The Titration of Trachoma and Inclusion Blennorrhoea Viruses in Cell Cultures

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SUMMARY

A technique is described for titrating trachoma and inclusion blennorrhoea viruses by counting the inclusions formed in HeLa cell monolayers. The method compares favourably in accuracy with other techniques used for the assay of viruses and is more reliable than titration in the chick embryo yolk sac.

INTRODUCTION

Hitherto trachoma and inclusion blennorrhoea viruses have been titrated only by inoculating suitable dilutions of a virus suspension into the yolk sac of embryonated eggs and recording either the death or infection of the embryos. However, since chick embryos vary in susceptibility to these viruses, this method is not satisfactory. Sometimes, even after a large inoculum, infection of the egg cannot be demonstrated (Collier, Duke-Elder & Jones, 1958). The finding that some strains of trachoma and inclusion blennorrhoea viruses induce cytoplasmic inclusions in cell cultures suggested an alternative method of titration (Furness, Graham, Reeve & Collier, 1960). The present paper describes a technique for assaying these viruses by counting the cytoplasmic inclusions formed in monolayers of HeLa cells.

METHODS

Viruses. The TE 55 strain of trachoma virus (T'ang, Chang, Huang & Wang, 1957) and the LB1 strain of inclusion blennorrhoea virus (Jones, Collier & Smith, 1959) were used.

HeLa cells. Stock cultures were propagated in 250 ml. bottles containing 10 ml. medium. The medium consisted of Hanks's balanced saline solution with the addition of 5% (v/v) tryptic digest broth, 0.5% (w/v) lactalbumin hydrolysate, 10% (v/v) horse serum, 2% (v/v) rabbit serum and 200 μ g. streptomycin/ml.; adjusted to pH 7.2-7.4 with 1.4% (w/v) NaHCO_3 . Serum was heated at 56° for 30 min.

When monolayers of HeLa cells had formed, they were washed in phosphate-buffered saline (PBS) without calcium or magnesium and separated by 3 ml. 0.02% (w/v) ethylenediamine tetra-acetic acid in PBS (Lwoff, Dulbecco, Vogt & Lwoff, 1955). The cell suspension was diluted tenfold in medium and dispensed in 10 ml. amounts into bottles or 2 ml. amounts into Leighton tubes, each containing a coverslip (Bello Glass Inc., Vineland, N.J., U.S.A.). The cultures were incubated at 37°.

Virus assay. When the HeLa cells had formed a monolayer in the Leighton tubes, the medium was changed and each of four tubes was inoculated with 0.1 or 0.2 ml. of a suitable virus dilution. After incubation for 72 hr. the coverslips were removed, washed in buffer pH 6.8, fixed in 95% (v/v) methanol in water for 1 hr. and stained with Giemsa May-Grünwald (8 ml. Giemsa; 4 ml. May-Grünwald; 80 ml. buffer, pH 6.8). The cells were differentiated for 60–90 min. in buffer, dried at 37°, cleared for 2 min. in xylene and mounted in DePeX (G. T. Gurr and Co. Ltd.). The cells were examined at a magnification of $\times 240$ and the inclusions counted. Only vesicles containing stainable material and distorting the nucleus were accepted as virus inclusions.

Estimation of inclusion forming units (IFU). By means of a micrometer slide, the area of a microscopic field was estimated to be 0.196 mm.² at a magnification of $\times 240$. The Leighton tubes had a rectangular well with a base measuring 11 $\times$ 39 mm., an area of 429 mm.² This was partly covered by a coverslip measuring 9 $\times$ 38 mm. The monolayer formed a continuous sheet of cells over both the coverslip and the exposed surface of the well not covered by the coverslip. Thus, the area of the monolayer exposed to infection with virus was equivalent to the surface area of the well of the Leighton tube, i.e. 429 mm.² The number of fields in this area equals

$$\frac{\text{total area of monolayer}}{\text{area of field}} = \frac{429}{0.196} \quad \text{or } c. 2200.$$

Assuming random adsorption of the virus over the surface of the monolayer, the number of IFU/ml. is given by the formula

$$\frac{\text{no. IFU counted}}{\text{no. of fields examined}} \times 2200,$$

adjusted by a factor determined by the volume and dilution of the inoculum.

Disruption of HeLa cells by ultrasonic vibrations. To disrupt the cells and release virus, the infected cells were scraped into the supernatant medium and transferred to a 50 ml. centrifuge tube cooled in ice water. The suspension was treated for 4 min. with ultrasonic vibrations by inserting the probe of an M.S.E. Mullard ultrasonic disintegrator with a power output of 60 W. at a frequency of 20 kc/sec.

Partial purification of virus in yolk sacs. After homogenizing the infected yolk sac for 1 min. in an M.S.E. blender with 4–5 ml. sucrose + potassium glutamate solution (Bovarnick, Miller & Snyder, 1950), the suspension was clarified by centrifuging at 1500 rev./min. for 10 min. The supernatant fluid was then centrifuged at 10,000 g for 90 min. in an angle head at 4° to deposit the virus which was finally resuspended in 4–5 ml. sucrose + potassium glutamate solution.

RESULTS

Infection of cells. Monolayers of HeLa cells in bottles were inoculated with 1 ml. purified infected yolk-sac suspension. After incubation for 6–18 hr. the medium was changed and the cells re-incubated. Both viruses produced some inclusions within 3–5 days. After 6–7 days the cells were resuspended in fresh medium and used to inoculate further monolayers. After about three serial passages, almost all the cells contained inclusions; these heavily infected cultures were used in these experiments.

Preparation of virus suspensions. Preliminary observations indicated that not all the virus was released spontaneously from the inclusions. The use of ultrasonic vibrations (USV) and freezing and thawing as a means of releasing virus was therefore investigated. Microscopic observations showed that USV disrupted normal cells within 3 min. Infected cells were therefore treated for 4 min. to ensure complete destruction of the cells and release of virus. To study the effect of USV on the infectivity of the released virus, the suspension was titrated and again treated with USV for 4 min. There was no alteration in its infectivity (Table 1). There was a decrease, however, after more prolonged treatment. Three cycles of freezing in solid CO₂ + ethanol followed by rapid thawing in a 37° water bath caused a significant loss of infectivity (Table 1). Disruption by USV was therefore adopted as the method of choice.

Table 1. *Effect of ultrasonic vibrations (USV) and three cycles of freezing and thawing (F & T) on the infectivity of trachoma and inclusion blennorrhoea viruses in HeLa cell monolayers*

Virus	Treatment to release virus from cells	No. IFU/ml. suspension	Treatment of released virus	No. IFU/ml. suspension
Trachoma	USV	8×10^3	USV	7.5×10^3
	USV	3×10^5	USV	1.6×10^5
	USV	9.5×10^4	USV	1.1×10^5
Inclusion blennorrhoea	USV	7.4×10^5	USV	6.5×10^5
	USV	5.2×10^5	USV	3.7×10^5
Trachoma	F & T	1.2×10^5	F & T	5×10^4
	F & T	4.4×10^4	F & T	8.4×10^3

Appearance of inclusions. To ascertain that all inclusions counted at time of assay were derived from virus in the original inoculum, monolayers were infected with a suitable dilution of virus and incubated; at 24 hr. intervals, four coverslips were selected at random, stained and examined. Although some inclusions could be seen after 24 hr., they were not readily identified until after 48 hr. when they were about the size of the cell nucleus. By 72 hr. the inclusions had enlarged, but had not significantly increased in number, indicating that a second cycle of infection had not occurred and that all the inclusions were derived from virus in the original inoculum. As a routine, therefore, results were read after 68–72 hr. since the inclusions were most easily counted at that time.

Random distribution of inclusions. An area of thirty consecutive fields along the length of the coverslip was arbitrarily chosen as the 'standard area' in which inclusions were counted. To determine whether the number of inclusions found in any one 'standard area' was a representative sample of the whole monolayer, several coverslips were picked at random and the inclusions in four different standard areas counted (a total of 120 different fields/monolayer). The number of inclusions found in each of the four standard areas was remarkably similar (Table 2). They seldom differed from the average for the 120 fields by more than 10%. This suggested not only that an area of thirty fields was adequate for our purpose, but also that the inclusions were randomly distributed. Random distribution on any coverslip was confirmed by comparing the observed distribution of inclusions in each field with

that expected from a Poisson series (Table 3) and testing the goodness of fit by the formula

$$\chi^2 = S \left\{ \frac{(O-E)^2}{E} \right\},$$

where S implies summation, O observed number and E calculated number of inclusions/field. There was a reasonable fit.

Table 2. *Variations in the number of inclusions counted in different areas of the same monolayer of HeLa cells*

	Monolayer no.*				
	1	2	3	4	5
Four 'standard areas' of 30 consecutive fields	124	133	135	138	309
	114	131	127	150	383
	118	102	116	139	317
	134	129	116	136	367
Av. no./30 fields	122.5	123.75	123.5	140.75	344
Difference between av. no. in 30 fields and:					
highest no. in 30 fields	11.5 (9.4 %)	9.25 (7.5 %)	11.5 (9.3 %)	9.25 (6.6 %)	39 (11.2 %)
lowest no. in 30 fields	8.5 (7.0 %)	21.75 (17.6 %)	7.5 (6.1 %)	4.75 (3.4 %)	35 (10.2 %)

* Monolayers 1-4 inoculated with the same virus dilution.

Table 3. *Poisson series fitted to inclusion counts*

No. of inclusions/field	Monolayer no.						
	1		2 and 3			4	
	Obs.	Calc.	Obs.	Obs.	Calc.	Obs.	Calc.
0-1	6	10.2	8	4	9.7	6	6.2
2	17	18.2	14	17	16.8	16	12.1
3	31	22.9	23	26	22.0	15	19.1
4	20	23.4	31	31	23.3	18	22.3
5	23	19.2	17	20	19.2	23	21.0
6	12	12.6	16	11	13.2	17	16.4
7	4	7.5	6	7	7.8	15	11.0
8 and over	7	6.2	5	4	6.7	5*	6.5*
9 and over	—	—	—	—	—	5	4.9
No. fields counted	120	120	120	120	118.7	120	119.5
Av. no. inclusions/ field	4.08		4.1	4.1		4.7	
Total no. inclusions counted	490		495	494		563	
	$\chi^2_{(6)} 7.75$		$\chi^2_{(6)} 5.74$	$\chi^2_{(6)} 9.11$		$\chi^2_{(7)} 5.02$	

* Eight inclusions only.

Optimum magnification for identifying inclusions. After incubation for 72 hr. inclusions can be recognized at magnifications of $\times 120$ and $\times 240$. The lower magnification gives a field 4 times larger than at $\times 240$, and its use might be advantageous when there are few infected cells. Counts made at both magnifications were, therefore, compared and were consistently lower at a magnification of $\times 120$ (Table 4). The higher magnification was therefore used as a routine.

Variations between replicates. The accuracy of titrations, using respectively 3 and 4 replicate coverslips, was compared. These results, together with the % standard deviation (% S.D.) and % standard error (% S.E.) are given in Table 5. The standard deviation was calculated from the formula

$$\text{S.D.} = \sqrt{\frac{S(x - \bar{x})^2}{N - 1}},$$

where S implies summation, $x - \bar{x}$ is the difference of each replicate from the mean and N the number of replicates; the % standard error (% S.E.) was calculated

Table 4. *Variations in the number of inclusions seen in the same monolayer at $\times 120$ and $\times 240$ magnifications*

Monolayer no.	No. of inclusions in 30 fields at:		
	$\times 120$ magnification		$\times 240$ magnification.
	Inclusions counted	Counts corrected to standard area*	Inclusions counted in standard area
6	34	8.5	11
7	45	11.25	19
8	92	23	30
9	140	35	47
10	142	35.5	51
11	309	77.25	103
12	317	79.25	120
13	367	91.75	126
14	383	95.75	141

* Thirty fields at $\times 120$ equals four standard areas.

Table 5. *Effect of the number of replicates on the % standard error (% S.E.) and % standard deviation (% S.D.)*

Average of three replicates			Average of four replicates		
Av./30 fields	% S.D.	% S.E.	Av./30 fields	% S.D.	% S.E.
21	20	11.6	21	21.9	11
43	16.5	9.5	44	17.5	8.8
47	18.5	10.7	46	16.3	8.2
47	13	7.5	122	12.9	6.5
66	36.8	21.2	167	16.5	32.8
116	44.8	25.9	245	18.0	9.0
121	15.7	9.0	454	17.8	9.0
236	20.8	11.4			
395	8.3	4.8			
399	28.2	16.3			
417	42.9	24.8			
471	19.2	11.1			

as % S.D./ $\sqrt{N}$. As would be expected, increasing the number of replicates did not greatly improve the accuracy of the method. The % S.E. was usually about $\pm 10\%$, although it might be as high as $\pm 25\%$; errors which are due to the inevitable sampling variations. When the S.E. is outside these limits it appears preferable to repeat the assay rather than attempt to increase accuracy by using more replicates.

These results indicated that within the limits tested (i.e. using four replicates, an average of 20–450 inclusions in 30 fields) the accuracy of the technique was not affected by the number of inclusions per field.

There was a linear relationship between concentration of virus and the inclusion count (Table 6).

Table 6. *Relationship between inclusion count and virus concentration*

Virus	Dilution factor	Count Av./30 fields	Count $\times$ dilution factor
Trachoma	10^0	430	430
	10^{-1}	61	610
	10^0	447	447
	10^{-1}	43	430
	10^0	395	395
	10^{-1}	44	440
	10^0	242	242
	$10^{-0.5}$	81	256
	10^{-1}	25	250
	$10^{-0.5}$	269	850
Inclusion blennorrhoea	10^{-1}	75	750
	$10^{-1.5}$	33	1040
	10^0	439	439
	10^{-1}	40	400

DISCUSSION

Before a virus can be studied in detail a quantitative technique for its assay must be developed. The feline and murine pneumonitis viruses have been titrated by counting the inclusions formed in explants of yolk sac (Weiss & Huang, 1954). Trachoma virus also induces lesions in such explants (Gordon, Quan & Trimmer, 1960). However, the use of HeLa cells is much less laborious. When a standard area of 30 fields is counted in each of four replicate monolayers at $\times 240$ magnification, a standard error of approximately $\pm 10\%$ may be expected; this compares favourably with the results obtained by Weiss & Huang (1954). The slight increase in accuracy obtained by examining a greater proportion of the monolayer, or by using more replicates, does not offset the extra expenditure of time and materials.

Within the limits investigated, i.e. 20–450 inclusions in 30 fields, the same standard error may be expected. Thus each series of monolayers measures a relatively wide range of virus titre. However, only suspensions containing more than 7×10^3 IFU/ml. can be assayed by the standard technique. To titrate lower concentrations, the size of the inoculum must be increased or a greater area of the monolayer examined. This cannot be achieved by using an optical system with a lower magnification and a correspondingly greater field area, as there is a significant decrease in the number of inclusions identified at the lower magnification.

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