STUDIES ON THE INTERACTION BETWEEN COXSACKIEVIRUS A9 AND HELA CELLS

II. MODE OF GROWTH OF COXSACKIEVIRUS A9 IN HELA CELL CULTURES AND THE EFFECT OF SULFATED POLYSACCHARIDE ON PLAQUE FORMATION

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SUMMARY: For the purpose of clarifying the mechanism of plaque formation in HeLa cell cultures by coxsackievirus A9, which does not show definite CPE in fluid cultures, we investigated the growth pattern of the virus in HeLa cells, comparing plaque (HeLa)-forming and non-plaque (HeLa)-forming viruses. It was revealed that the yield of both viruses per cell was nearly the same, but non-plaque (HeLa)-forming virus was far less efficient in infecting HeLa cells. Dextran sulfate was effective in releasing more virus from cells, when HeLa cell cultures were infected with plaque (HeLa)-forming virus, but not in cultures infected with non-plaque (HeLa)-forming virus. From these experimental results, the mechanism by which plaques are formed in HeLa cell cultures by coxsackievirus A9 was discussed.

INTRODUCTION

Coxsackievirus A9 (CA 9 virus) has been reported to be noninfective to HeLa cells. McLaren, Holland and Syverton (1960) reported that the resistance of HeLa cells to CA 9 virus infection is correlated with the absence of the receptor active in the primary adsorption of this virus. Takemoto and Habel (1959) showed that the resistance of HeLa cells to CA 9 virus infection was relative, since virus carrier cultures could be established and even when a relatively large amount of virus was present, only a small proportion of the cells was infected at any time.

In a previous report (Matsumoto et al., 1976), it was shown that most of the fresh isolates of CA 9 virus in MK cells formed plaques in HeLa cell cultures, although they did not show CPE in fluid cultures.

To get more information on the interaction between CA 9 virus and HeLa cells, experiments were carried out to characterize the plaque(HeLa)-forming virus* and the non-plaque(HeLa)-forming virus* by comparing their growth

* The explanation of these viruses appeared in the previous report (Matsumoto et al., 1976).
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pattern in HeLa cells, the efficiency of infecting HeLa cells, as well as the effect of dextran sulfate on the growth of virus. The present report deals with the results obtained.

MATERIALS AND METHODS

Cells and media: These items were described in the previous report (Matsumoto *et al.*, 1976).

Viruses: CA 9 virus (strain 64-M641; hereafter abbreviated as 641) isolated from a throat swab of a patient suffering from febrile illness was used. The third passage of virus in MK cells and its two derivatives were employed for the experiments. One derivative (56M) was obtained by three consecutive plaque purification in HeLa cells and the other without plaque(HeLa)-forming ability (17M) was obtained by two consecutive plaque purification in MK cells. Poliovirus type 1 (Mahony) grown in MK cells and HeLa cell-adapted herpes simplex virus (HF strain) at the 176th passage were also used. The latter was received by courtesy of Dr. K. Yoshino, Institute for Medical Sciences, University of Tokyo.

Antisera: Rabbit serum against herpes simplex virus type 1 (anti-HF virus) was received from Dr. K. Yoshino and that against poliovirus type 1 was prepared in our laboratory. Horse antiserum to CA 9 virus was received from Dr. J. L. Melnick, Baylor College of Medicine, Houston, U.S.A.

Dextran sulfate: The sodium salt of dextran sulfate (DS), molecular weight 2×10^6 , was purchased from Pharmacia, Uppsala, Sweden.

Agar: Bacto agar (Difco) and agarose (Nakarai and Wako) were used at a final concentration of 1.5% and 0.9%, respectively.

Agar extract: Three per cent Bacto agar gel in distilled water was broken up with a spatula in a centrifuge tube and centrifuged at 12,000 rpm for 30 min. The clear supernatant was used as a 3% agar extract.

Growth experiment: HeLa cell bottle (200 ml) cultures containing 1.5 to 3×10^6 cells were infected with 0.3-ml amounts of each of serial ten-fold dilutions of virus, two bottles being used per dilution. After 1.5-hr adsorption at 36 C, each culture was washed three times to remove unadsorbed virus, and treated with 0.5 ml of antisera (40 units/0.1 ml) for 20 min. The culture was again washed, added with 15 ml of YLE medium (bicarbonate 0.22%), and incubated at 36 C. To observe the effect of DS on the growth of virus in fluid cultures, DS at a final concentration of 100 μ g/ml was added to maintenance medium. Cultures were observed daily for the appearance of CPE and 1 ml of culture fluid was harvested from each bottle at specified intervals. Infectivity of each pooled sample was determined by plaque titration in MK and HeLa cells. The procedures of plaque assay appeared in the previous report (Matsumoto *et al.*, 1976).

Immunofluorescence: Six-week old, female albino mice were immunized with a 20% homogenate of the torso of suckling mice infected with the prototype strain. Three intraperitoneal injections, 0.5 ml each, were given to each

mouse at 3-day intervals, and 1.0 ml of the antigen was inoculated 3 weeks after the first injection, being followed by intraperitoneal inoculation of Ehrlich ascitic tumor cells the next day. Ascitic fluid harvested 7 days later served as antibody for dye conjugation after the procedures of Kawamura (1969). Aliquots of two coverslip cultures of HeLa cells infected with CA 9 virus were harvested at specified time intervals, fixed with acetone for 20 min at room temperature and stained for immunofluorescence. The specificity of staining was checked by the blocking of fluorescence in the infected coverslips pretreated with the international reference serum against CA 9 virus.

Assay of infective centers: The number of infective centers was determined by a modification of the method of Simon and Kirvaitis (1965). Pairs of HeLa cell monolayers were infected with appropriate virus dilutions. After 1 hr at 36 C, the monolayers were washed with PBS and treated with antiserum. Then the cells were dispersed with PBS containing 0.02% EDTA and 0.025% trypsin. The cells taken from each group of two bottles were suspended in 2 ml of maintenance medium and diluted further; 0.2 ml each of appropriate dilutions was added to MK cell monolayers. After 30 min at 36 C, the monolayer was covered with 2 ml of nutrient medium containing 1% agar. After it had solidified, an additional 5 ml of ordinary agar medium was added carefully so as not to disturb the initial agar surface. Care was taken to complete the procedure within 3 hr after infection, so that newly produced virus did not disturb the plaque count.

Adsorption test: To examine the effect of dextran sulfate (DS) on virus adsorption, the stock virus was diluted with YLE medium containing 100 $\mu\text{g/ml}$ of DS and inoculated into three bottles per variable adsorption time. After 15, 30, 60 and 120 min, overlay medium was added; plaques were counted 3 to 4 days after inoculation.

Single cycle growth experiment: HeLa cell tube cultures were washed twice with PBS and 0.2 ml of a virus suspension (Moi, 2 PFU(HeLa)/cell) was inoculated into each. After one hour adsorption of virus at 36 C, the cell sheet was washed with PBS and 1.0 ml of YLE medium with or without 100 $\mu\text{g/ml}$ DS was added. At specified intervals, three tubes from each group were taken out; fluid was collected, pooled and centrifuged at 1,500 rpm for 10 min. The supernates served for the assay for free virus. After removal of fluid, cells were washed three times with PBS and 1.0 ml of maintenance medium was added (YLE, bicarbonate 0.22%). After freezing and thawing for three times, the cell suspension was harvested and centrifuged at 2,500 rpm for 20 min. The supernates were assayed for cell-associated virus.

Fig. 1. Growth curves of coxsackievirus A9 in HeLa cell cultures.

The upper charts (Fig 1A) show the growth curves of a plaque (HeLa)-forming virus, strain 641 MK3. The middle charts (Fig. 1B) show those of a plaque (HeLa)-purified virus (56M)

RESULTS

Growth Curve of Plaque(HeLa)-forming Virus and Non-plaque(HeLa) Forming Virus in HeLa Cell Cultures

To investigate the growth pattern of CA 9 virus in HeLa cells under liquid medium, three strains, 641, 56M and 17M, were chosen as shown in the "MATERIALS AND METHODS". The infectivity of these viruses in terms of PFU/0.2 ml in MK and HeLa cells is shown in Fig. 1. Serial ten-fold dilutions of the stock virus were inoculated into HeLa cell cultures, and 1.0-ml aliquots of infected culture medium harvested at specified time intervals were assayed in MK and HeLa cell cultures in parallel. When assayed by PFU in MK cell cultures, virus in the medium, in general, started to rise at 9 hr post inoculation(p.i.), indicating a significant release of progeny virus. The minimal effective inoculum dose in terms of PFU (MK cell) showing an evidence of significant virus growth in HeLa cells was $10^{3.3}$ PFU/0.2 ml for 641 strain, $10^{1.9}$ PFU/0.2 ml for 56M strain and $10^{3.6}$ PFU/0.2 ml for 17M strain. As shown in Fig. 1, the pattern of the growth of 17M strain in HeLa cell cultures was different from that of the other two viruses. With the latter two viruses containing plaque(HeLa)-forming virus, infectious virus assayed by plaques either in MK cells or in HeLa cells increased gradually to reach a peak 5 to 7 days, p.i., although infectivity assayed in MK cells was always higher than that assayed in HeLa cells. On the contrary, with 17M strain containing no detectable plaque(HeLa)-forming virus, a growth pattern similar to the one-step growth curve was obtained with a peak titer mostly one day p.i. when virus was assayed in MK cells, whereas increase of plaque(HeLa)-forming virus was hardly observed. The results thus indicated that the amount of plaque(HeLa)-forming virus contained in an inoculum was critical for the growth of progeny viruses of similar characteristics in HeLa cell cultures.

Immunofluorescence of Infected HeLa Cells

To study further the time course of CA 9 virus infection in HeLa cell fluid cultures, the direct fluorescent antibody technique was applied. Two strains of CA 9 virus, 641 and 17M, used in the preceding experiment were inoculated into HeLa cell cultures on coverslips. Cells with specific fluorescence were first seen 4 hr p.i. and increased in number by 6 to 7 hr p.i. Fluorescence was seen at this time in single cells (Fig. 2, photos. A and B). From 24 to 48 hr p.i. clusters of fluorescent cells were observed in cultures infected with the plaque-

obtained from the original virus. The lower charts (Fig. 1C) show those of a non-plaque (HeLa)-forming virus (17M MK2) derived from the original virus. MK or HeLa cells were infected with various dilutions of virus; 10^{-1} dilution ($\circ$ — $\circ$); 10^{-2} dilution ($\bullet$ — $\bullet$); 10^{-3} dilution ($\square$ — $\square$); 10^{-4} dilution ($\blacksquare$ — $\blacksquare$); 10^{-5} dilution ($\triangle$ — $\triangle$); 10^{-6} dilution ($\blacktriangle$ — $\blacktriangle$); 10^{-7} dilution ($\times$ — $\times$).

The infectivity of each virus used for inoculation is shown by an open column (PFU/0.2 ml in MK cells) or a shadowed column (PFU/0.2 ml in HeLa cells).

(HeLa)-forming virus, strain 641 (Fig. 2, Photo. C). In cultures infected with the non-plaque(HeLa)-forming virus, strain 17M, however, fluorescent cells were no more detectable by 24 hr p.i. The results may suggest that infection by the former virus spread to a limited number of neighboring cells, while further transmission of infection did not take place from single cells primarily infected with the latter virus.

*Effect of Antiserum in Agar Overlay on the Development
of Plaques by CA 9 Virus in HeLa Cell Cultures*

The results of the preceding immunofluorescence experiment appeared to suggest that the mode of spread of 641 strain in HeLa cell cultures was characterized by cell-to-cell transmission of virus, which is usually seen with herpes viruses, but not with enteroviruses (Black and Melnick, 1955; Stoker, 1958). To solve the problem, effect of immune serum incorporated in agar overlay was examined. Poliovirus and herpes simplex virus served as controls. HeLa cell bottle cultures each were infected with 0.2 ml of a virus suspension containing approximately 50 PFU(HeLa) per 0.2 ml. After adsorption of virus at 36 C for 90 min, each culture was washed three times with PBS and overlaid with 5 ml of agar medium containing the homologous antiserum at a specified concentration. The results were read 4 days p.i. with CA 9 virus or poliovirus, 7 days p.i. with herpes simplex virus. The presence of antiserum did not influence the plaque formation by herpes virus but reduced greatly the number and size of plaques formed by poliovirus and CA 9 (data not presented). This may exclude the possibility of cell-to-cell spreading of CA 9 virus in HeLa cell cultures.

Yield of Coxsackievirus A9 in HeLa Cells

To investigate the different patters of growth of plaque(HeLa)-forming and non-plaque(HeLa)-forming viruses in HeLa cell cultures, the following experiment was designed. As a plaque(HeLa)-forming virus, the 641 strain harvested from HeLa cell cultures 48 hr p.i. was used and the stock virus of the 17M strain served as a representative of non-plaque(HeLa)-forming virus. Pairs of HeLa cell monolayers were each inoculated with 0.2 ml of a specified dilution of each virus. After adsorption of virus and treatment of cultures with immune serum, they were maintained under serum-free maintenance medium and virus yield 8 hr p.i. was determined by plaque count in MK cell cultures. Infective centers were also assayed in MK cell cultures. As shown in Table I, no significant difference was observed between the two viruses in the total or per cell yield of infectious virus 8 hr p.i. if the amount of virus for inoculum was controlled so

Fig. 2. Immunofluorescence of HeLa cells infected with coxsackievirus A9.

- A. Photographed 6 hr after infection with a plaque (HeLa)-forming virus (64-M641 MK3).
- B. Photographed 6 hr after infection with a non-plaque (HeLa)-forming virus (17M MK3).
- C. Photographed 24 hr after infection with a plaque (HeLa)-forming virus (64-M641 MK3).

TABLE I

Comparison of the per cell yield of infectious virus in HeLa cell cultures by plaque (HeLa)-forming virus and non-plaque (HeLa)-forming virus

Virus	Exp. No.	Inoculum PFU/0.2ml ($\log_{10}$)		Infective center	Total yield	Yield/ cell (PFU)	Adsorption efficiency
		HeLa	MK	MK (PFU)	MK (PFU)	8 hr	
64-M641 MK3	I	1.3	3.7	1.3	5.0	3.7	$1:10^{2.4}$
	II	2.3	4.7	2.4	6.0	3.6	$1:10^{2.3}$
66-17M MK2	I	—*	5.6	1.5	4.2	2.7	$1:10^{4.1}$
	II	—	6.6	2.3	5.9	3.6	$1:10^{4.3}$

Plaque (HeLa)-forming virus, strain 641, was the released virus from HeLa cells shown in Fig. 1A (a). Non-plaque (HeLa)-forming virus was 17M MK2 virus obtained by two passages in MK cells after MK plaque purification.

Adsorption efficiency was calculated dividing the inoculum by the number of infective center.

* —: plaques were not produced in HeLa cell cultures inoculated with undiluted virus.

that the number of infective centers was nearly the same in cultures infected with either virus. The efficiency of adsorption of virus to HeLa cells was very low with both viruses, above all that of non-plaque(HeLa)-forming virus being from one-fiftieth to one-hundredth of that of plaque(HeLa)-forming virus. These results may suggest that the different patterns of growth of these two viruses in HeLa cell cultures might be accounted for by the difference in the efficiency of adsorption of virus to host cells.

Effect of Sodium Dextran Sulfate (DS) on the Growth of CA 9 Virus and the Development of CPE in HeLa Cell Cultures

Previous experiments showed that CA 9 viruses multiplied in HeLa cell cultures under fluid medium producing a considerably high yield of virus per cell when titrated in MK cells. The question remained why CA 9 virus could form plaques in HeLa cell cultures under agar overlay, while no definite CPE could be observed in infected cultures under fluid medium. As mentioned above, low efficiency of adsorption of CA 9 virus to HeLa cells was considered to be responsible for this apparently inconsistent phenomenon. In order to get more information on this point, the effect of sulfated polysaccharide contained in agar was investigated. Sodium dextran sulfate (DS) was used for these experiments as a substitute for sulfated polysaccharide in agar.

The first experiment was designed to elucidate the relation between concentrations of DS and the development of CPE by CA 9 virus in HeLa cell fluid cultures. As shown in Fig. 3, DS enhanced CPE by 63-G13-5 strain of CA 9 virus as its concentration increased. When 100 $\mu\text{g/ml}$ DS was added to the medium, the infectivity in terms of TCID_{50} increased so much as to approximate that expressed in terms of PFU. The effect of DS was not observed with a non-plaque(HeLa)-forming virus, 17M.

The effect of DS on the growth of CA 9 virus in HeLa cell cultures was then investigated by incorporating the drug in the maintenance medium during multi-cycle growth of the virus. As shown in Fig. 4, in plaque(HeLa)-forming virus, the amount of virus released up to 9 hr p.i. was almost identical with or without DS. At 24 hr p.i. and thereafter enhanced virus release was observed in the cultures with DS and 4 to 5 days later the titer of free virus was nearly $3 \log_{10}$ higher than that in the control cultures without DS. Cytopathogenic effects were observed in the cultures with DS 3 days p.i. and most of the cells were destroyed in 7 days. In the cultures without DS, however, cell destruction was very limited up to this time. On the other hand, the effect of DS was not observed on the growth of non-plaque(HeLa)-forming virus (Fig. 4).

These results indicate that DS enhanced the growth of plaque(HeLa)-forming CA 9 virus in HeLa cell cultures, while the drug showed neither enhancing nor inhibitory effect on the growth of non-plaque(HeLa)-forming CA 9 virus in the same cell cultures.

*Effect of DS on the Intracellular Growth and Release of
CA 9 Virus in HeLa Cell Cultures*

It was shown that DS has an enhancing effect on the growth of plaque(HeLa)-forming virus in HeLa cell cultures. In order to determine at which stage of

Fig. 3. Effect of dextran sulfate on the appearance of CPE by coxsackievirus A9 in HeLa tube cultures.

Maintenance media containing various concentrations of DS were added to monolayers of HeLa tube cultures after washed with PBS. Serial ten-fold dilutions of a plaque (HeLa)-forming virus, strain 63-G13-5 (●—●), and a non-plaque(HeLa)-forming virus, strain 17M MK2 (○—○) were inoculated at 0.2 ml/tube. Results (CPE) were read 7 days after inoculation. The broken line (.....) shows the PFU (HeLa)/0.2 ml of 63-G13-5 virus strain.

Fig. 4. Effect of DS on the growth of coxsackievirus A9 in HeLa cell cultures. Infectivity of the supernates of culture fluids harvested at specified intervals p.i. in the presence (●—●) or absence of DS (○—○) was assayed in HeLa (left) or MK (right) cell cultures.

the growth of virus DS was effective, adsorption tests were carried out. It was found that DS did not enhance virus adsorption to HeLa cells (data not presented). Then the effect of DS on the single cycle growth of CA 9 virus was studied. Figure 5 shows the time course of the production of cell-associated virus and free virus in HeLa cell cultures with or without DS. In both cultures, the increase of cell-associated virus was the same. However, at 10 hr p.i. and thereafter more virus was released in cultures with DS than in cultures without DS. These results suggest that DS has no effect on the intracellular growth of virus but some enhancing effect on the release of virus from infected HeLa cells.

Effect of DS on Plaque Formation by CA 9 Virus in HeLa Cell Cultures

The effect of DS on the plaque formation by CA 9 virus in HeLa cell cultures was then investigated. Various concentrations of DS (from 25 $\mu\text{g/ml}$ to 100 $\mu\text{g/ml}$) were added to the overlay medium containing 1.5% Bacto agar or 0.9% of agarose. As shown in Table II, DS did not influence plaque formation under Bacto agar. However, when agarose was used as a substitute for Bacto

Fig. 5. Effect of DS on the growth and release of coxsackievirus A9. The maintenance medium of infected HeLa cell tube cultures was harvested at indicated times p.i. (strain, 64-M641 MK3), cells were then washed three times with PBS and YLE medium was added to prepare the suspension of cell-associated virus. Infectivity was assayed by the plaque method in HeLa cells. Cell-associated virus in the absence of DS (○—○), Cell-associated virus in the presence of DS (●—●), Fluid virus in the absence of DS (△—△), Fluid virus in the presence of DS (▲—▲).

TABLE II

Effect of DS on plaque formation by coxsackievirus A9 in HeLa cell cultures

Virus strain	Agar used	DS in overlay ($\mu\text{g/ml}$)				
		0	25	50	70	100
64-M641 MK3	Bacto agar	3.0	3.3	3.4	3.2	3.5
	agarose	—*	3.3	3.3	3.5	3.4
66-17M MK2	Bacto agar	—				—
	agarose	—				—

Infected HeLa bottle cultures (2-oz bottle) were overlaid with nutrient medium containing Bacto agar or agarose. Plaque number was read 4 days after inoculation.

* —: plaques were not produced in HeLa cell cultures inoculated with undiluted virus.

agar, plaque formation was observed only when DS was added. Agar extract was also effective when added to agarose overlay as shown in Table III.

With non-plaque(HeLa)-forming virus, plaque formation was not observed even under overlay medium containing DS at 100 $\mu\text{g/ml}$ (Table II).

TABLE III

Effect of agar extract on plaque formation by coxsackievirus A9 in HeLa cell cultures

Virus	Conc. of agar extract in overlay medium (%)	PFU/0.2 ml (log ₁₀)	Size (mm)* ± SD
64-M641 MK3	0	<2.0	
	0.125	2.5	4.9±0.87
	0.25	2.3	4.4±1.03
	0.5	2.6	7.7±1.04

* Average of 20 plaques.

DISCUSSION

Experiments were carried out to differentiate between plaque(HeLa)-forming and non-plaque(HeLa)-forming CA 9 viruses. It was revealed that there are marked differences between them, when examined in HeLa cell cultures; the pattern of growth of virus, transmission of matured virus to neighbouring cells, efficiency of adsorption to cells, and the effect of DS on the appearance of CPE, release of matured virus into medium and plaque formation under agar overlay.

From these results, a scheme of the mechanism of plaque formation by CA 9 virus in HeLa cell cultures has been proposed as shown in Fig. 6.

If monolayer cultures of HeLa cells are infected by plaque(HeLa)-forming virus and an efficient infectious center is formed, a considerably large amount of progeny virus will be produced from this cell, that is $10^{3.6}$ PFU/cell as measured by titration in MK cells. Although infectivity of progeny virus to HeLa cells is much lower than the infective units titrated in MK cells, it is possible that progeny virus can infect at least neighbouring cells, as shown by immunofluorescence. In fluid cultures, however, the infectivity of progeny virus to HeLa cells is too low to induce CPE unless release of matured virus from cells is enhanced by DS. It was suggested that plaque formation by CA 9 virus in HeLa cell cultures under Bacto agar overlay was also accounted for by the effect of sulfated polysaccharide in agar, because plaques were not formed under agarose-overlay medium, unless DS or agar extract was incorporated. On the other hand, non-plaque(HeLa)-forming virus produces nearly the same amount of virus as plaque(HeLa)-forming virus does, but due to a much lower efficiency of adsorption of progeny virus to HeLa cells, the spread of infection to neighbouring cells might be extremely difficult.

The experiments concerning the treatment of cell cultures with DS before inoculation of virus were carried out by Takemoto and Spicer (1965) and Toyoshima and Vogt (1969). Takemoto and Spicer (1965) reported that the produc-

Fig. 6. Scheme of the plaque formation by CA 9 virus in HeLa cell cultures.

tion of herpes virus in treated HeLa cell cultures was significantly less than that in untreated control cells. However, Toyoshima and Vogt (1969) reported that the treatment of chick embryo fibroblasts with DS for one hour before infection had no effect on focus formation by Rous sarcoma virus. We also observed that the pretreatment of HeLa cells with 100 $\mu\text{g}/\text{ml}$ DS for one hour, 2 hr or one day before infection with CA 9 virus had no effect on the plaque formation.

As to the mechanism of the enhancing activity of DS on plaque formation, few reports have appeared. Takemoto and Liebhaver (1962) reported that enhanced plaque formation was observed with type 1 (Mahony) poliovirus under agar overlay containing DS, and that in the presence of DS, viruses were released from the cells earlier and in greater amounts than in the control cultures, as we have observed with plaque(HeLa)-forming virus in HeLa cell cultures.

Concerning the susceptibility of HeLa cells to CA 9 virus it should be answered why the efficiency of infection by CA 9 virus is so low that the rate of infection is one in $10^{2.3}$ MK cell infectious units with plaque(HeLa)-forming virus and one in $10^{4.3}$ or less with non-plaque(HeLa)-forming virus. In the present studies it was not answered whether the low efficiency of infection was due to the low adsorption rate of virus particles to cells or to the low efficiency of penetration or uncoating of adsorbed particles. It will also be necessary to find whether or not any clones of HeLa cells fully susceptible to plaque(HeLa)-forming CA 9 virus could be selected. If such clones could be obtained, further studies would be possible to elucidate the difference in the structure of the coat proteins of virions of plaque(HeLa)-forming and non-plaque(HeLa)-forming virus.

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REFERENCES

- BLACK, F. L. AND MELNICK, J. L. (1955): Micro-epidemiology poliomyelitis and herpes-B infections. Spread of the viruses within cultures. *J. Immunol.*, *74*, 236-242.
- KAWAMURA, A. JR. (1969): Fluorescent antibody techniques and their application. University of Tokyo Press, University park press.
- MATSUMOTO, M., NAKANO, M., NISHIKAWA, F. AND TAGAYA, I. (1976): Studies on the interaction between coxsackievirus A9 and HeLa cells. I. Plaque-forming ability of coxsackievirus A9 in HeLa cell cultures. *Japan. J. Med. Sci. Biol.*, *29*, in press.
- MCLAREN, L. C., HOLLAND, J. J. AND SYVERTON, J. T. (1960): The mammalian cell-virus relationship. V. Susceptibility and resistance of cell in vitro to infection by Coxsackie A9 virus. *J. Exptl. Med.*, *112*, 581-594.
- SICKLES, G. M., MUTTER, M., FLORINO, P. AND PLAGER, H. (1955): Recently classified types of coxsackie virus group A. Behavior in tissue culture. *Proc. Soc. Exptl. Biol. Med.*, *90*, 529-531.
- SIMON, E. D. AND KIRVAITIS, J. L. (1965): Quantitative infection of chick fibroblast cells with low multiplicities of Newcastle disease virus. *Virology*, *26*, 377-378.
- STOCKER, M. G. P. (1958): Mode of intercellular transfer of herpes virus. *Nature*, *29*, 1525-1526.
- TAKEMOTO, K. K. AND HABEL, K. (1959): Virus-cell relationship in a carrier culture of HeLa cell and Coxsackie A9 virus. *Virology*, *7*, 28-44.
- TAKEMOTO, K. K. AND LIEBHABER, H. (1962): Virus-polysaccharide interactions. II. Enhancement of plaque formation and the detection of variants of poliovirus with Dextran Sulfate. *Virology*, *17*, 499-501.
- TAKEMOTO, K. K. AND SPICER, S. S. (1965): Effect of natural and synthetic sulfated polysaccharides of viruses and cells. *Ann. New York Acad. Sci.*, *130*, 365-373.
- TOYOSHIMA, K. AND VOGT, P. K. (1969): Enhancement and inhibition of Avian Sarcoma viruses by polycations and polyanions. *Virology*, *38*, 414-426.