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A HIGHLY EFFICIENT METHOD OF CELL INFECTION WITH ENCEPHALOMYOCARDITIS VIRUS RNA

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Summary. — A method for infecting Krebs-II cells with encephalomyocarditis virus RNA based on treatment of the cells with DEAE-dextran at high concentration was elaborated. The specific infectivity of virion RNA was 3×10^8 plaque-forming units per ug, i. e. comparable to the specific infectivity of the whole virus. A high proportion of the cell population could be infected by this method.

Key words: encephalomyocarditis virus; infectious RNA; DEAE-dextran; Krebs-II cells

Introduction

The infectivity of RNA preparations isolated from various viruses depends greatly on the conditions of infection. When added directly to the cells, RNA has a very low specific infectivity (about 10 plaque-forming units-PFU-per μg). To increase the specific infectivity of RNA, several methods were developed, including osmotic shock (Koch *et al.*, 1960; Montagnier and Sanders, 1962), treatment of the cells with dimethyl sulphoxide (Amstey, 1966), cytochalasin B (Koch and Opperman, 1975) and polycations (Pagano and Vaheri, 1965; Koch and Bishop, 1968). In the latter instance, diethyl aminoethyl- (DEAE-) dextran has been usually used. When the cells are treated with these polycations, RNA infectivity increases approximately $10^3 - 10^4$ — fold but still remains considerably lower than the infectivity of the whole virus.

For the determination of encephalomyocarditis (EMC) virus (genus *Cardiovirus*) RNA infectivity only one method based on the use of osmotic shock has been reported (Montagnier and Sanders, 1962). Its limitations include comparatively low specific infectivity and the non-physiological nature of the treatments used. I attemted, therefore, to treat the cells with polycations. In selection of the optimal conditions some differences from the poliovirus RNA — HeLa cells system were found. Under the selected conditions the infectivity of EMC virion RNA was comparable to that of the whole virus.

Materials and Methods

Preparation of virion RNA. EMC virion RNA (vRNA) was prepared by phenol extraction from virus purified by the simplified method of Kerr and Martin (1972). To the clarified virus containing fluid with a titer of 0.5 — 3 × 10^{10} PFU/ml was added 1/25 volume of a protamin sulphate (Serva) solution (50 mg/ml) in 50 mM Tris and after 20 min of incubation at 4 °C, the precipitate was separated. Polyethylene glycol 6000 (Serva) was added to the supernantant up to 5% concentration; after 30 min the precipitated virus was pelleted at 10,000×g for 10 min. The pellet was suspended in a small (1/40 of the initial virus volume) volume of a 50 mM Tris-HCl solution, pH 7.6, containing 10 mM 2-mercaptoethanol. After the addition of sodium dodecyl sulphate (SDS) up to a 1% concentration the virus was reprecipitated with polyethylene glycol at 20 °C. RNA extracted from the virus purified in this way was precipitated with 2 volumes of ethanol and stored as an ethanol suspension at —10 °C. Sedimentation in sucrose density gradient showed this RNA to be homogeneous with a sedimentation coefficient of 35 S. When labelled with 5-^{3}H-uridine, the specific radioactivity of this RNA was within the range from 5 to 20 × 10^3 count/min per μg.

The replicative form of RNA (RF RNA) was prepared from Krebs-II ascites cells 5 hr after inoculation at a multiplicity of 10 PFU/cell. The cells were suspended at a concentration of 3 × 10^7 cells/ml in 0.01 M sodium acetate buffer, pH 5.1, and then SDS to a 0.5% concentration and an equal volume of phenol satured with the same buffer at 4 °C and heated to 100 °C were added. The mixture was heated in a water bath until disappearance of turbidity (about 60 °C) and shaken for 3 min. Then it was cooled at 4 °C and the phases were separated by centrifugation for 10 min at 10,000xg. The aqueous phase was extracted once more at room temperature. The resulting RNA was precipitated with 2 volumes of ethanol. The precipitate was dissolved in the extraction buffer and an equal volume of 4 M LiCl was added. After 16 hr the precipitate formed was separated and soluble RNA was chromatographed on a Sepharose 2B column equilibrated with 25 mM EDTA solution, pH 7.0 containing SDS. RNA eluted in the void volume of the column was freed from traces of single-stranded RNA and DNA by chromatography on a cellulose CF-11 column (Franklin, 1967). RNA purified in this way was stored as an ethanol suspension at —10 °C. Its specific radioactivity was the same as that of vRNA.

Determination of RNA infectivity. Krebs-II ascites cells were washed 3 times with Earle's solution and suspended in PSM buffer (0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.3, 0.001 M magnesium acetate) to a concentration of 1.36 × 10^8 cells/ml; DEAE-dextran (mol. wt. 2 × 10^6, Pharmacia) solution (100 mg/ml) was added to a final concentration of 2.5 mg/ml. After 3 min, 0.4 ml of RNA solution in STE buffer (0.1 M NaCl, 0.01 M Tris-HCl, pH 7.5, 0.0001 M EDTA) was added to 1.1 ml of the cell suspension. RNA was left to adsorb under shaking for 60 min at room temperature. Then the infected cells were mixed with an equal volume of 1% nutrient agar overlay heated at 70 °C (Shirman *et al.*, 1973) and distributed in 1.2 ml volumes on the surface of a solid agar layer in plastic Petri dishes. After 2 days of incubation in a 5% CO_2 atmosphere at 37 °C the plaques were counted. RNA concentration was measured by radioactivity, and specific infectivity per μg RNA was calculated based on the known value of specific radioactivity.

Results

When assaying EMC vRNA infectivity by the method of Bishop and Koch (1969) based on pretreatment of the cells with poly-L-ornithine and DEAE-dextran and cocultivation of the infected cells with the indicator culture, the specific infectivity was low (approximately 10^3 PFU/μg) and the number of plaques formed did not depend linearly on RNA concentration. Such a result may have been due to the toxic effect of RNA. To reduce it, the concentration of the infected cells could be increased. But this was prevented by aggregation of the cells pretreated with poly-L-ornithine at high cell concentrations.

Like Bishop and Koch (1967) I found that the addition of poly-L-ornithine did not affect the infectivity of RF RNA but, as distinct from the results of

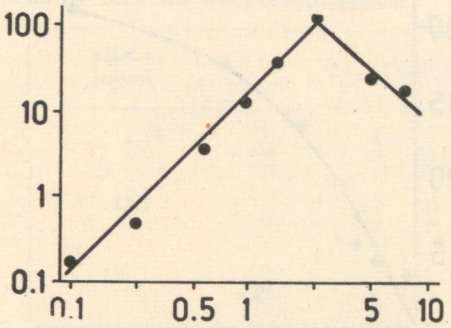

Fig. 1.
Relationship between specific infectivity of RF RNA (PFU per count/min; ordinate) and DEAE-dextran concentration (μg/ml; abscissa)

these authors, in my system it did not enhance vRNA infectivity. This finding made it possible to markedly increase the concentration of cells to be infected, up to a maximum of 10^8 cells/ml (at higher concentrations the suspensions became too viscous). At the same time, indicator cells were no more used, i. e. the infected cells were directly seeded on the agar layer to determine the number of plaques.

With increasing cell concentration the optimal concentration of DEAE-dextran should evidently change. To this end washed cells were suspended in PSM buffer to a concentration of 1.36×10^8 cells/ml. Different concentrations of DEAE-dextran were added to several portions of cells; after 5 min of incubation at room temperature, 1.1 ml cell samples were mixed with 0.4 ml RNA solution in STE buffer. After 30 min adsorption at room temperature, the cells were seeded on the agar layer as described in "Materials and Methods". RF RNA specific infectivity was directly proportional to the square of DEAE-dextran concentration (Fig. 1). The optimal DEAE-dextran concentration was 2.5 μg/ml. At higher concentrations plaques become indistinct, probably because of the toxic effect of the polycation. The results obtained in vRNA infectivity assay were similar.

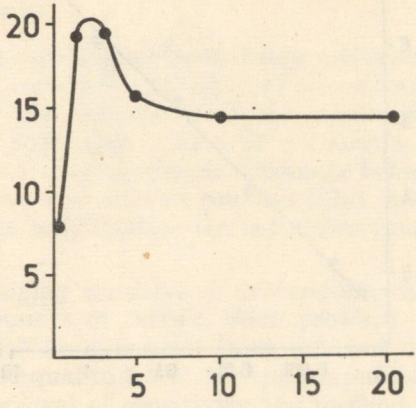

Fig. 2.
Relationship between specific infectivity of RF RNA and the time of pretreatment of cells with DEAE-dextran
Abscissa: time in min; ordinate: specific infectivity (PFU/μg $\times 10^{-5}$)

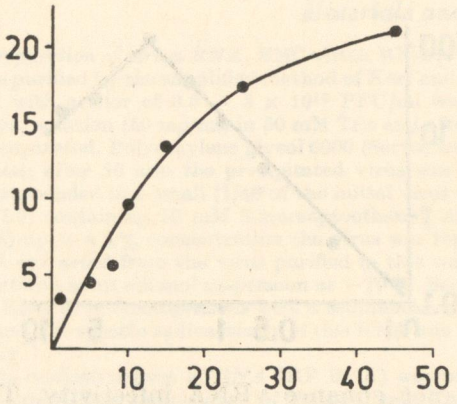

Fig. 3.

Relationship between the specific infectivity of RF RNA and the time of RNA adsorption by sensitized cells
Abscissa and ordinate as in Fig. 2.

In the next experiments, the optimal time of cell sensitization (the time of cell contact with DEAE-dextran before the addition of RNA) and RNA adsorption was determined (Figs 2 and 3). The optimal time of sensitization was 3 min. RNA adsorption was complete within 45—60 min. Under these conditions the specific infectivity of vRNA was 3×10^8 and that of RF RNA 2×10^6 PFU/μg.

In quantitation of RNA infectivity it is very important to retain the linear relationship between the amount of input RNA and the number of plaques formed in the maximum range of concentrations. In other words, the toxic effect of RNA should be avoided. To detect any deviations from the linear relationship at high RNA concentrations, cells were infected with various amounts of RF RNA and in those cases where the expected number of plaques was too high, the infected cells were appropriately diluted with a suspension

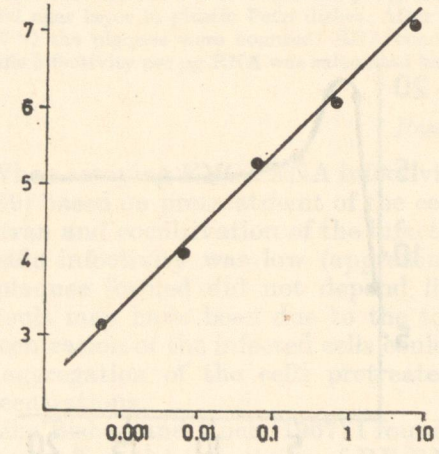

Fig. 4.

Relationship between the number of infectious centres formed and the amount of input RNA. Abscissa: RF RNA concentration (μg/ml); ordinate: log PFU/ml

Table 1. The influence of carrier RNA on RF RNA infectivity determinations

rRNA μg/ml		No. of plaques	rRNA μg/ml		No. of plaques
0	I	1, 1	1.17	I	1, 2
	II	20, 12		II	17, 15
0.0117	I	1, 2	11.7	I	1, 2
	II	16, 20		II	12, 17
0.117	I	2, 1	117	I	2, 1
	II	11, 10		II	17, 19
				III	0, 0

I, II, III: RF RNA concentrations — 0.05, 0.5 and 0 count/min per ml, respectively.

of uninfected unsensitized cells. The results (Fig. 4) showed that the linear relationship was retained up to the maximum concentration used (7 μg/ml). Since RF RNA is available in limited amounts, determination of the minimal toxic concentration in such an experiment is impossible. In an attempt to determine the minimal toxic concentration ribosomal RNA (rRNA) from Krebs-II cells was used. Despite the fact that rRNA toxicity may differ from that of vRNA or RF RNA, this experiment was of interest because infectivity of RNA has frequently to be assayed in mixtures containing a great excess of cellular RNAs. The results of titration of RF RNA in the presence of various concentrations are presented in Table 1. No toxic effect of rRNA was observed up to the maximum concentration used (117 μg/ml).

A combination of high specific infectivity of RNA with a lack of the toxic effect permits synchronous infection of the entire cell population. This was confirmed in an experiment (not show) in which at least 70% of the cells were infected.

Discussion

The specific infectivity of EMC vRNA determined by the proposed method of 3×10^8 PFU/mg corresponds to the infectivity of 0.1% of all molecules. A comparable value was reported for the infectivity of the whole EMC virus (Burness, 1970). The finding of such high values is of interest for several reasons, primarily because the lack of a significant difference between the specific infectivity of the whole virus and that of purified RNA means that, under certain conditions, the latter may initiate the infectious process as effectively as whole virus.

The proposed method proved to be highly sensitive in determining RNA infectivity in the presence of large amounts of carrier. Such problems are frequently encountered in studies on RNAs extracted from infected cells. Taking the RNA content in one cell to be equal to 2.5×10^{-5} μg (unpublished data), it may be calculated that at this level of sensitivity the method can

detect 1 molecule of infectious RNA in 2×10^6 cells. Besides, the high efficiency of infection makes it possible to study the RNA-induced infectious process under conditions of synchronous infection.

The observed square relationship between the specific infectivity of RNA and DEAE-dextran concentration was noteworthy. I found a similar relationship on analysis of the date reported by Bishop and Koch (1967). This indicates that this relationship is not a peculiarity of the present system. The implication of this observation is obscure; it may be assumed that in the cell — RNA interaction DEAE-dextran is involved as in a second order reaction.

Another interesting observation was the ratio of specific infectivities of vRNA and RF RNA. In the present system the former was by 3 orders of magnitude more infectious than the latter, whereas in the system of poliovirus RNA — HeLa cells the reverse is true (Bishop and Koch, 1967). I assume that in the latter case vRNA infectivity was somewhat reduced, possibly because of the presence of a higher nuclease activity. The lower infectivity of RF RNA as compared with that of vRNA may possibly reflect the fact that the initiation of infection by RF RNA requires the functioning of some mechanism including cell transcription (Koch *et al.*, 1967; Perez-Bercoff *et al.*, 1974). Another alternative is a limited penetration of RF RNA into the cell. The investigation of these problems is now under way.

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