CYTOPATHIC INDEX: A NEW METHOD FOR THE QUANTITATIVE EVALUATION OF TISSUE CULTURE INFECTIVITY OF DIFFERENT VIRUS STRAINS

D. BALDUCCI, G. ANDREONI, G. B. GORI, L. CASTELLI, AND G. MANCINI

Laboratorio di Microbiologia dell’Istituto Superiore di Sanità, and Clinica delle Malattie dell’Università di Roma, Rome, Italy

Received for publication July 29, 1959

In the analysis of various properties of pathogenic bacteria most investigators have paid special attention to the morphology of the colonies, antigenic characteristics, and virulence. Similar methods are now used in virus research except that the morphological studies concern plaques, not colonies.

The plaque under agar technique, introduced by Dulbecco (1952) into animal virology, has been used to distinguish western equine encephalomyelitis virus from Newcastle virus, vesicular stomatitis from foot-and-mouth disease virus (Sellers, 1955), and poliomyelitis from Coxsackie and ECHO viruses (Hsiung and Melnick, 1957). Plaque size varies from one strain to another within the same type of poliovirus (Dubes, 1956) and variations have been noted among mutants of the same strain (Dulbecco and Vogt, 1955).

At the present time, the study of antigenic characteristics seems to be the most important tool for systematic virus classification. By this method 3 distinct immunologic types of poliomyelitis viruses (Bodian et al., 1949) and several distinct types of Coxsackie A and B, ECHO, adenovirus, etc., have so far been found. With regard to poliovirus, qualitative differences in antigenic constitution among heterotypic strains and quantitative differences among intratypic strains can be demonstrated, according to Wenner et al. (1956).

The third property of importance in virus classification is virulence, particularly virulence for animals. Of special interest at present is the relative capacity of different viruses to infect cell cultures. (Deinhardt and Henle, 1957; Guerin and Guerin, 1957; Warren and Cutchins, 1957). Actually this property could be also interpreted as “differential virulence for tissue cultures,” particularly if the virus propagation on each single tissue culture is analyzed quantitatively.

The purpose of the present investigation is to study the propagation of polioviruses and adenoviruses in different types of human and animal cells, and to evaluate the tissue culture infectivity of these viruses by means of a new cytopathic index.

MATERIAL AND METHODS

Cells. The following animal cells were used: second passage kidney epithelial cells of Macaca mulatta (rhesus monkey); second passage kidney epithelial cell of Cercopithecus aethiops sabueus (African green monkey); a monkey heart cell line (Salk). The human cells used were derived from the following lines: kidney epithelial cell (Rita); HeLa epithelial cell (Gey); KB epithelial cell (Eagle).

Cultures. The cells were grown in Kolle bottles, treated with trypsin, and transferred to tubes, seeding 125,000 cells for each tube. The medium used for the six types of cells consisted of the following: calf serum, 10 per cent; lactalbumin hydrolyzate 5 per cent stock solution, 5 per cent; Hanks’ solution, 85 per cent.

The tubes were incubated at 37°C for 48 hr, the growth medium was removed, and the monolayer was washed with Hanks’ solution. Each tube received 1.75 ml of maintenance medium before the inoculation: bovine albumin 10 per cent stock solution, 1 per cent; horse serum, 1 per cent; lactalbumin hydrolyzate 5 per cent stock solution, 10 per cent; Earle’s solution, 88 per cent.

Viruses. The virus strains used were the poliovirus strains of Italian vaccine (type 1 Brunhenders (Gear, 1956)-type 2 MEF1-type 3 Saukett); the poliovirus avirulent strains of Sabin (type 1 L Sc, 2ab-type 2 P 712, Ch, 2ab-type 3 Leon, 12 a,b) and Koprowski (type 1 Wch-type 3 Fx). For comparison, we also used the prototype strains of adenovirus types 1-2-3-5-7.
Example of calculation of linear regression

<table>
<thead>
<tr>
<th>Days</th>
<th>Log TCID₅₀*</th>
<th>Deviations from Mean</th>
<th>Squares of Deviations</th>
<th>Products of Deviations $xy$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.75</td>
<td>-2</td>
<td>-2.70</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>1.75</td>
<td>-1</td>
<td>-1.70</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>4.25</td>
<td>0</td>
<td>0.80</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>5.00</td>
<td>1</td>
<td>1.55</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>5.50</td>
<td>2</td>
<td>2.05</td>
<td>4</td>
</tr>
</tbody>
</table>

Sum...15  17.25  10  12.75
Mean...3  3.45

$*$ Log of tissue culture infective dose-50 per cent.

Example of calculation of cytopathic index

<table>
<thead>
<tr>
<th>Virus Strain</th>
<th>Rhesus Monkey Kidney</th>
<th>HeLa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Log TCID₅₀*</td>
<td>Regression coefficient</td>
</tr>
<tr>
<td>Polio type 1 Brunhenders...</td>
<td>5.62</td>
<td>1.03</td>
</tr>
<tr>
<td>Polio type 2 MEF₁...</td>
<td>5.94</td>
<td>1.13</td>
</tr>
<tr>
<td>Polio type 3 Saukett...</td>
<td>5.50</td>
<td>2.16</td>
</tr>
</tbody>
</table>

$*$ Log of tissue culture infective dose-50 per cent.
All the virus strains were grown in rhesus monkey cells, treated once with fluorocarbon (Halonen et al., 1958) diluted 10⁻¹, distributed into several containers and then stored at -30 C. For each preparation of the seeding virus a single container was thawed out, used, and then discarded. A volume of 0.25 ml of each virus dilution, from 10⁻¹ to 10⁻⁴, was inoculated into the cultures in at least four tubes. The tubes were read every day for 5 days by the same person. Cytopathic effect was considered present when at least 50 per cent of the cells were destroyed.

**Cytopathic index.** The evaluation of virus-cell relationships up to the present time has usually been based upon the 50 per cent infectivity ratio calculated at the end of the experiment when the development of the cytopathic activity of the virus is at its end or very near it. In our opinion this is too static a measure and could be improved by calculating the rate of development of cytopathic effects. By inoculating the virus dilutions in tissue cultures as described and reading the cultures at least once each day, it is possible to follow the course of the cytopathic activity. It is clear that the progression of its activity is not uniform (figure 1). The growth patterns of viruses on the same types of cells show clear differences (figure 2). The best way to evaluate the average

![Figure 3. Spectrum of cytopathic indices for five poliovirus strains determined in six types of cells.](image)

**TABLE 3**

<table>
<thead>
<tr>
<th>Virus Strain*</th>
<th>Date of Expt</th>
<th>Log TCID₅₀</th>
<th>Log TCID₅₀ Avg</th>
<th>Regression Coefficient</th>
<th>Regression Coefficient Avg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polio 1 Brunhenders CT 474</td>
<td>1-10-58</td>
<td>5.00</td>
<td>5.30</td>
<td>0.97</td>
<td>1.03 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>1-29-58</td>
<td>5.70</td>
<td>5.90</td>
<td>1.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2-18-58</td>
<td>5.20</td>
<td>5.25</td>
<td>1.09</td>
<td></td>
</tr>
<tr>
<td>Polio 1 Brunhenders CT 837</td>
<td>9-12-58</td>
<td>6.00</td>
<td>6.00</td>
<td>0.87</td>
<td>0.96 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>9-26-58</td>
<td>6.00</td>
<td>6.00</td>
<td>1.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10-10-58</td>
<td>5.25</td>
<td>5.75</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>Polio 2 MEF, CT 474</td>
<td>1-10-58</td>
<td>5.60</td>
<td>5.81</td>
<td>1.08</td>
<td>1.13 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>1-7-58</td>
<td>6.00</td>
<td>6.00</td>
<td>1.16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2-18-58</td>
<td>5.90</td>
<td>5.90</td>
<td>1.15</td>
<td></td>
</tr>
<tr>
<td>Polio 2 MEF, CT 838</td>
<td>10-12-58</td>
<td>5.75</td>
<td>5.75</td>
<td>1.00</td>
<td>1.06 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>11-5-58</td>
<td>5.80</td>
<td>5.80</td>
<td>1.14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11-18-58</td>
<td>5.50</td>
<td>5.50</td>
<td>1.03</td>
<td></td>
</tr>
<tr>
<td>Polio 3 Saukett CT 474</td>
<td>1-12-58</td>
<td>6.00</td>
<td>6.00</td>
<td>2.13</td>
<td>2.13 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>1-20-58</td>
<td>6.00</td>
<td>6.00</td>
<td>2.15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2-18-58</td>
<td>6.00</td>
<td>6.00</td>
<td>2.20</td>
<td></td>
</tr>
<tr>
<td>Polio 3 Saukett CT 838</td>
<td>2-15-59</td>
<td>6.00</td>
<td>6.00</td>
<td>2.16</td>
<td>2.16 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>2-3-59</td>
<td>6.00</td>
<td>6.00</td>
<td>2.15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2-15-59</td>
<td>6.00</td>
<td>6.00</td>
<td>2.20</td>
<td></td>
</tr>
</tbody>
</table>

* CT 474, etc., are identification numbers.
† Log of tissue culture infective dose-50 per cent.
increase of tissue culture infectious dose-50 per cent (TCID₅₀) involves determination of the products of the pairs of variables, \( X = \) number of days, \( Y = \) average TCID₅₀, where \( X \) is the independent variable or abscissa. \( Y \) is the dependent variable or ordinate, and \( x \) and \( y \) are deviations from the means of \( X \) and \( Y \), respectively. The rate \( \Sigma xy/\Sigma x^2 \) is known as the regression coefficient, and is an estimate of the increase of TCID₅₀ with time (table 1).

Regression coefficient or \( K = \Sigma xy/\Sigma x^2 = 12.75/10 = 1.275 \). By the following method it is possible to compare the values obtained with different viruses on different types of cell cultures: For each virus suspension, record the log TCID₅₀ in rhesus kidney cell as 10. Find the “rhesus kidney cell coefficient” \( \theta \) by subtracting the measured log TCID₅₀ on rhesus kidney cell from 10. Add each of these rhesus kidney cell coefficients to the experimental values of TCID₅₀ obtained in the other types of cell to get the single adjusted value for each type of cell. Multiply this value by the regression coefficient. The product is the cytopathic index (table 2).

**TABLE 4**

<table>
<thead>
<tr>
<th>Virus Strain*</th>
<th>Rhesus Monkey Kidney</th>
<th>HeLa</th>
<th>KB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polio 1 Brunhenders</td>
<td>10.30</td>
<td>4.43</td>
<td>5.90</td>
</tr>
<tr>
<td>Polio 1 Brunhenders</td>
<td>13.20</td>
<td>5.52</td>
<td>10.64</td>
</tr>
<tr>
<td>M 13*</td>
<td>11.30</td>
<td>3.67</td>
<td>4.84</td>
</tr>
<tr>
<td>Polio 2 MEF₁</td>
<td>12.50</td>
<td>5.62</td>
<td>13.50</td>
</tr>
</tbody>
</table>

* M 13 and M 28 are identification numbers.

**RESULTS AND DISCUSSION**

This method could be useful for evaluating either the virulence of a virus strain on various types of cells or the susceptibility of one type of cell to various virus strains. The two aspects are both very important: the first, in the field of *in vitro* markers and the second, in relation to the problem of improving virus cultivation. Our method is not reliable for small variations, but we find it very useful because it can be easily carried out, requiring only standard tube cultures of any type of trypsinized cells.

Figure 3 shows a spectrum of cytopathic indices for five poliovirus strains determined on six types of cells. There are differences within the intratypic poliovirus strains, but we do not yet know how permanent these differences are. It is noteworthy that titers and regression coefficients of different partially purified preparations of the same poliovirus strains show small differences by our method (table 3).

We cannot say now that a particular spectrum is correlated with a degree of neurovirulence of the poliovirus strains, but further investigations may clear up this point. Two poliovirus strains passed in rhesus monkeys and reisolated from their blood in monkey kidney culture without further passage showed an increase of cytopathic indices in three types of cell cultures (table 4). This experiment suggests that the properties of virus growing *in vivo* may become modified, and that the cytopathic index is a reliable and easy method for determining such differences. This method makes it easy to compare the virulence of viruses in tissue culture or the susceptibility of cells to different viruses. Each of the poliovirus strains used in our experiments has had higher cytopathic indexes in the
various cell types used than the adenovirus proto-
type 1-2-3-5-7 (figure 4).

This quantitative study of virus-cell relationships could prove to be particularly useful if a parallel relationship should be established be-
tween virus virulence for man and animals, and
virus virulence for one or more tissue culture
systems.

ACKNOWLEDGMENT

We wish to thank Mr. Joseph Ehrenreich, Wis-
tar Institute of Anatomy and Biology, Philadel-
phia, for his help in the preparation of the manuscript.

SUMMARY

A method for the quantitative evaluation of
tissue culture infectivity of virus strains is pre-
sented which takes into account the rate at which
the tissue culture infective dose increases with
time during the test. A cytopathic index may be
calculated for each of various virus strain-cell
combinations, involving determination of re-
gression coefficients which estimate the rate of
development of the tissue culture infective dose-
50 per cent (TCID50), and adjustment of the
TCID50 by a factor based on the infectivity of
the same virus in a reference tissue culture (rhesus
monkey kidney).

An experimental study of the propagation of
several strains of poliovirus and adenovirus in
different types of human and animal cells is de-
scribed; and cytopathic indices are calculated
for the various combinations. Cytopathic indi-
ces may possibly be useful as in vitro markers
and as aids in the study of methods for improving
virus cultivation.

REFERENCES

Bodian, D., Morgan, I. M., and Howe, H. A.
1949 Differentiation of type of poliomyelitis
viruses. III. The grouping of fourteen

strains into three basic immunological types.

Deinhardt, F. and Henle, G. 1957 Studies
on the viral spectra of tissue culture lines of

Dubes, G. R. 1956 Differences among strains of
poliomyelitis viruses in plaque size on monkey

Dulbecco, R. 1952 Production of plaques in
monolayer tissue cultures by single particles
U.S., 38, 747–752.

Dulbecco, R. and Vogt, M. 1955 Biological
properties of poliomyelitis viruses as studied
by the plaque technique. Ann. N. Y. Acad.
Sci., 61, 790–800.

Gear, J. 1956 The South African poliomyelitis

Guérin, L. F. and Guérin, M. M. 1957 Sus-
ceptibility of pig kidney tissue cultures to

Halonen, P., Hufnagel, R. J., and Turner, H.
C. 1958 Preparation of ECHO complement
fixing antigens in monkey kidney tissue cul-
ture and their purification by fluorocarbon.

Huang, G. D. and Melnick, J. L. 1957 Mor-
phologic characteristics of plaques produced
on monkey kidney monolayer cultures by
enteric viruses (poliomyelitis, Coxsackie and

Sellers, R. F. 1955 Growth and titration of
the viruses of foot-and-mouth disease and
vesicular stomatitis in kidney monolayer

Warren J. and Cutchins, E. C. 1957 General
characteristics and viral susceptibility of
bovine embryonic tissue cultures. Virology,
4, 297–304.

Wenner, H. A., Kamitsuka, P., and Lena
han, M. 1956 A comparative study of type 2
poliomyelitis viruses. II. Antigenic differ-
ences relating to 18 type 2 strains. J. Im-
munol., 77, 220–231.