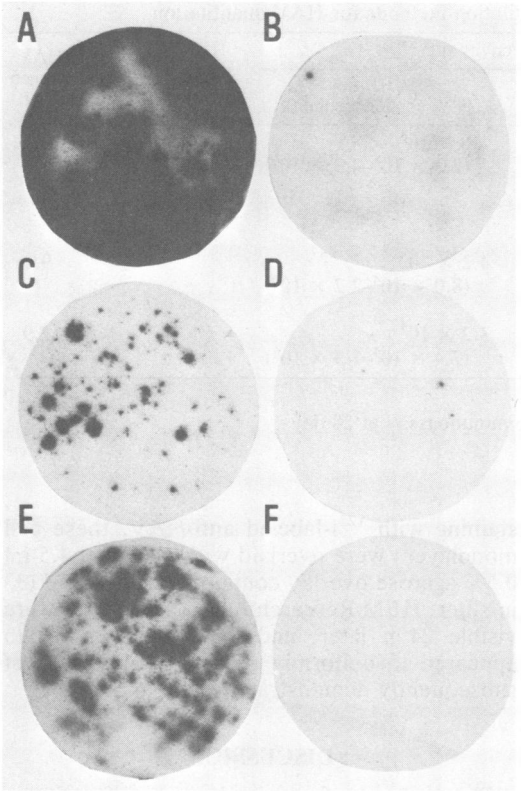

FIG. 4. Immunological specificity of RIFA procedure. (A and B) Autoradiograms of cultures incubated for 28 days after inoculation with HM-175 virus. At the time of staining with ^{125}I-labeled anti-HAV, the ^{125}I-labeled anti-HAV was diluted in 10% preinfection (A) or postinfection (B) serum from a chimpanzee inoculated with the MS-1 strain of HAV. (C through F) Autoradiograms of cultures incubated for 14 days after inoculation with HM-175 virus preincubated with preinfection (C) or postinfection (D) human hepatitis A serum or with preinfection (E) or postinfection (F) serum from an owl monkey infected with PA-33 strain HAV.

gous preinfection serum. Similarly, HM-175 radioimmunofoci did not develop when virus was first incubated with postinfection (but not preinfection) serum collected from an owl monkey infected with PA-33 strain HAV (Fig. 4E and F), nor did PA-21 radioimmunofoci develop when virus was first incubated with HM-175 immune serum (data not shown).

Kinetics of radioimmunofocus development. The development of radioimmunofoci over a period of 21 days after the inoculation of BS-C-1 cells with HM-175 virus is shown in Fig. 5. Petri dish cultures were inoculated with 80 RFU of virus and harvested on days 1, 7, 14, 21, and 30. No foci were visible in cultures harvested on day 1, indicating that viral replication was essential to focus development and that exposed grains were not related to input viral antigen. By day 7, a few scattered foci of antigen were barely visible on the autoradiograms, whereas by day 14, foci were well developed and could be readily counted. By day 21, foci had expanded considerably, and overlapping foci prevented accurate quantitation. Cultures held for 30 days revealed only confluent foci (data not shown). Subsequent experiments, therefore, were harvested at 14 days.

Relative sensitivity of RIFA and end-dilution methods of HAV quantitation. The viral contents of the HM-175 and PA-21 virus seed preparations, determined by the RIFA method, were compared with those estimated by a tube infectivity method based on the in situ radioimmunoassay detection of HAV antigen in acetone-fixed cell sheets and with the antigen content of the original virus seed preparation as determined by solid-phase radioimmunoassay

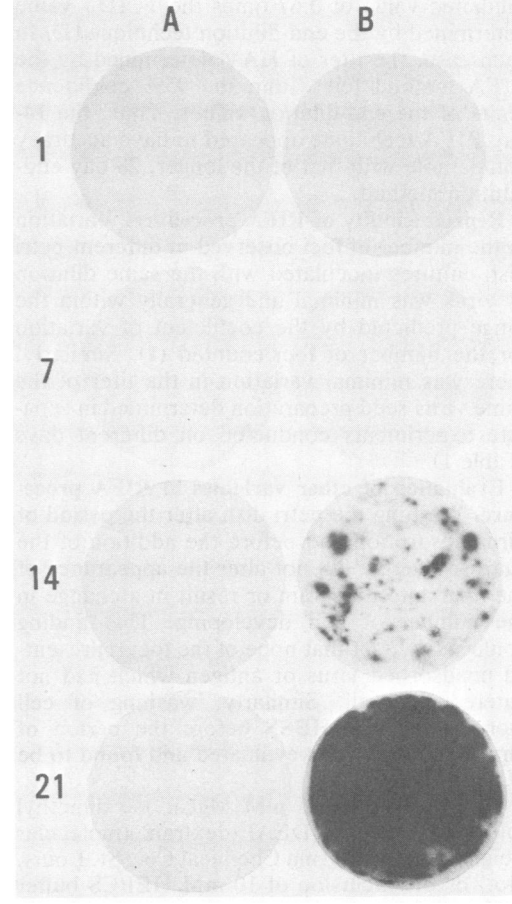

FIG. 5. RIFA autoradiograms prepared 1, 7, 14, and 21 days after the inoculation of BS-C-1 cell cultures with HBSS (A) or approximately 80 RFU of HM-175 virus (B).

TABLE 1. Sensitivity of RIFA and end-dilution methods for HAV quantitation

Virus seed prepn	HAV infectivity titer		HAV antigen (P/N)
	RFU/ml ($\times 10^5$)[a]	$TCID_{50}$/ml (95% confidence limit)[b]	
HM-175	3.2 3.7 3.3 3.7	1.0×10^6 (2.3×10^5–4.3×10^6)	14.5
PA-21 (no. 1)	2.5 3.7	4.2×10^5 (8.0×10^4–2.2×10^6)	9.4
PA-21 (no. 2)	2.2	3.2×10^5 (7.4×10^4–1.3×10^6)	12.9

[a] Values obtained in independent RIFAs at 14 days.
[b] Determined by end-dilution method with in situ radioimmunoassay at 28 days.

(Table 1). The titer of HAV in three different viral seed preparations, measured in terms of RFU per milliliter, was close to the theoretically predicted value of 0.67 times the $TCID_{50}$ value determined by the end-dilution technique (1). In each case, the titer of HAV determined by the RIFA method fell within the 95% confidence limits of the end-dilution values. Thus, the 14-day RIFA technique appeared to have accuracy comparable with that of the longer, 28-day end-dilution method.

Reproducibility of RIFA procedure. Variation in the number of foci observed in different petri dish cultures inoculated with the same dilution of virus was minimal and generally within the range predicted by the coefficient of variation for the number of foci counted (1). Similarly, there was minimal variation in the titer of the same virus seed preparation determined in separate experiments conducted on different days (Table 1).

Evaluation of other variables in RIFA procedure. Washing the petri dish after the period of virus adsorption and before the addition of the agarose overlay did not alter the appearance of the final autoradiogram or result in a change in the number of foci developing. This finding confirms the fact that none of the foci represented unadsorbed virus or antigen which had not entered the cell. Similarly, washing of cell monolayers with HBSS before the period of virus adsorption was evaluated and found to be of no benefit.

The addition of 25 mM MgCl, 1% dimethyl sulfoxide, or 2% DEAE-dextran (molecular weight, 500,000; Sigma Chemical Co., St. Louis, Mo.) or the inclusion of 10 mM HEPES buffer (GIBCO) into the agarose overlay neither increased the quantity nor improved the quality of radioimmunofoci developing in BS-C-1 cells infected with HM-175 virus. Before fixation and staining with ^{125}I-labeled anti-HAV, these cell monolayers were overlaid with a second 4.5-ml 0.5% agarose overlay containing neutral red (33 mg/liter; HEM Research Inc.). No plaques were visible 24 h later, and the neutral red stain appeared to uniformly reduce the intensity of subsequently demonstrated radioimmunofoci.

DISCUSSION

Plaque assays for animal viruses, first introduced by Dulbecco (3), offer significant advantages over end-dilution methods of viral titration and thus have achieved widespread use in the field of virology. Such an assay would be especially useful for studies involving HAV, given the exceptionally slow rate of growth of this virus in cell culture (2, 12). Thus far, this has not been possible, given the noncytopathic nature of HAV. In this paper, however, we describe a modification of the plaque assay method in which foci of infected cells are detected by staining with radiolabeled anti-HAV followed by autoradiography. This modification results in a new method for the quantitation of HAV which retains many of the advantages of the plaque assay.

The RIFA procedure fulfills most of the criteria suggested for a reproducible viral plaque assay (1). No radioimmunofoci develop in the absence of HAV, and the number of radioimmunofoci observed in each culture follows a linear relationship with the dose of virus inoculated (Fig. 2 and 3). This linear relationship confirms that each radioimmunofocus occurs as the result of a single infectious unit of HAV (5), which we have termed an RFU. Repeated assays of the same virus stock in separate experiments were in close agreement with each other (Table 1), and within individual experiments, the distribution of foci among the various culture

dishes was consistent with a Poisson distribution. The specificity of the immunologically based detection system was proven both in direct competition (blocking) experiments and by the fact that the development of foci could be prevented by the prior neutralization of virus with specific antisera (Fig. 4). The only suggested criterion for a plaque assay not met by the RIFA procedure is the reisolation of the virus from individual foci (1). This task may not be possible given the fact that the RIFA procedure involves the removal of the overlay, the fixation of cells with acetone, and the detection of antigen with labeled antibodies.

The RIFA procedure retains the major advantages of the plaque assay. It is highly accurate, yet conserves cells and is more rapid than existing end-dilution methods of HAV titration (2, 12). The enhanced rapidity of the assay is probably related more to the sensitivity of the detection procedure in locating small foci of infected cells than to improved replication of virus under agarose. This assumption is based on an examination of Fig. 5, in which the appearance of antigen closely follows that which we have observed with immunofluorescence. Titration of virus by the RIFA method required only a 14-day incubation (followed by 4 to 6 days for autoradiography), compared with 21 to 28 days for the end-dilution method. Nonetheless, the titer of virus obtained with the RIFA method, in terms of RFU per milliliter, was close to the expected value of 0.67 times the $TCID_{50}$ titer (1). Although the sensitivity of the RIFA method might be enhanced by prolonging the period of viral replication to 21 or 28 days, this results in considerable enlargement of the radioimmunofoci (Fig. 4A and 5) with resultant overlapping of foci and decreased accuracy. It is possible, however, that use of smaller inocula, larger-diameter culture vessels, and longer incubation periods might further enhance the sensitivity of the RIFA procedure.

The RIFA procedure provides a practical, relatively rapid, yet highly accurate method of quantifying HAV. In addition, this new procedure should result in an improved ability to measure neutralizing antibody and thus allow a more critical examination of the degree of antigenic variation existing between different strains of HAV. Equally important, however, is that the concept of immune autoradiographic detection of foci of viral replication might also be applied profitably to the quantitation of other noncytopathic viruses.

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