AN AGAR CELL-SUSPENSION PLAQUE ASSAY FOR ISOLATED VIRAL RNA

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The standard techniques for assay of infectious viral nucleic acids are based on a change in the tonicity of the medium surrounding the cells. These procedures commonly involve the use of cell monolayers (Colter and Ellem, 1961), since cells in suspension do not resist osmotic shock as well as cells attached to glass or plastic petri dishes. Suspended mammalian cells will adsorb as much as 80% of a given quantity of isolated viral RNA (Borriss and Koch, 1964), but the resulting infective centers usually have been assayed with cell monolayers (Ellem and Colter, 1960). We wish to report on an agar-cell suspension assay for infectious viral RNA which is based on the observation (Koch et al., 1966a) that infection of suspended HeLa cells by poliovirus RNA can be stimulated as much as ten thousandfold by exposing the cells, either before or after the addition of RNA, to polybasic substances, such as methylated serum albumin, poly-lysine, poly-ornithine and DEAE-dextran. Ryser and Hancock (1965) have previously reported that polyamino acids stimulate the uptake of serum albumin by sarcoma-180 cells. The use of DEAE-dextran to augment the infectivity of poliovirus RNA for HeLa cell monolayers was first described by Vaheri and Pagano (1965).

Materials

Strain S_3 HeLa cells were grown at cell densities between 3 and 5×10^5/ml in Eagle's medium supplemented with 5% horse serum.

Poly-L-ornithine (New England Nuclear Corporation), molecular weight 45,000, was used as a 100 µg/ml solution in PSM.* Stock solutions of DEAE-dextran (Pharmacia), molecular weight approximately 2 x 10^6, were prepared in 0.1X PSM at 100 µg/ml. Other requirements for the assay include: 1) a 4.2% solution of Difco "Special Agar-Noble" in distilled water, 2) petri dishes (10 cm diameter) containing a 15 ml "basal layer" of 1% agar in SM-S, and 3) 1.5X SM-S for the agar-cell suspension layer.

Single-stranded poliovirus RNA was obtained from highly purified Mahoney Strain poliovirus (Levintow and Darnell, 1960) by phenol extraction at 60°C, or by MAK column chromatography of RNA isolated from infected cells by phenol extraction (Koch and Kubinski, 1964). Poliovirus-induced double-stranded RNA (Baltimore et al., 1964; Bishop et al., 1965) was obtained by extracting the RNA from infected cells with phenol at 60°C, precipitating it with one molar sodium chloride to remove high molecular weight single-stranded RNA, and chromatographing the supernatant on an MAK column. In principle, this procedure follows that of Amman, Delius and Hofschneider (1964) for the isolation of "replicative form" from phage M 12-infected E. coli. Details of the purification and physiochemical characteristics of the product will be described elsewhere (Bishop et al., 1966).

Methods

The cells from 200 ml of a suspension culture are sedimented (1300 rpm for 3 minutes, International PR-2 Centrifuge), washed with 100 ml of PSM, and resuspended in PSM at a cell density of 3-4 x 10^7/ml. This cell suspension is mixed with an equal volume of the poly-ornithine solution and incubated with continuous shaking in a 37°C

*Abbreviations used: PSM, .15M NaCl, .02M sodium phosphate buffer, pH 7.2, and 10^{-3}M $MgCl_2$; SM, standard Eagle's medium for suspension cultures; SM-S, the same, supplemented with 2% horse serum; MAK, methylated albumin kieselguhr.

water bath for 5 minutes, then diluted twentyfold in PSM. The diluted cells are added in 0.6 ml aliquots to tubes containing 0.1 ml of a given RNA dilution (made in the DEAE-dextran solution). After incubation at 37°C for 5 minutes, 0.1 ml of the poly-ornithine solution is added to each cell-RNA mixture, and incubation at 37°C continued for another 60 minutes. One volume of molten 4.2% agar and two volumes of 1.5X SM-S are combined (final agar concentration of 1.4%), distributed in 1 ml aliquots to capped Wasserman tubes, and maintained at 56°C.

A second lot of cells is sedimented as before, resuspended in SM-S to give approximately 1.5×10^7 cells/ml, and 0.7 ml samples added at each tube of RNA incubation mixture to serve as "indicator cells". The contents of each tube are then added to one of the Wasserman tubes containing 1.4% agar in SM-S. This mixture is stirred lightly and immediately poured onto a petri dish containing the basal layer. The top agar layer solidifies in 20 minutes. The plates are inverted and incubated in a numidified 5% CO_2 atmosphere at 37°C. Plaques are first visible after 28 hours, and can be counted (up to 200 per plate) after 42 hours without any staining procedure.

Characteristics of the Assay System

Dose-response curves for the effect of four polycations on the infectivity of single-stranded poliovirus RNA are shown in Figure 1. All of the substances tested have toxic effects on the cells, causing aggregation and reduction in cloning efficiency. This may explain the sharp decrease in RNA titers at high polycation concentrations. As shown in Table 1, both single and double-stranded RNA will induce a small number of infective centers when assayed in PSM alone. Poly-ornithine, used at optimal concentrations, causes an increase of more than 10^5 in the titer. DEAE-dextran, when used alone, yields an intermediate rise in the titer, but has no additive effect in combination

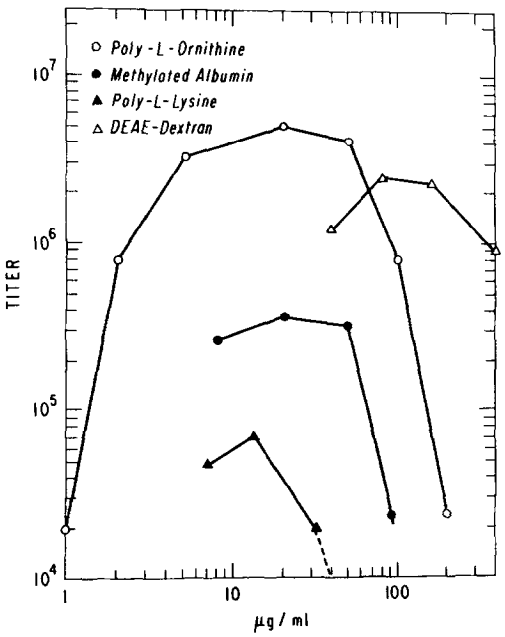

Figure 1. Dose-response curves for the effect of polycations on the infectivity of single-stranded poliovirus RNA assayed in suspended cells. The procedure used was that described in the text, except that poly-lysine and methylated albumin were added only after exposure of the cells to RNA.

TABLE 1

Effect of Poly-L-ornithine and DEAE-dextran on the Titer of Infectious Poliovirus RNA

Polycation	Titer (pfu/ml)	
	Single-stranded RNA	Double-stranded RNA
None	5×10^1	4×10^2
Poly-ornithine	2×10^7	3×10^7
DEAE-dextran	5×10^4	1.5×10^7
Poly-ornithine and DEAE-dextran	2×10^7	3×10^7

Titrations of standard RNA preparations were performed as described in the text. Where indicated in the table, one or both of the polycations were omitted from the procedure

with poly-ornithine; it is included in the assay because of its ability to protect RNA against the action of ribonuclease, as reported by Vaheri and Pagano (1965), and confirmed in our laboratory (unpublished observation).

There is a linear relationship between RNA concentration and plaque-forming units over a three-log range (Fig. 2), but RNA concentrations greater than 3 μg/ml inhibit the formation of infective centers; a detailed analysis of this latter phenomenon will be presented elsewhere (Koch et al., 1966b). After infection with RNA, the cells can be diluted up to one thousandfold without reduction in the number of infective centers, but it is advisable to add horse serum to a final concentration of 5% before making the dilutions in SM-S.

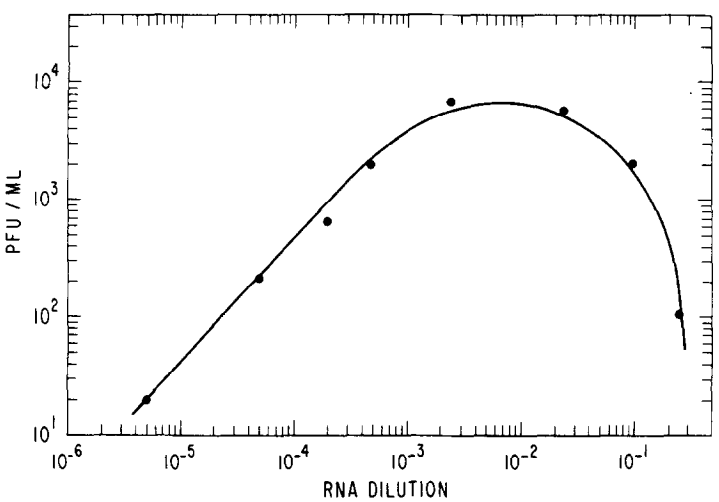

Figure 2. Relationship between RNA concentration and plaque-forming units. A 300 μg/ml solution of single-stranded poliovirus RNA was diluted as indicated, and assayed as described in the text.

Poliovirus-induced double-stranded RNA, purified as described in Methods, is infectious when assayed by this technique, although preliminary results suggest that the conditions necessary for maximum titers differ in certain respects from those required for single-stranded polio RNA. The specific infectivities of these two RNA's

are 3×10^4 to 1×10^5 pfu per μg of single-stranded RNA, and 2 to 5×10^5 pfu per μg of double-stranded RNA. The precision of the assay for any single set of determinations is ± 20%. Larger variations have been observed on a day-to-day basis which can be attributed, at least partially, to changes in cell growth conditions.

Conclusion

We have described a relatively simple and rapid assay for infectious viral RNA which permits the use of suspended cells in an isotonic environment. Infective centers are detected with "indicator cells" which need not be exposed to the same conditions as the cells which are initially infected. The observation that polybasic compounds augment the infectivity of purified viral RNA may be applicable to other nucleic acid-host cell systems.

Acknowledgment

Many of the technical details of the assay procedure are derived from Dr. J. Maizel's unpublished modification of Cooper's agar-cell suspension assay for whole infectious virus (Cooper, 1961).

REFERENCES

Amman, J., Delius, H., and Hofschneider, P. H., J. Mol. Biol., 10, 557 (1964).
Baltimore, D., Becker, Y., and Darnell, J. E., Science, 143, 1034 (1964).
Bishop, J. M., Summers, D. F., and Levintow, L., Proc. Natl. Acad. Sci. U.S., 54, 1273 (1965).
Bishop, J. M., Quintrell, N., and Koch, G., in preparation (1966).
Borriss, E., and Koch, G., Z. Naturforschg., 19b, 32 (1964).
Colter, J. S., and Ellem, K. A. O., Ann. Rev. Microbiol., 15, 219 (1961).
Cooper, P. D., Virology, 13, 153 (1961).
Ellem, K. A. O., and Colter, J. S., Virology, 11, 434 (1960).
Koch, G., and Kubinski, H., Z. Naturforschg., 19b, 683 (1964).
Koch, G., Quintrell, N., and Bishop, J. M., Fed. Proc., 25, 652 (1966a).
Koch, G., Quintrell, N., and Bishop, J. M., in preparation (1966b).
Levintow, L., and Darnell, J. E., J. Biol. Chem., 235, 70 (1960).
Ryser, H. J.-P., and Hancock, R., Science, 150, 501 (1965).
Vaheri, A., and Pagano, J. S., Virology, 27, 434 (1965).