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INFLUENCE OF THE COMPOSITION OF OVERLAY MEDIA ON THE PLAQUE FORMATION BY SOME ECHOVIRUSES

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Summary. — Plaque formation under the overlay with agar, starch, methyl- (M-) cellulose or carboxymethyl- (CM-) cellulose was studied in 6 clones of echoviruses 7 and 13. The use of agar and starch yielded identical results in the majority of clones. Smaller or no plaques were formed under overlays with M- or CM-cellulose.

Introduction

The method of the negative colonies or plaques with the use of agar overlay (Dulbecco, 1952; Dulbecco and Vogt, 1954, 1955) has become widely used in virological research. However, sulphated polysaccharides present in agar can inhibit the reproduction of a number of viruses, echoviruses included (Simon and Dömök, 1963; Barron and Karzon, 1965; Rouhandeh *et al.*, 1965). At the same time, while working with these viruses, some authors succeeded in getting good results by substituting starch of M-cellulose for agar in the overlay (De Maeyer and Schonne, 1964; Dömök and Simon, 1966; Bulatov, 1967*a*, *b*).

The aim of the present work was a comparative study of the plaque formation by clones of echoviruses 7 and 13 under the overlay of the different composition.

Materials and Methods

Viruses. Six clones of echovirus types 7 and 13, obtained by plaque selection under agar (Podoplekin, 1967), were investigated. The clones differed from one another in plaque size and represented a homogeneous population in this respect.

Cell culture. The viruses were propagated and tested in human embryo cell cultures cultivated in 100-ml flasks (WAKO, Japan). As growth medium, Hanks' solution supplemented with 0.5% of lactalbumin hydrolysate (LAH) and 10% of bovine serum was used.

Overlay media. Four types of overlay were used: with 1.5% Bacto-agar (Difco Laboratories, U.S.A.); with 20% hydrolyzed starch (Starch plant, Zaspensk, U.S.S.R.); with 1% M-cellulose (Institute of synthetic resins, Vladimir, U.S.S.R.); with 1% CM-cellulose (sodium salt; Factory of photographic materials "Dinamo", Leningrad, U.S.S.R.). The rest of the overlay ingredients was identical in all cases: tenfold concentrate of Earle's solution — 18.6%; thrice distilled water — 64%; bovine serum — 4%; aminopeptide (hydrolysate of cattle blood proteins) — 5%; 1:1000 solution of neutral red — 3%; and 7.5% solution of sodium bicarbonate — 5.4%. An equal volume of the 3% agar solution or 40% starch solution or 2% M- or CM-cellulose solution was added to this mixture. In a number of cases, specified in the text, 0.5 mg/ml protamine sulphate (SPOFA, Czechoslovakia) was added to the overlay. The final results of the plaque titration were read after 72 hours of incubation at 37° C.

Haemagglutination test. The method was already described (Podoplekin, 1963).

Isolation of infectious ribonucleic acid (RNA). The method of Wecker *et al.* (1962) as modified by Podoplekin and Ivanova (1966) was used for RNA extraction and infection of cells.

Table 1. Titres and plaque sizes of clones of echoviruses 7 and 13 under overlays of different composition

Echovirus			Titre (PFU/ml) under overlay containing				Plaque size (mm) under overlay containing			
Type	Strain	Clone	Agar	Starch	M-cellulose	CM-cellulose	Agar	Starch	M-cellulose	CM-cellulose
7	85/4	529	4.0×10^6	4.5×10^5	3.3×10^6	5.0×10^6	3—4	3—4	0.5—0.7	1.5—2
7	85/4	532	1.5×10^6	1.5×10^5	no plaques	0.7×10^6	1—1.5	1.0—1.5	—	0.5—0.7
7	17—VI	156	1.1×10^7	2.0×10^7	no plaques	no plaques	0.5—0.7	1.5—2.0	—	—
7	17—VI	225	3.5×10^7	1.2×10^7	no plaques	2.2×10^7	3—4	3—4	—	0.5—0.7
13	1859	536	6.3×10^7	7.2×10^7	5.8×10^7	6.5×10^7	4—5	4—5	0.5—0.7	1.5—2.0
13	1859	538	2.4×10^7	2.1×10^7	1.6×10^7	2.8×10^7	2.5—3.0	2.5—3.0	0.3—0.5	2.5—3.0

Study of the inhibitory action of M-cellulose. Cell monolayers, grown in flasks, were infected with large doses of virus (10—15 plaque forming units -PFU- per cell), incubated for 15 minutes at 37° C and thoroughly washed. Then 5 ml of a 0.5% solution of LAH was added to one group of flasks. Into the other group, the same volume of medium with 1% M-cellulose was added. After given intervals of time, the medium was removed and the monolayer washed; then maintenance medium of another composition was added, or the cells were taken off from the glass by 0.02% ethylendiamine tetraacetate solution. The amount of cells was calculated. Then the cells were centrifuged, resuspended in distilled water, subjected to three cycles of freezing and thawing, and assayed for virus (the cells intended for RNA extraction were not subjected to freezing and thawing).

Results and Discussion

It was found (Table 1) that the numbers of plaques formed by all the tested viruses were practically the same both with agar and starch overlay. The sizes of the plaques were also the same, except clone 156 of echovirus 7, in which the plaque diameter under starch overlay was 3 times greater than under agar. The addition to agar overlay of 0.5 mg/ml protamine sulphate increased the plaque size of the mentioned clone 2—2.5 times. The plaque size of all the other clones remained unchanged in the presence of this substance. Consequently, the difference in plaque diameter of clone 156 under agar and starch was due to the inhibiting action of sulphated polysaccharides of the agar, which can be neutralized by protamine sulphate.

The overlay with M-cellulose considerably (6—8 times) reduced the plaque size in a number of the viruses tested. The rest of the viruses formed no plaques. If plaques were present, the titre of viruses reached the same values as under the overlay with agar and starch.

The overlay with CM-cellulose did not change the plaque size of clone 538. The diameter of the plaques was reduced 2—6 times in clones 536, 529, 532 and 225. Plaque formation was not observed in clone 156. The plaque numbers under the overlay with CM-cellulose were the same as under agar or starch.

The morphology of the plaque formed under the overlay with agar or M- or CM-cellulose was the same: the plaques were transparent with smooth edges. Under the overlay with starch, some viruses formed indistinct plaques whose edges were not smooth.

The mechanism of the inhibiting action of M-cellulose on plaque formation was examined in detail on echovirus 7 (strain 17-VI, clone 25). The quantitative aspects of the formation of infectious viral RNA, haemagglutinins and full virus in cells incubated with 0.5% solution of LAH and in the medium with 1% M-cellulose were studied.

For these determinations, two time intervals, namely 4 and 6 hours after infection, were chosen. At 4 hours, under the multiple infection of human embryo cells by the viruses tested, the quantity of haemagglutinins is minimal and at 6 hours the maximal titre of infectious virus is reached (Podoplekin, 1968).

It was observed (Table 2) that, under the multiple infection of the cells, the titre of the virus at the same time intervals was with LAH 70—200 times higher than in the medium containing M-cellulose. At the same time, the titre

Table 2. Titres of infections virus, RNA and haemagglutinins of echovirus clone 225 in cells incubated in media of different composition

Medium and time of incubation	Titre per 10^5 cells			PFU
	Infectious virus (PFU)	RNA (PFU)	HA units	HA units
LAH 4 hr	4.5×10^4	1×10^1	32	1406
LAH 6 hr	2×10^6	6.5×10^2	128	15625
MC 4 hr	6×10^2	1.8×10^1	16	37.5
MC 6 hr	9×10^3	2.2×10^2	128	74.2
LAH 2 hr + MC 2 hr	2×10^3	n. d.	16	125
LAH 2 hr + MC 4 hr	5.9×10^5	n. d.	64	9219
LAH 4 hr + MC 2 hr	8.6×10^5	n. d.	64	13437
MC 2 hr + + LAH 4 hr	7.5×10^5	n. d.	64	11712
MC 4 hr + LAH 2 hr	5×10^5	n. d.	128	3906

LAH — medium with 0.5% lactalbumin hydrolysate; MC — medium with 1% methyl-cellulose; n. d. — not done.

of infectious RNA, extracted by phenol from the infected cells, was practically the same in both cases.

The composition of medium did not influence the accumulation of viral haemagglutinins. Their titres at equal intervals after infection were approximately the same for LAH and M-cellulose media (32 and 16 units/10^5 cells at 4 hours; 128 and 128 units at 6 hours after infection). In LAH medium, for one unit of haemagglutinins there were 1406 and 15625 PFU of virus at 4 and 6 hours after infection, respectively. In M-cellulose medium, these values were only 37.5 and 74.2 PFU.

When the infected cells were incubated for 2 hours with LAH and then for 2 or 4 hours with M-cellulose, this ration was equal to 125 and 9219 respectively. On incubating the cells for 4 hours with LAH and for 2 hours with M-cellulose, the ratio was 13437. After incubation of the infected cells for 2 or 4 hours with M-cellulose and subsequently for 2 and 4 hours with LAH, the indicated ratio was equal to 11712 and 3906, respectively.

Therefore we may assume that M-cellulose did not interfere with synthesis of viral RNA and the proteins (haemagglutinins) of the viral capsid, but that it markedly inhibited the process of formation of complete virions. This effect was more marked between the 2nd and 4th hour of the infection and less so during the first 2 hours and after the 4th hour.

The mechanism of the phenomenon described is not yet clear, bur the data obtained allow an explanation of the cause of the diminution of the plaque sizes under the overlay with M-cellulose. Since plaque formation is a multistage process of the spread of infection from one cell to another, the velocity of infection spread and consequently the number of the infected cells for unit of time is diminished under the conditions of inhibition of infectious virus synthesis in each of these cells. This number may be so small that some-

times (in our case with clones 532, 156 and 225) macroscopically detectable focuses of dectruction are not found.

A significant diminution of the plaque size of the virus of vesicular exanthema of swine or complete absence of plaques under an overlay with 1.5% M-cellulose was noted by Zee Yuan Chung *et al.* (1967). Only after reducing the amount of M-cellulose in the overlay up to 1.05% were these authors able to obtain distinct plaques with all types of this virus tested.

We found no data concerning the inhibiting action of M-cellulose against echoviruses. Bulatov (1967*a*) considers that the titration of echoviruses under the overlay with M-cellulose gives the same results as titration under agar overlay. He does not give any comparative data as to the size of plaques under different overlays. Moreover, Bulatov worked with non-cloned virus populations and with another tissue culture (monkey kidney cells). Therefore, it is impossible to compare his results with the present ones.

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