Plaque Assay Method for Adenovirus Type 5 with the Culture of HEK Cells Synchronously Infected with the Virus.

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ABSTRACT: The mixture of antiserum and antigen at each adequate dilution was added the seed virus contained X × 10^4 PFU per ml. The mixture was diluted upto the concentration contained 50 to 100 plaques per ml per a dish and 2 ml of the last dilution was made. The last dilution of the mixture was added equal volume of HEK cell suspension contained 2 or 3 ×10^6 cells per ml and shaked at 37°C for 20 or 30 minutes. One ml of these mixture of antiserum, antigen and seed virus was plated into a dish, and 4 ml of maintainance medium contained 0.5% calf serum and 0.75% carboxymethylcellulose was added and spread over in a dish. After incubation for 5 or 6 days, the cell sheet was fixed with 10% formaldehyde saline solution and washed and stained with Gimsa solution. The plaques formed in this method were clear and easy to count. The activity of antigen A, C and P of adenovirus type 5 for the blocking antibody against purified adenovirus type 5 was studied with the application of this plaque assay method.

For the quantitative examination of animal viruses, the plaque assay method was extremely estimated since Dulbecco (1952) had reported it. The plaque formation of animal viruses was usually performed with the agar overlay method. However, the advantaged knowledge of the inhibitory effect of agar on the cell growth and the plaque formation of viruses was shown by Takemoto and Liedhaver (1961). In contrast, the use of carboxymethylcelluolse instead of agar in the overlay medium was shown to assure the uniform results for the plaque formation of certain viruses by Hotchin (1955) and Rapp et al. (1959).

Recently, the plaque formation of dengue viruses and certain group B arboviruses under the carboxymethylcellulose overlay medium was performed and demonstrated excellent results by Schulze and Schlesinger (1963) and Makino and Mifune (1975).

For the plaque assay method, many workers usually used a monolayer of cells, not suspended cells, at the initial inoculation of the virus. It was, however, found that when the adequate number of suspended cells was infected with the virus and plated into

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dishes, the plaque formation in the cell sheet was able to observe after the incubation for several days in this study. Furthermore, the block test was able to perform such as follows: the suspended cells were infected synchronously with the virus survived from the neutralization reaction with the excess antibody in the mixture of antiserum and antigen. This method is useful for the application of block test of the antiserum with antigens. In this paper, the results of the block activity of antigen A, C and P of adenovirus type 5 will be described.

MATERIAS AND METHODS

Seed virus: Adenovirus type 5, AD 75 strain, was propagated in HEK cell culture and the seed virus was partially purified by equilibrium density centrifugation in cesium chloride as described by Russell et al. (1967).

Cells: A line of human embryo kidney cells (HEK) cultured in Eagle's medium containing 10% tryptose phosphate broth and 10% calf serum for the growth medium and 0.5% calf serum for the maintainance medium were used.

Antisera: Guinea pig antiserum perpared against the partially purified virus was kindly given by Dr. Pereira.

Antigen A, C and P : Soluble antigen obtained by the cesium chloride density gradient centrifugation was fractionated with DEAE Sephadex A50 column chromatography. The column was eluted with increasing molarities of sodium chloride in a step-wise gradient as stated by Pereira (1967).

P antigen was prepared as described by Russell et al. (1967). RK 13 cells infected with adenovirus type 5 in the presence of cytosine arabinoside at a concentration of 20 ug per ml was harvested at 12 to 14 hours after incubation. Infected cells were washed with phosphate buffered saline and scrapped them. The pellet of cells obtained by centrifugation was disrupted by sonication at low temperature and centrifuged at 98,000g for one hour. Such a supernatant fluid obtained from infected cells was the early extract called P-antigen as described by Russell et al. (1967).

Neutralization test: Serial dilutions of antiserum were added the equivalent virus solution contained 1.5×10^4 PFU per ml. After the mixture was kept at room temperature for one hour, it was diluted upto 1: 100 and added the equivalent cell suspension consisted of 2 or 3×10^6 cells per ml. These mixture were also shaken at 37°C for 30 minutes and each 1 ml of mixtures was plated into dishes with 5 cm in diameter. Each dishes was added 4 ml of the overlay medium of Eagles solution contained 0.5% calf serum and 0.75% carboxymethylcellulose. After incubation for several days, the cell sheets were fixed with 10% formaldehyde saline solution and stained with Gimsa solution for the observation of plaques. The neutralization titer of the antiserum was determined by the ordinal method of the calculation of plaque reduction. Table 1 shows the method of neutralization technique using suspended cells for the initial inoculation of the virus.

Table 1. Method for neutralization test

1. 0.2 ml of antiserum at the twofold dilution was added 0.2 ml of $x \times 10^4$/ml of virus dilution.
2. 0.2 ml of diluent without antiserum was added 0.2 ml of $x \times 10^4$/ml of virus solution for the calculation of the original virus titer.
3. Mixtures of virus and antiserum or diluent were kept at room temperature for 60 minutes. They were diluted up to 1:100, and the last dilution consist of 2 ml in each tubes. In each the last dilution of mixtures, the seed virus will be contained 50 to 100 plaques per a dish.
4. Preparation of the cells: Monolayer cells of HEK was trypsinized, washed and prepared adequate numbers of bijou in which 2 ml of cell suspension were contained 2.0×10 cells per ml.
5. Each 2 ml of HEK cell suspension were added 2 ml of diluted mixture of virus and antiserum or diluent, and they were shaken at 37°C for 30 minutes.
6. One ml of mixtures consisted of cells, antiserum and virus was plated into a petri dish. Two petri dishes were used for each dilution.
7. Four ml of maintaince medium contained 0.5% calf serum and 0.75% carboxymethylcellulose (CMC) was added and spread out in each dishes.
8. After incubation at 37°C for 5 or 6 days, discarding the maintainance medium in each dishes, cells were fixed with 10% formaldehyde saline solution for 30 minutes. After washing the cells, they were stained with Gimsa solution and the number of plaques was counted.

Block test: The excess antibody in the mixture of antiserum and antigen was determined by the proportion of the plaque reduction of the seed virus. Table 2 shows the method for the technique of the block test.

Table 2. Method for block test

1. 0.2 ml of a certain dilution of antiserum was added 0.2 ml of antigen tested, and the mixture was kept at room temperature for 60 minutes.
2. Mixtures of antiserum and antigen were added each 0.2ml of $x \times 10^4$ /ml of virus solution and kept at room temperature for 60 minutes.
3. For the calculation of the original virus titer, the diluent was added instead of the antigen in stage 2.
4. Further techniques were followed as similar as the method described in Table 1.

Adsorption test: Two ml of the seed virus of 1.5×10^2 PFU per ml was added 2 ml of suspended HEK cells of 2.29×10^6 cells per ml. The mixture in the serial tubes was shaken at 37°C for various time. The cells were washed three times with phosphate buffered saline by low speed centrifugation, and resuspended in 2 ml of Eagles medium contained 0.5% calf serum. Then, each 1 ml of cell suspension was spread over in two dishes. After one hour incubation in the CO_2 atmosphere, 4 ml of overlay medium consisted of Eagles solution contained 0.5% calf serum and 0.5% carboxymethylcellulose was added and incubated for seaeral days. The observation of plaques was performed, after the cell sheets were fixed and stained as described above.

RESULTS

1. Number of cells and concentration of calf serum in the overlay medium contained 0.75% carboxymethylcellulose

One ml of HEK cell suspension solution at various concentrations was plated into a dish with 5 cm diameter and covered with 4 ml of the overlay medium and kept at 37°C in the CO_2 atmosphere for several days. As seen in Table 3, the destruction of cells was observed in cases of the cell number counted 4×10^6 cells per ml or more over. The adequate number of cells for the formation of cell sheets was found in cases of 1 to 3 $\times$ 10^6 cells per ml in the overlay medium contained 0.5% calf serum and 0.75% carboxymethylcellulose.

Table 3. Conditions of cell number and calf serum concentration for the growth of HEK cells in the overlay medium contained 0.75% carboxymethylcellulose

cell number per ml	serum concentration	
	0.5 %	1.0 %
	condition of monolayer cells	
1 × 10^6	good and thin	good and thin
2 × 10^6	good and thin	good and thin
3 × 10^6	good but thick	some cells destructed
4 × 10^6	some cells destructed	most cells destructed
5 × 10^6	most cells destructed	most cells destructed
6 × 10^6	most cells destructed	most cells desctucted
7 × 10^6	most cells destructed	most cells destructed
8 × 10^6	most cells destructed	most cells destructed

Overlay medium was used the miture of equivalent volume of 199 and Eagles basal medium containing 0.75% carboxymethylcellulose.

2. Serum concentration in the overlay medium

HEK cells infected synchronously with adenovirus type 5 as described in the method, and one ml of them was plated into a dish and covered with 4 ml of the overlay medium contained various concentrations of calf serum. After the incubation for six days in the CO^2 atmosphere, the cell sheets were fixed and stained as described in the method. As seen in Table 4, the adequate number and size of plaques was obtained in the overlay medium contained 0.5% calf serum.

3. Adsorbtion test

Eighteen ml of 2.29×10^6 HEK cells per ml suspended with Eagles medium contained 0.5% calf serum was added 2 ml of adenovirus type 5 contained 1.5×10^3 PFU per ml. The mixture was divided into 10 tubes and shaken in the water bath at 37C for various times.

Table 4. Influence of serum concentration in overlay medium contained 0.75% carboxymethyl cellulose on plaque formation of adenovirus type 5

Cell number	serum concentration in CMC		
	0.5%	1.0%	2.0%
	plaque count		
2.4×10^6/ml	255 ; 297	137 ; 148	66 ; 69
	average number of plaques		
	276	148	68

Remarks: The abbreviation of CMC means the overlay medium contained 0.75% carboxymethylcellulose.

At each time, the virus adsorbed onto HEK cells was examined as described in the method. As seen in Table 5, it was found that the virus was adsorbed onto the cells at a level of 90% or more during 20 minutes.

Table 5. Adsorption test of purified adenovirus type 5 on HEK cells

adsorption time at 37°C (min.)					
0	10	20	30	60	120
plaque number					
average number given in experiment 1					
227	89	108	124	116	119
percent of adsorption					
(0%)	(76%)	(92%)	(100%)	(100%)	(100%)
average number given in experiment 2					
121	106	106	116	119	124
percent of adsorption					
(0%)	(87%)	(91%)	(98%)	(98%)	(100%)

Remarks: Seed virus was used at the titer of 1.5×10^3ml PFU and cell number was given at 2.29×10^6/ml in each experiment as seen in case of O mimnte in Table.

4. Neutralization test

As seen in Table 6, the neutralization titer of the antiserum of guinea pig immunized with purified adenovirus type 5 was given at the level of 1 : 320 in the proportion of 83% in the plaque reduction using the technique described in the method.

Table 6. Neutralization test of antiserum against purified virion of Adenovirus type 5 with homolgous antigen

antiserum dilution	plaque number	average number
1 : 40	1 , 1	1
1 : 80	2 , 3	3
1 : 160	2 , 2	2
1 : 320	8 , 12	10
1 : 640	43 , 47	45
1 : 1280	54 , 57	55
1 : 2560	56 , 62	59
maintainance medium	59 , 54	57

Remarks: Seed virus was applied the titer of 1.5×10^4/ml PFU and cell number was given at 3.0×10^6/ml in this experiment.

5. Block test of the antiserum with the antigen A (hexon)

From the result of neutralization test, the antiserum dilution contained 4 units (1 : 80) of the neutralization titer was used for the block test. As seen in Table 7, the activity of the hexon antigen for the blocking antiserum was indicated at the level of 1 : 160 in the proportion of 81% in the plaque reduction.

Table 7. Block test of the antiserum against purified adenovirus type 5 with the antigen A (hexon)

antiserum dilution	antigen dilution	plaque number	average number
1 : 80	1 : 20	59 , 42	50
〃	1 : 40	49 , 46	48
〃	1 : 80	44 , 42	43
〃	1 : 160	48 , 43	46
〃	1 : 320	4 , 6	5
〃	1 : 640	4 , 2	3
〃	1 : 1280	2 , 2	2
maintainance medium		53 , 48	51

Remarks: The seed virus was used at the titer of 1.8×10^4 PFU per ml and the number of cells was given 2.8×10^6 cells per ml in this experiment.

6. Block test of the antiserum with the antigen C (fiber)

Though it seems to be slightly blocked the antiserum with the original concentration of the antigen C, it was considered that the antigen C did not completely block the antiserum.

Table 8. Block test of antiserum against purifiedvirion of Adenovirus type 5 with antigen C

antiserum dilution	antigen C dilution	plaque number	average number
1 : 80	1 : 1	18 , 22	25
〃	1 : 5	5 , 7	7
〃	1 : 10	4 , 6	5
〃	1 : 20	7 , 4	6
〃	1 : 40	7 , 3	5
〃	1 : 80	8 , 6	7
〃	1 : 160	7 , 9	8
maintainance medium		73 , 79	76

Remarks: Seed virus was applied at the titer of 2.4×10^4/ml PFU and cell number was given 2.3×10^6/ml in this experiment.

7. Block test of the antiserum with P-antigen

It was obviously found that the P-antigen has not the activity to block the antiserum against the purified virus.

Table 9. Block test of antiserum against purified adenovirus type 5 with the P-antigen induced in HEK cells infected with adenovirus type 5

antiserum dilution	antigen dilution	plaque number	avarage number
1 : 80	1 : 1	8 , 7	8
〃	1 : 5	9 , 10	9
〃	1 : 10	9 , 14	12
〃	1 : 20	6 , 2	4
〃	1 : 40	5 , 5	5
〃	1 : 80	3 , 5	4
〃	1 : 160	3 , 7	5
maintainance medium		136 , 122	129

Remarks: Seed virus was used at the titer of 3.9×10^4 PFU per ml and the number of cells was given 2.4×10^6 per ml in this experiment.

DISCUSSION AND SUMMARY

Since Takemoto and Liebharber (1961) had reported the inhibitory effect of ager on the cell growth and the plaque formation of certain animal viruses, the carboxymethylcellulose overlay medium was frequently used for the plaque assay method. In fact, the plaque formation of dengue viruses under the carboxmethylcellulose overlay medium was observed without the decrease in number and size as described by Makino and Mifune in our laboratory. However, it had been demonstrated by Russell et al. (1967) that the adsorption of the virus onto the suspended cells was earlier and efficiently advanced than that on monolayer cells. When the suspended HEK cells infected with adenovirus type 5 was plated into dishes, the plaque formation in the cell sheets was observed under the constant condition.

It was demonstrated by Wilcox and Ginsberg (1963) and Kjellen and Pereira (1968) that the production of adenovirus type 5 neutralizing antibody is mainly induced by the antigen of hexon. It was also found that the antigen of hexon was able to completely block the antiserum against purified virus with using the block test in this study. In contrast, though it seemed to block slightly the antiserum with the antigen C, it was considered that the antigen C may have not enough capacity for the neutralization of the antiserum against purified virus. These findings were supported from the experiments carried out by Kjellen and Pereira. It was also demonstrated in this study that the P-antigen induced in HEK cells infected with adenovirus type 5 could not block the antiserum against purified virus.

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同時感染した HEK細胞を用いたアデアウイルス５型のプラーク法

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適宜に階段稀釈した抗血清とX×10^4 PFUのウイルスを混じ室温１時間放置した後，直ちに１：100まで稀釈する．この稀釈液と等量の維持に浮游させた２～３×10^6/mℓの HEK 細胞とを混和し，37℃ 30分間軽く振盪する．径５cm のシヤレーに１mℓずつ入れ，細胞をガラス面に拡げ，0.5%仔牛血清，0.75%カルボオキシメチールセルローズを含んだ維持液を加え蔽う．５日ないし６日目に細胞をギムザ液で染色し，プラークを算える．以上が抗血清の中和抗体の測定法である．抗血清と抗原（分画その他）を加え充分反応させた後，更らに X×10^4 PFU のウイルスを追加し室温 60分放置し，以後の手順は上記の方法に従う，以上が抗原による抗血清中の抗体のブロック能を知る方法である．以上の方法を用いて，アデノウイルス５型の抗血清に対するA抗原，C抗原及びP抗原のブロック能を検査した．抗血清が完全にブロックされたのはA抗原によってのみであり，C抗原及びP抗原は抗血清のブロック能を有しないことを知った．

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