A STUDY OF THE FACTORS WHICH AFFECT THE PLAQUE ASSAY

FOR ADENOVIRUS

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ABSTRACT

A STUDY OF THE FACTORS WHICH AFFECT THE PLAQUE ASSAY FOR ADENOVIRUS

The factors that affect the plaque assay of adenovirus type 5 in KB cells have been elucidated and the optimum features were combined to form an optimum plaque assay system.

In the agar overlay, horse serum, at 10%, was found to be optimum for plaque formation. Neutral red inactivated adenovirus type 5 photodynamically and also inhibited virus multiplication in the dark. Noble agar at 0.6% allowed the formation of the most plaques and auto-claved agar, which contains sulphated polysaccharides, did not inhibit plaque formation by adenovirus, compared to agarose. Proteose peptone was found to be an essential supplement to Eagle's minimum essential medium (MEM) for plaque formation by adenovirus type 5 on KB cells. The pH of the agar overlay was found to be critical and maximum plaque numbers were obtained at pH values between 7.1 and 7.2.

Adsorption of virus to cell monolayers was not influenced by pH in the range 6.6 and 7.5. The efficiency of virus adsorption was apparently increased by culturing KB cells in the presence of kanamycin, but decreased in the presence of DEAE-dextran.

The optimum conditions for assay of type 5 adenovirus allowed the formation of plaques by adenovirus types 3, 8 and 12.

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INTRODUCTION

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It is generally agreed that plaque formation is the most accurate method for assaying virus. However, plaque formation by adenoviruses is relatively difficult to achieve. The plaques require a minimum of 8 days to form and may take up to 28 days before the maximum plaque numbers are reached. This phenomenon is due to the inherent slow growth cycle of the adenovirus. Kjellen (1961) showed that the newly formed virions are not released from the host cells until very late in the growth cycle, thereby delaying the infection of the surrounding cells. This presents the problem of keeping the cell monolayer cultures alive under the agar overlay for the long period required for plaque formation. Due to this difficulty, many researchers have been unable to obtain plaques with adenovirus and therefore have resorted to the less accurate but more easily performed quantal end-point technique for virus assay.

Although some workers have utilized the plaque technique for assaying adenovirus, little work has been done to investigate the factors that affect plaque formation. Due to the necessity for having an accurate assay system for adenovirus, this study was undertaken to define the conditions under which consistent and reproducible results can be obtained, and to elucidate some problems relating to plaque formation by adenoviruses. Type 5 adenovirus was chosen for the study because it is one of the small number of serotypes which forms relatively large plaques in serial heteroploid human cell lines (e.g. Hela, KB).

LITERATURE REVIEW

LITERATURE REVIEW

Dulbecco (1952) introduced the plaque assay for animal viruses as a direct analogy with the plaque assay of bacteriophages (Ellis and Delbruck, 1939). The aims are to assay virus infectivity more accurately and to express virus concentrations in quantitative terms rather than quantal units.

A typical plaque assay consists of the inoculation of a statistically adequate yet easily countable number of infectious virus (say 30-100) particles onto a complete cell monolayer. Each particle is allowed to replicate under conditions where the resulting lesion remains local, and the lesions are finally counted. It has been shown for poliomyelitis viruses (Dulbecco and Vogt, 1954) that each lesion is caused by a single infective particle (or aggregate of particles not divisible by dilution) and this allows the calculation of the infective particle content of the original inoculum. Using this fact, the plaque technique could be used for the isolation of clones of virus from single infective particles for genetic studies and the plaque character used as a genetic marker. The recovery of virus from single plaques provides another way for virus purification.

I. PLAQUE FORMATION BY ADENOVIRUS

Adenovirus plaques were first produced by Bonifas and Schlesinger in 1959. They studied the nutrient requirements for plaque formation by adenovirus and discovered that the plating efficiency was enhanced by increasing the concentration of arginine in the overlay

medium. Larger clearer plaques and a ten-fold or greater increase in plating efficiency resulted when 0.4 to 1.0 mM arginine replaced the 0.1 mM concentration of Eagle's basal medium. There was insufficient arginine in Eagle's basal medium for optimum plaque formation by adenovirus.

Rouse, Bonifas and Schlesinger (1963) found that under conditions of Mycoplasma (henceforth referred to as PPLO) contamination, arginine depletion was greatly accelerated in KB cell cultures. The early exhaustion of arginine was accompanied by a low plating efficiency for adenovirus type 2 plaques. In the presence of increased arginine, PPLO contaminated cells yielded plaque titers as high as those on uncontaminated cells. Therefore, PPLO contamination interfered with the viral infectious process by depriving the cell-virus complex of a single essential nutrient, arginine.

In a later paper, Rouse and Schlesinger (1967) discovered that of all amino acids in Eagle's basal medium, only arginine is essential for the synthesis of infectious progeny virus in KB cells infected with type 2 adenovirus. Their results show that when arginine was restored to the infected KB cell cultures after 28-32 hours of deprivation, infectious virus was made without detectable lag and increased exponentially at the "normal" rate. Using the techniques of immunofluorescence and hemagglutination, they demonstrated that "early" antigen and proteins associated with the hexon and penton fiber subunits were synthesized during the arginine free period. They therefore proposed that arginine plays a specific role in a late synthetic step essential for adenovirus maturation.

Consequently, the depletion of arginine by PPLO in cell

cultures would certainly affect the plaquing efficiency of the adenovirus.
Therefore, it is necessary to control the PPLO growth in contaminated
cell cultures.

Most PPLO are resistant to the commonly used antibiotics in
cell culture e.g. Penicillin and streptomycin. However, in the search
for effective control of PPLO, Brown and Officer (1968) found that
kanamycin was one of the best antibiotics for the control of PPLO con-
tamination, due to the fact that it was the least toxic of the antibi-
otics tested to animal cells in culture and consequently could be used at
high concentrations without killing the cells in the process.

In an attempt to isolate an early/large plaque variant from the
wild type 5 adenovirus, Kjellen (1963) obtained a genetically stable
mutant. The first plaques of the genetically stable variant could be
seen on the 4th day after infection and the numbers increased up to 8 or
9 days, after which there was no further increase in plaque numbers. He
also reported that DEAE-D, when incorporated into the agar overlay, did
not enhance plaque formation.

Green et al. (1967) studied the plaquing efficiencies of puri-
fied human adenoviruses in a KB clonal subline. He found that the adeno-
viruses have an efficiency of plating (eop) smaller than one i.e. the
virion:PFU ratio ranged from 11:1 for adenovirus type 3 to 2,300:1 for
adenovirus type 25. The ratios were based on particle number derived
from chemical analysis rather than the more commonly employed electron
microscope counts. The results obtained were not corrected for adsorp-
tion efficiency variations which do exist e.g. the ratios for types 2 and
5 were 33/1 and 20/1 respectively. For adenovirus type 2 (Ad 2) 85-95%

of the PFU are adsorbed to KB cell monolayers in 60-120 minutes (Philipson, 1967); for adenovirus type 5 (Ad 5) 40-50% are adsorbed in 2 hours (Lawrence and Ginsberg, 1967). Nevertheless, the adsorption efficiency of most of the viruses studied is unknown.

Different serotypes of adenovirus produce plaques of varying sizes. This may be due to the inherent genetic character of the virus. Consequently, the plaque technique provides another method for the characterization and classification of the adenoviruses. Accordingly, Green (1967) tabulated the adenoviruses according to their plaque characteristics. Under the conditions used, the human adenoviruses formed three groups according to plaque size. Adenovirus types 1, 2 and 5 produced large plaques (5 mm.) visible 8-11 days after infection: Ad 12, 18, 31, 11, 14, 16 and 21 formed small plaques (0.5 mm.) 12 to 15 days after infection; and the remaining serotypes formed intermediate-sized plaques (1 to 2 mm.).

Since the plaque is the outcome of an initial encounter of single cells with single virions (Schlesinger, 1969), viral replication is highly asynchronous (Strohl and Schlesinger, 1965 a,b). Moreover, the release of the infectious adenovirus form the host cells is extremely slow. This may explain the observations made by Kjellen (1961) that the plaque numbers increased at a regular rate until about 21 days, after the first plaques were seen on day 8 with adenovirus types 4 and 5.

II. FACTORS AFFECTING PLAQUE ASSAY

1. Factors affecting virus adsorption

In the study of the rate of Ad 5 adsorption to MAS-A or MAS-A1

cells (human bone marrow), Kjellen (1961) found that about 50% of the viruses which form plaques attached after 30-60 minutes, using a 0.3 ml. inoculum on 60 mm. petri plates with 10^6 cells each. For adenovirus type 2, Philipson (1967) reported that 85-95% of the PFU were adsorbed to KB cell monolayers in a period of 60-120 minutes. Their results showed that under the respective conditions, the adsorption rates for adenovirus types 5 and 2 were approximately exponential over the 1 hour adsorption period.

The rate of adsorption also depends to a large extent on the volume of the inoculum. It has been found, for poliovirus, that the rate of adsorption is markedly affected by the volume of the virus inoculum, the virus adsorption rate being inversely proportional to the volume of virus inoculum (Taylor and Graham, 1961). However, they found that a small volume of virus inoculum, e.g., 0.1 ml., would not readily permit uniform distribution of virus over the plate; but when a larger volume of virus containing fluid was used over the monolayer to improve the distribution of virus, the rate of virus adsorption was markedly decreased. Consequently, the efficiency of plating increased with decreasing adsorption volumes. This phenomenon was also described by Bachrach et al. (1957) for foot-and-mouth disease virus.

As a result, a compromise has to be made between the application of small volumes of virus inocula for maximum virus adsorption and the use of larger volumes to permit uniform distribution of virus over the entire cell monolayer.

The effects of DEAE-D on virus adsorption

The plaque titre of certain infectious preparations of nucleic acid, e.g. poliovirus RNA, from animal viruses has been greatly enhanced (up to 100,000-fold) by diethylaminoethyl-dextran (DEAE-D)(Pagano and Vaheri, 1965). However, the addition of DEAE-D to inocula of whole particles either enhances or reduces the plaque titre depending on the virus (Kim and Sharp, 1969). They found that for poxviruses (rabbitpox and vaccinia) the dextran made insignificant improvement in adsorption of the virus particles to the cells, as determined by direct electron microscopic counts of unadsorbed virus, though there was a 12-fold increase in the plaque numbers of rabbitpox virus. This observation implies that in the absence of dextran, some of the virus that adsorbed to the cells were unable to initiate plaques. The DEAE-D particles were not aggregated with the virus particles nor were the virus particles induced to aggregate when observed in the electron microscope. There-fore, they proposed that plaque enhancement by dextran may be a protec-tive action of the dextran particles upon freshly uncoated DNA within the cell vesicle, since the size of the dextran particles is comparable with the virus particles and the particles are probably present at the time of uncoating of the virus in the cell vesicles. However, no evidence was presented to support the proposal. The actual mode of action of DEAE-D on virus adsorption is still dubious.

2. Effects of variations in the composition of the overlay medium on plaque formation

a) Sera

Reports by different researchers have established the fact that

the ability of viruses to form plaques is dependent on both the type and amount of serum in the agar overlay.

Philipson (1961) investigated the effects of different types of sera (chick, calf and rabbit), in varying concentrations, on the production of Ad 2 plaques. He found that an overlay medium in which the serum components consisted of 1% calf and 2% chicken serum appeared to produce the largest number of plaques. No explanation was given for his finding. However, the results show that with increasing concentrations of each serum (with the exception of chicken serum alone), there was a corresponding reduction in plaque numbers. This may indicate the presence of virus inhibitors in the sera used.

Green et al. (1967) modified this overlay medium to contain 6% chicken serum and 6% horse serum instead of calf serum, which was used in the growth medium. This modification was needed because calf serum prevented the plaque formation of adenovirus types 12 and 18. This may be due to a number of reasons. Firstly, there may be some type-specific inhibitors to adenovirus types 12 and 18 in the calf serum, which were absent or present in smaller quantities in the chicken and horse sera. Secondly, there may be additional nutrients in the chicken and horse sera which were absent in the calf serum, though this is less likely.

Lawrence and Ginsberg (1967) used 15% chicken serum and Herman (1968) used 20% fetal bovine serum for their adenovirus plaque assay systems.

In addition to heat-stable virus inhibitors, Smull and Ludwig (1969) discovered a heat-labile factor in fresh bovine serum which inhibited plaque formation by poliovirus in HeLa cell monolayers when the host cells were subcultured several times in growth medium containing the

fresh bovine serum prior to their inoculation with virus.

Due to this unpredictable presence of non-specific virus inhibitors in sera from different sources, some workers have preferred to eliminate serum from the overlay altogether and instead have substituted 0.5% gelatin (Simpson and Hirst, 1961), or 0.25% skim milk (Stinebaugh and Melnick, 1962), or 0.1% serum albumin (Cooper, 1955).

The foregoing review indicates that it is necessary to test different batches of sera for the presence of virus inhibitors before using them for plaque assay, due to the fact that some batches of sera may contain inhibitors against the viruses.

b) Volume of agar overlay

The volume of the agar overlay strongly influences plaque formation by animal viruses. For example, Vogt et al. (1957) showed that an increase in the thickness of the overlay from 0.8 mm. to 1.6 mm resulted in a marked decrease in plaque numbers of poliomyelitis viruses. A further increase in the thickness from 1.6 mm. to 3.2 mm. had a similar inhibitory effect. He, therefore, proposed that a constant volume of agar-overlay and an accurate leveling of the plates during the overlaying were necessary to avoid a variability in the efficiency of plating (eop) both from plate to plate and within the same plate.

Along similar lines, Baron et al. (1961) studied the influence of the thickness of the agar overlay on plaque formation. He demonstrated that the size and number of plaques of Newcastle disease virus diminished as the depth of the agar overlay increased. This may be due to a reduction in the oxidative metabolism of the host cells. To determine whether this effect was due to the diminishing concentration of oxygen in the

deeper portions of the tube, the oxygen concentration was increased by maintaining infected and overlaid cultures in an atmosphere of oxygen. As a result, plaques developed at a much greater depth in the presence of oxygen than in air. Moreover, different viruses were found to require different amounts of oxygen for their growth. Therefore, a thick agar overlay reduces the oxygen available to the cells, which in turn inhibits the multiplication of the virus.

Plaque development may also be inhibited if the overlay is too thin (Dougherty, 1964). The lack of nutrients for the virus and cells was suggested as a possible cause. The optimal thickness of the agar overlay was found to be between 4-8 mm. in a 50 mm. petri dish.

c) Inhibitors in agar

Autoclaved agar is known to contain sulphated polysaccharides which inhibit the formation of some viruses, for example, arboviruses (Liebhaber and Takemoto, 1961), and encephalomyocarditis virus (Takemoto and Nomura, 1960). However, they do not affect the wild type poliovirus (Nomura and Takemori, 1960). Although several postulates have been made as to the mechanism of inhibition by sulphated polysaccharides on viral growth, none of them are universally accepted yet.

Consequently, some workers have attempted to use other solidifying agents, for example, clotted plasma (Mandel, 1958; Underwood, 1959) and methyl cellulose (Schulze and Schlesinger, 1963; Scherer, 1964; Salim, 1968).

Scherer (1964) obtained more plaques, with Japanese B encephalitis virus, when 1.6% methyl cellulose was used instead of 1.5% agar. He concluded that methyl cellulose was the enhancing agent compared to

agar. His conclusion, however, may be erroneous because the high con-
centration of agar used (1.5%) may be inhibitory to plaque formation by
the virus compared to lower concentrations of agar, e.g., 0.6%. This
was shown to be true for influenza virus (Sugiura and Kilbourne, 1965).
Since Scherer used only one concentration of agar, his result is subject
to possible misinterpretation.

Autoclaved agarose, which is free of sulphated polysaccharides
has been widely used in plaque assay work. Bergold and Mazzali (1968)
replaced Noble agar (1.2%) and DEAE-D with 0.5% agarose for the plaque
assay of 52 arboviruses. They found that agarose had the same enhancing
effects as agar with DEAE-D on plaque formation. Moreover, the standard
overlay prepared with 0.5% agarose was much clearer, which allowed
plaque counting without any staining of the BHK21 (baby hamster kidney),
Vero (green monkey kidney) and Wl-38 (human diploid lung) cell sheets.
However, Ventura (1968) found no significant differences, either in
plaque numbers or in size, between the use of agar and agarose, for
plaque formation by Venezuelan encephalitis virus.

The plaque sizes of mengo virus variants under agar, agarose,
methyl cellulose and agar with polyanions were examined by Campbell and
Colter (1965). The plaque size of EMC virus was greatly enhanced when
methyl cellulose was used instead of agar; but the results concurrently
showed that methyl cellulose was not a good medium for use with the
mengo virus-L (mouse) cell system due to the small sizes of plaques
obtained with it. Both M-(medium plaque) and S-(small plaque) mengo
virus variants produced much larger plaques under agarose than they do
under the same concentration of agar. However, L-(large plaque) mengo

produced smaller plaques under agarose than under agar. This contradic-
tory result was explained as being due to the stiffer gels produced by
agarose compared to agar at similar concentrations. If this greater
gelling capacity affected L-mengo virus unfavourably, it is difficult to
understand why it has an opposite effect on the M- and S- mengo viruses.

The incorporation of several polyanions in agar overlays was
shown to have little or no effect on the sizes of plaques produced in
L-cell monolayers by L- and S- mengo, whereas M- mengo plaque size was
greatly affected. There appears to be no simple relationship between the
sulfate content of the polysaccharides tested and their inhibitory or
enhancing effects on M-mengo plaque size. This variability could tenta-
tively be explained as the result of differing sensitivities of each
variant to an agar inhibitor.

Liebhaber and Takemoto (1961) first showed that polycations,
e.g. DEAE-D, could neutralize the inhibitory effect of sulphated agar
polysaccharides on plaque formation by encephalomyocarditis virus. The
plaque size enhancement was accomplished with a concentration as low as
50 μg./ml. of the DEAE-D, with no further increase in plaque size at
higher concentrations. On the other hand, Sugiura and Kilbourne (1965)
demonstrated that concentrations of more than 400 μg./ml. of dextran
were toxic to cells (clone 1-5C-4 derived from a variant line of Chang's
human conjunctival cell). The DEAE-D had a markedly favorable effect on
development of plaques with A1/CAM and Swine/S-15 influenza viruses,
both of which failed to produce visible plaques without DEAE-D.

It was soon found that these polycations could also remove the
inhibitory effects of agar polysaccharides on m-(plaque size) mutants of

poliomyelitis, coxsackie A9 and B4, ECHO 5, 6, 8, 11, 26, 32, foot-and-mouth disease, mengo, group B arbo and herpes viruses (Miles and Austin, 1963; Brown and Packer, 1964).

However, the use of polycationic substances does not always result in improvement of plaque formation by viruses under an agar overlay. Kjellen (1963) found that DEAE-D had no effect on plaque formation by Ad 5.

Wallis and Melnick (1968) claimed that the enhancement of plaque number and size by DEAE-D and protamine is not due to their binding of sulfated polysaccharides in agar as has been assumed before. In the case of simian adenovirus, enhancement by protamine is due to the presence of arginine in digested protamine. Herpes (JES) and echovirus types 3, 4, 5 and 6 are known to be susceptible to agar inhibitors. However, they were found to be enhanced by cationic polymers even under starch gel and methyl cellulose overlays, which are free of polyanions, compared to the respective overlays without cationic polymers.

In virus diffusion experiments, Wallis and Melnick found that cationic polymers enhance the diffusion of virus through agar or starch gel. Therefore, plaque enhancement seems to be the result of the gel becoming positively charged so that viruses can move effectively through it. Where starch gel and methyl cellulose enhanced plaque formation with viruses known to be inhibited under agar, it was because of higher viscosity of the agar gel. When the consistency of the agar gel was reduced from 1.5% to 0.4%, the same plaque counts and sizes were observed under all three overlays. However, the data does not eliminate the possibility that the lowering of the agar concentration also lowers some