OUKA
The University of Osaka
Institutional Knowledge Archive

Title	A Study on the Number of Infectious Particles Necessary for "B" type Inclusion Formation
Author(s)	Kamahora, Juntaro; Toyoshima, Kumao; Nii, Shiro et al.
Citation	Biken's journal : journal of the Research Institute for Microbial Diseases. 1959, 2(4), p. 345-352
Version Type	VoR
URL	https://doi.org/10.18910/83128
rights	
Note	

The University of Osaka Institutional Knowledge Archive : OUKA

https://ir.library.osaka-u.ac.jp/

The University of Osaka

BIKEN'S JOURNAL 2, 345-352 (1959)

A Study on the Number of Infectious Virus Particles Necessary for "B" Type Inclusion Formation

JUNTARO KAMAHORA, KUMAO TOYOSHIMA, SHIRO NII AND SHIRO KATO

Department of Pathology, The Research Institute for Microbial Diseases, Osaka University, Osaka

(Received for publication, December 15, 1959)

SUMMARY

The "B" type inclusion of ectromelia virus has been considered a definite indicator of pox virus infection in the cell. The relationship between the number of "B" type inclusion bearing cells and the number of infectious virus particles was studied from the statistical point of view using free Ehrlich ascites tumor cells and tissue culture L cells infected with ectromelia virus. Further the relationship between the number of "B" type inclusions in a single cell and the multiplicity of the infection was investigated. Our results revealed that one infectious virus particle is sufficient to form one "B" type inclusion in a single cell.

INTRODUCTION

As to the number of the virus particles which could initiate an infection of a cell, a one particle theory was presented by Parker (1938) using vaccinia virus in rabbit skin. Further the theory that "one virus particle for one plaque" was reported by Dulbecco and Vogt (1954). Since then, the concept that a cell infection would be initiated by one virus particle has been generally admitted.

The significance of the viral inclusions in the multiplication of pox group viruses has been regarded as fundamental since the discovery of "B" type inclusion bodies in ectromelia virus (Kato *et al.*, 1955). Lately the "B" type inclusion of ectromelia virus was found by Coons' methed to be not only Feulgen positive but also the site of virus antigen (Kameyama *et al.*, 1959). As Toyoshima *et al.* (1957) previously reported, statistically one inclusion cell seems to be initiated by one infectious virus particle. The present study was intended to confirm this fact.

Several reports have been made on the relation between ectromelia inclusion formation and the virus growth using Ehrlich ascites tumor cells and tissue culture cells from our laboratory (Hagiwara *et al.*, 1955; Hagiwara and Kamahora, 1956; Furusawa *et al.*, 1959; Ikegami *et al.*, 1959; Toyoshima *et al.*, 1959). During these experiments, cells were found both in Ehrlich tumor cells and in tissue culture cells which contained multiple rather than single "B" type inclusions. The number of the "B" type inclusions sometimes amounted to more than twenty in a single cell, especially when a high virus titer was used for inoculation. It is likely that there might be some correlation between the number of "B" type

inclusions and the virus titer of the inoculum. It is well known that there are cases in which two different types of viruses grow independently in tha same cell. For instance, a double infection of ectromelia virus and another species of virus, e.g. herpes simplex virus, has been observed (Nii,unpublished).

Also a double infection of two strains of ectromelia virus was observed by Hagiwara taking advantage of morphological difference of their "A" type inclusions. In line with this, it seems most probable that a certain number of the same inclusions in a single cell are initiated by a corresponding number of infectious particles entering the cell.

MATERIALS AND METHODS

1. *Virus*

Mouse passaged ectromelia virus (Hampstead strain) was used. In the experiments with Ehrlich ascites cells, infected liver emulsion was employed using the same technique as previously described, while in the experiments with tissue culture L cells, Daifron (fluorocarbon) purified ectromelia virus which had 2×10^7 plaque forming units (PFU)/ml was used (Nii, 1959). The details of the method of purification of ectromelia virus by Daifron was given in the previous paper (Kameyama *et al.*, 1959).

2. *Mice DD strain was used.*

3. *Ehrlich ascites tumor cell*

Erhlich ascites tumor cells were used after washing with phosphate buffered saline. About 2×10^8 cells/ml were inoculated intraperitoneally into each mouse.

4. *Tissue culture*

L cells 5.5×10^6 were dispersed in a large prescription bottle with ten milliliters of LA-YH (Hanks' saline with 0.5 per cent lactalbumin hydrolysate and 0.1 per cent yeast extract) containing 5 per cent ox serum. *Preparation of antiserum:* According to the method described by Furusawa *et al.* (1957) the ascties fluid of hyperimmunized mice was used as the antiserum against the virus after heating at 56°C for one hour.

5. *Mouse LD 50*

Samples were serially diluted ten fold. Each of the four mice in one group was injected with 0.2 ml of the diluent. After an observation period of 10 days, the LD 50 was determined by Reed and Muench's method.

Abbreviations used:

"B"; a "B" type inclusion.

"B"s; "B" type inclusions.

"B" cell; a "B" type inclusion bearing cell.

"Bc"; a small round compact form of "B" type inclusion which is characteristic of an early stage of development.

ETC; an Ehrlich ascites tumor cell.

RESULTS

1. *The number of infectious virus particles necessary for one "B" cell*

The virus material was diluted as follows; ×1, ×2, ×4 and ×10. Two volumes of each diluent were mixed with one volume of ETC. 1.5 ml of the mixture was injected into the mouse intraperitoneally. Two mice were injected with each mixture. Eighteen hours after inoculation, the ascites was tapped and a smear

preparation made of it. An eighteen hour period was chosen since the inclusion cell curve in the first cycle usually reached a maximum about this time. The percentage of "B" cells was counted. The value is represented by fractions, *e.g.*, 0.73, 0.56 *etc.* as shown in Table 1.

The relationship between multiplicity of infection (s) and the fraction of infected cells is considered to fit a Poisson's distribution curve. By taking the appearance of "B" as a positive phenomenon in this Poisson's distribution curve the number of virus particles needed for one "B" cell was calculated.

If as a minimum, one infectious particle is enough to form a "B" cell, the fraction of "B" cells will be equal $Pr \geqq 1$ in the formula (2). Similarly if two or more infectious particles are required to form a "B" cell, the "B" cell percentage will be equal to $Pr \geqq 2$ in the formula (3). The situation with three or more particles will be derived from the formula (4).

$$Pr = \frac{S^r e^{-s}}{r!} \quad \cdots\cdots (1)$$

$$Pr_{\geqq 1} = 1 - e^{-s} \quad \cdots\cdots (2)$$

$$Pr_{\geqq 2} = 1 - e^{-s}(1+S) \quad \cdots\cdots (3)$$

$$Pr_{\geqq 3} = 1 - e^{-s}\left(1+S+\frac{S^2}{2}\right) \quad \cdots\cdots (4)$$

$$\left[P_o = e^{-s}\right]$$

Pr; Probability of cells being infected by r number of particles.
S; Multiplicity of infection.

According to formulae (2), (3) and (4) multiplicities of infections (S-values) were calculated on each assumption (Table 1). Under these conditions, the product of the dilutions times the multiplicities must be equal in all the dilutions.

Table 1. Statistical analysis of virus particles required for formation of inclusion cells

Virus		ETC	Fraction of "B" cells	Multiplicities (calculated)					
				if ≧ 1		if ≧ 2		if ≧ 3	
Dilution	ml	ml		S	S× dilution	S	S× dilution	S	S× dilution
× 1	1	0.5	0.73	1.30	1.3	2.6	2.6	3.8	3.8
× 2	1	0.5	0.56	0.80	1.6	1.8	3.6	2.9	5.8
× 4	1	0.5	0.33	0.40	1.6	1.1	4.4	2.0	8.0
× 10	1	0.5	0.12	0.13	1.3	0.57	5.7	1.2	12.0

S: Multiplicity of infection calculated by Poisson's distribution function from fraction of inclusion cells.
Fraction of inclusion cells were determined 18 hours after infection.

As shown in Table 1, the products are different when calculations are made according to the formulae (3) and (4), while formula (2) was found most adequate to this calculation. It was thus suggested that one "B" cell could be formed by at least one infectious virus particle ($r \geqq 1$). The mean value of the products of dilution times S indicates the multiplicity of infection in the original dilution. Then the product of this mean value times the cell number which was counted by means of a hemocytometer, will represent the total number of effective virus particles (V). In Table 2, the titer of the total effective virus is compared with the mouse LD50 of the same samlpe. The ratio of V to LD50 is several to 1. Since LD50 was determined by serial ten fold dilutions, a precise comparison was impossible.

Table 2. Comparison of total effective virus in EV-ETC system with mouse LD_{50}

Total effective virus (V)	Mouse LD_{50}
1.9×10^8	$10^{7.8}$ LD_{50}
1.5×10^8	$10^{7.3}$ LD_{50}
1.4×10^8	$10^{7.2}$ LD_{50}
1.2×10^8	$10^{7.3}$ LD_{50}
0.7×10^8	$10^{7.0}$ LD_{50}

Table 3. Partial neutralization test in EV-ETC system

Antiserum dilution	Fraction of* "B" cells	Exp. / Control
× 1	0.017	0.02
× 4	0.20	0.26
× 16	0.44	0.57
× 64	0.50	0.65
× 256	0.74	0.96
Control	0.77	1.00

* Fractions of "B" cells were determined 18 hours after infection.

2. *Effect of antibody*

The effect of the antiserum upon the infectious virus was examined using fractions of "B" cells. Serial four fold dilutions of the antiserum were made and each diluent was mixed with an equal volume of virus material *in vitro*. The mixture was incubated at 37°C for an hour, and half volume of ETC was added. 2.5 ml of each final mixture was inoculated intraperitoneally into mice. For each diluent two mice were used. 18 hours after inoculation, the fractions of "B" cell were counted (Table 3). The neutralizing index of the antiserum used for this experiment was 3.5 as judged by the ordinary neutralization test with mice.

3. *Multiple infection and the morphological changes induced by it*

Two or more "B"s, sometimes even more than twenty are often observed in single cell both in smear preparations of infected liver cells and infected cultured L cells. An attempt was made to see what causes this phenomenon. Infected ETC was prepared under the same condition as in Exp. 1. 6 hours after infection, consecutive smear preparations were made at 2 hour intervals. Then the proportion of early stage "B" cells was examined. The results are shown in Table 4. C1, C2 and $C \geqq 3$ show cells which include 1, 2 and 3 or more compact "B"s respectively. Up to 16 hours after infection, the ratio of C1/C2+C3 is relatively constant. Eighteen hours after inoculation, the ratio begins to change. Considering the period of the

Table 4. Distribution of numbers of inclusions in single cell

Time hrs. / Incl.	6	8	10	12	14	16	18	20	22
C 1	23	9	14	16	13	14	24	19	13
C 2	6	2	4	4	3	4	13	9	10
C≧3	4	0	2	1	2	1	3	7	6
R C	26	44	37	40	38	32	37	32	36
D	13	76	186	214	283	341	375	470	546
Total	72	131	243	275	339	402	452	537	616

C_1, C_2 and $C_{\geqq 3}$ show 1, 2 and ≧3 "B" containing cells.
RC shows cells which contain relatively compact "B".
D shows cells which contain diffuse "B".

lst infectious cycle in this system, it was suggested that 18 hours after infection the "Bc" of the 2nd infectious cycle started to be formed. Thus only the proportion of the "Bc" cell within 16 hours were used for the following calculation. The "Bc" cells were compared with the probability of multiple infection calculated from the multiplicity of infection by the Poisson's distribution formula (1). Multiplicity of infection was estimated from the total effective virus (V) by the method described in Exp.1. The fractions of "Bc" cells were calculated from the following formulae.

$$Pr=o = 1 - I$$

$$Pr = I \times \frac{\Sigma Cr}{\Sigma C}$$

I; Fraction of total "B" cells 18 hours after infection.
Cr; Total number of cells with r number of "Bc" within 16 hours of infection per 1,000 ETC.
C; The sum total of Cr within 16 hours.

The distributions of "Bc" cells at three dilutions of the original virus material were compared with the probability of multiple infection. As shown in Table 5, a considerable coincidence was observed between the multiplicity of infection and the number of "Bc" in a single cell. These results suggest that one "Bc" is formed by a single infection and their multiple appearance in a single cell coincides with multiple infection.

4. *Experiment using tissue culture L cells and purified ectromela virus*

The L cell and ectromelia virus system has been extensively studied in our laboratory. Recently a one step growth curve has been depicted using this system. According to this, ten hours have been found to be sufficient for the appearance

Table 5. Relationship between multiplicity of infection and number of inclusion bodies in single cells

Multiplicity	0.63 / cell		0.315 / cell		0.16 / cell	
Incl. per cell	pr	Pr	pr	Pr	pr	Pr
0	0.55	0.53	0.74	0.73	0.84	0.85
1	0.32	0.34	0.21	0.23	0.15	0.14
2	0.084	0.106	0.038	0.036	0.012	0.011
≧3	0.040	0.026	0.012	0.004	0.000	0.001
Total	0.994	1.002	1.000	1.000	1.002	1.002

"pr" shows experimental results.
"Pr" shows probability calculated by Poisson's distribution function.

of "B" in almost all cells in the first step. As virus material, a Daifron-purified virus suspension was used. The virus dilution was made as follows, 5^1, 5^2 and 5^3. Three milliliters of each diluent was incoulated into large prescription bottles containing about 5.2×10^6 cells. After incubation for an hour and a half at 37°C, the cell sheets were washed three times with Hanks' balanced salt sloution to remove residual virus and then maintenance media was added. After another 6 and a half hours of incubation at 37°C, the cells were scraped off, suspended by vigorous pipetting, and packed by centrifugation. Then smear preparations of the cells were made, fixed with methanol and stained with Giemsa solution. The fraction of "B" of each dilution was tabled and the corresponding "S" values were calculated by the same method. As shown in the tables (Table 6 and 7) a striking correspondence was observed between the values. This strongly suggests that one infectious virus particle is enough to produce one "B" cell, and multiple inclusions are the result of multiple infection with an equal number of virus particles.

Table 6. Virus particles required for formation of "B" cell in tissue culture

Virus dilution*	Fraction of "B" cells	S	S × dilution
× 1	0.96	3.2	/
× 5	0.90	2.3	12
× 25	0.38	0.48	12
× 125	0.089	0.094	12

* Virus: Daifron purified virus was used.
S: Multiplicities of infection calculated by Poisson's distribution function from fraction of "B" cells.
Fraction of "B" cells were determined 8 hours after infection.

Table 7. Relationship between multiplicity of infection and number of inclusion bodies in single tissue culture cell

Multiplicity	0.48		0.094	
Inclusion per cell	pr	Pr	pr	Pr
1	0.30	0.30	0.084	0.086
2	0.055	0.070	0.007	0.004
≧ 3	0.017	0.011	0.001	0.0001

pr: experimental results.
Pr: probability calculated by Poisson's distribution function.

DISCUSSION

The relationship between the number of infectious virus particles and the number of "B" was studied from a statistical viewpoint. The results showed first that as a minimum, one infectious virus particle was enough to produce one "B" cell, and secondly that mutiple "B"s in a single cell might be formed by a corresponding number of infectious virus particles which had entered the cell almost simultaneously. The interference between active virus particles does not occur in this case, contrary to the case with inactivated virus previously reported.

In the present analysis, one of the most probable factors which might be responsible for errors in the computation of the "B" cell percentage might be the proliferation of the cells. As we reported before, infected cells ceased to multiply in the systems employed in this experiment. Uninfected cells seem to have a lag phase of growth and mitotic figures were rarely observed during the short observation period used in the present study. More convincing proof was obtained by the experiments with ultraviolet rays. Irradiation by ultraviolet rays caused the cessation of cell proliferation for a considerable period and the percentage of inclusions in previously irradiated cells was not found to differ essentially from that of nonirradiated cells.

Our experiment was concerned only with the relationship between the number of infectious virus particles and "B" formation. The relation between the number of infectious virus particles and the total number of virus particles including noninfective virus particles has not yet been determined. The factors which might be involved in this determination depend upon the method of purification of the virus, the source of the virus material, the density of virus particles, and the kind of cell or animal used for virus titration.

Our experimental results on the effect of anibody upon the formation of viral inclusions, revealed that antibody with a neutralizing index of 3.5 could suppress the appearance of the inclusion cells to almost one hundredth of the cotrol. One of the main factors which might be involved in this might be the difference between total neutralization and partial neutralization. The fact that nonlinear parallelism existed between the concentration of antibody and its suppression of inclusion formation might show the complexity of the function of the antibody *in vivo*.

REFERENCES

Dulbecco, R. and Vogt, M. (1954). One-step growth curve of WEE virus on chicken embryo cells grown *in vitro* and analysis of virus yield from single cells. *J. Exptl. Med.* **99,** 183–199.

Furusawa, E., Kameyama, S., Kim, S., Iwa, K., Oketani, J. and Miyagawa, T. (1958). Multiplication of ectromelia virus in culture of strain L cells. *Virus* **8,** 499–503.

Hagiwara, K., Kato, S., Furusawa, E., Baba, E. and Kamahora, J. (1956). The mechanism of the growth of ectromelia virus propagated in the ascites tumor cells. II. Studies on the growth curves *in vivo* and *in vitro*. *Med. J. Osaka Univ.* **7,** 561–569.

Hagiwara, K. and Kamahora, J. (1956). The mechanism of the growth of ectromelia virus propagated in the ascites tumor cells. III. Morphological observations on the growth cycles *in vivo* and *in vitro*. *Med. J. Osaka Univ.* **7,** 571–588.

Hagiwara, K. unpublished.

Ikegami, N., Kato, S. and Kamahora, J. (1959). A study on the inhibitory activity of 5, 6-Dichloro-1-β-ribofuranosyl benzimidazole (DRB) and proflavin on the one step growth cycle of the mousepox virus (ectromelia virus) in L cell. *Biken's J.* **2,** 215–219

Kameyama, S., Takahashi, M., Toyoshima, K., Kato, S. and Kamahora, J. (1959). Studies on the inclusion bodies of ectromelia virus using fluorescent antibody *technique*. *Biken's J.* **2,** 341–344.

Kato, S., Hagiwara, K. and Kamahora, J. (1955). The mechanism of the growth of ectromelia virus propagated in the ascites tumor cells. I. Study on the inclusion bodies of ectromelia virus. *Med. J. Osaka Univ.* **6,** 39–50.

Nii, S. (1959). Plaque formation and some cytopathogenic characteristics of ectromelia virus and vaccinia virus on mono layers. *Biken's J.* **2,** 195–206.

Nii, S. unpublished.

Parker, R. F. (1938). Statistical studies of the nature of the infectious unit of vaccine virus. *J. Exptl. Med.* **67,** 725–738.

Parker, R. F., Bronson, L. H. and Green, R. H. (1941). Further studies on the infectious unit of vaccinia. *J. Exptl. Med.* **74,** 263–281.

Toyoshima, K., Furusawa, E. and Kamahora, J. (1958). Interference between active and ultraviolet-inactivated viruses of ectromelia in Ehrlich ascites tumor cells. *Med. J. Osaka Univ.* **8,** 299–313.

Toyoshima, K., Kato, S. and Kamahora, J. (1959). A study of inclusion formation in the first cycle of ectromelia virus infection in ascites tumor cells in the abdominal cavity of rats. *Biken's J.* **2,** 221–226.

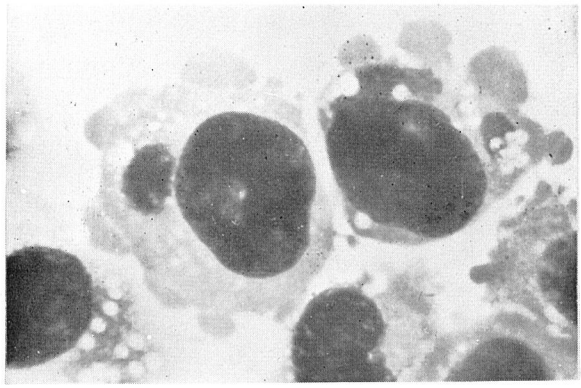

Fig. 1. Two Ehrlich ascites tumor cells bearing rather developed "B" type inclusions.

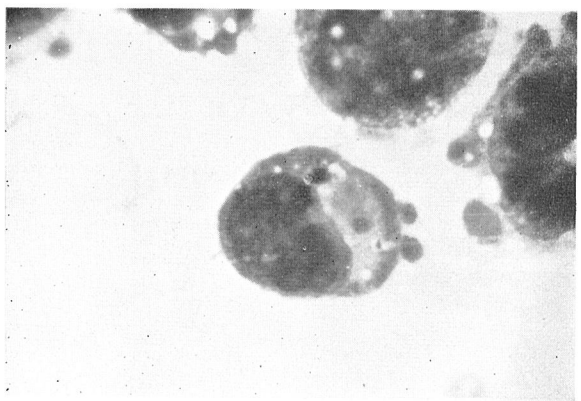

Fig. 2. An Ehrlich ascites tumor cell having three compact "B" type inclusions.

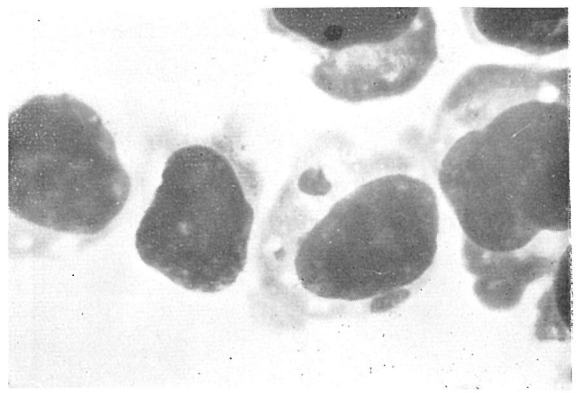

Fig. 3. An Ehrlich ascites tumor cell having two typical compact "B" type inclusions.

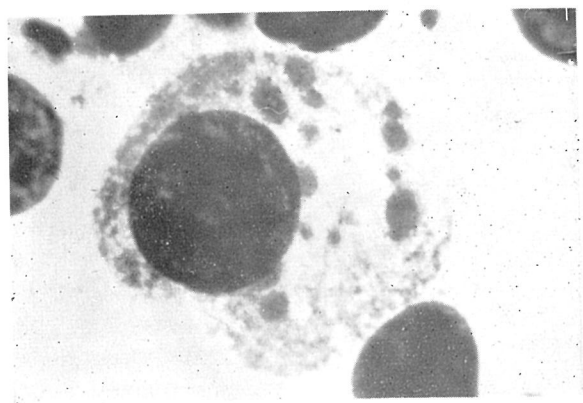

Fig. 4. Multiple "B" type inclusions in a single L cell.