One-step Growth Curves for Inclusion Blennorrhoea Virus in HeLa Cell Monolayers

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SUMMARY

When incubated in tissue-culture medium, inclusion blennorrhoea virus lost infectivity for HeLa cells at 37° but not at 30°. The rate of adsorption of virus to HeLa cell monolayers was dependent on temperature and on the volume of the inoculum. When the volume of medium was minimal, the virus was adsorbed before a detectable proportion was inactivated. Adsorption was complete after 7–8 hr. at 30° and 5–6 hr. at 37°.

At 30° the intracellular virus went into eclipse and maturation was retarded. When the temperature was raised to 37° maturation was rapid so that, for practical purposes, replication was synchronized during this period. In singly infected HeLa cells, at 37° infective virus was not detected for 22–23 hr., after which the progeny increased exponentially until at 34–38 hr. 35–60 infectious particles/infectious cell were formed. The total number of particles/inclusion seen in Giemsa-stained preparations exceeded the number of infectious units/inclusion.

After 42–48 hr. infective virus was found in the supernatant and the number of intracellular infectious units began to decrease. Unlike vaccinia, another large virus, the virus progeny did not directly infect adjacent cells.

INTRODUCTION

The technique for assaying inclusion blennorrhoea and trachoma viruses in HeLa cell monolayers described by Furness, Graham & Reeve (1960), slightly modified, was used to study the adsorption and intracellular growth of inclusion blennorrhoea virus in HeLa cell monolayers.

METHODS

Virus. The LB1 strain of inclusion blennorrhoea virus (Jones, Collier & Smith, 1959) was grown in HeLa cell monolayers in 250 ml. Pyrex feeding bottles (Furness *et al.* 1960). Suspensions were prepared by resuspending monolayers in 10 ml. medium 68–72 hr. after infection, transferring them to a 50 ml. centrifuge tube cooled in ice water and releasing the virus by treatment for 4 min. with ultrasonic vibrations. For this purpose a stainless-steel probe of an M.S.E.—Mullard ultrasonic disintegrator with a power output of 60 W. at a frequency of 20 kc./sec. was inserted in the cell suspension. The suspensions contained $2.5\text{--}5 \times 10^7$ infectious units/ml.

HeLa cell monolayers. These were grown in Hank's balanced saline solution

containing 0.5% (w/v) lactalbumin hydrolysate, 10% (v/v) human serum, 5% (v/v) calf serum and 100 μ g./ml. streptomycin; the sera were inactivated by heating at 56° for 1 hr. HeLa cell monolayers grown on coverslips in Leighton tubes were used in all quantitative experiments (Furness *et al.* 1960).

Virus assay. The medium covering the HeLa cell monolayers was replaced with 0.25 ml. fresh medium and infected with 0.1 ml. of a suitable dilution of virus. The virus was allowed to adsorb for at least 6–7 hr. at 37°. Thereafter 1.5 ml. medium was added to each monolayer. After incubation for 62–66 hr., the coverslips were removed, stained with Giemsa and the inclusions in thirty fields counted at a magnification of $\times 240$ (Furness *et al.* (1960). Cultures were incubated at 37° unless otherwise stated.

One-step growth curves. Monolayers covered with 0.25 ml. medium were infected with 0.1 ml. of a virus dilution capable of infecting between 300 and 800 of the cells in an area of thirty fields after adsorption for 2 hr. at 30°.

After the virus had been allowed to adsorb to the monolayers for 2 hr. at 30° unadsorbed virus was removed by washing with phosphate buffered saline (Dulbecco & Vogt, 1954) at 30°. To count the number of cells infected, five monolayers were covered with 2 ml. medium and the inclusions counted after incubation for 68–72 hr. at 37°. The remaining monolayers were covered with 1 ml. medium and incubated at 37° to allow maturation of the virus. Released and intracellular inclusion forming units were determined during replication as follows. At intervals, four monolayers were chosen at random and the supernatants pooled and titrated to obtain the number of infectious units released from the cells. The cells themselves were resuspended in a total of 4 ml. medium, disintegrated with ultrasonic vibrations and assayed to determine the number of intracellular infectious units.

RESULTS

The effect of temperature on the viability of inclusion blennorrhoea virus. To assess the rate of inactivation of virus at 37°, 4 ml. of a suitable dilution of virus prepared in medium at pH 6.7–7.1 were incubated in $\frac{1}{4}$ oz. screw-capped bottles in a water-bath. At intervals 0.1 ml. samples were titrated in HeLa cell monolayers covered with 1 ml. medium. No virus was inactivated in 8–10 hr. at 30°, whereas over 80% of the virus was killed in 6 hr. at 37°. The virus was not protected by increasing the serum content of the medium from 15 to 45% (Table 1).

Adsorption of virus to HeLa cell monolayers. To determine the degree of inactivation during the adsorption period, monolayers covered with 1 ml. medium were

Table 1. *The loss of infectivity of inclusion blennorrhoea virus for HeLa cells at 37°*

Suspending medium. Culture medium containing (% serum)	Inoculum (no./ml.)	% infectious units surviving after incubation at 37° for		
		2 hr.	4 hr.	6 hr.
15	2.8×10^6	86	28	14
15	6.7×10^6	40	33	21
15	3.0×10^6	48	21	10
30	3.0×10^6	50	24	8
45	3.0×10^6	44	22	7

inoculated with 0.1 ml. of a suitable dilution of virus and incubated at 30° or 37° to allow the virus to adsorb to the cells. At intervals, four monolayers were freed from unadsorbed virus by washing with phosphate buffered saline, covered with 2 ml. medium and incubated for 68–72 hr. at 37° to enable adsorbed particles which were still infectious to form inclusions. Compared with the infectious units adsorbed in 8 hr. at 30°, only half the virus formed inclusions at 37° indicating that some infectious units were inactivated. The method of titration of inclusion blennorrhoea virus in HeLa cell monolayers (Furness *et al.* 1960) did not therefore determine the total number of infectious units in the original suspension.

Fig. 1. The rate of adsorption of inclusion blennorrhoea virus by HeLa cells at 30° and 37°.

Since the efficiency of adsorption of polio-virus to cell monolayers is increased by decreasing the volume of the supernatant fluid (McLaren, Holland & Syverton, 1959; Furness, 1961), the experiment was repeated with 0.25 ml. medium; the total volume after inoculation thus being only 0.35 ml. This modification increased the efficiency of adsorption. Similar number of inclusions were formed after adsorption for 7–8 hr. at 30° and 5–6 hr. at 37° (Fig. 1), indicating that the infectious units were adsorbed from this volume before significant inactivation occurred. These results do not appear consistent with the finding that over 80 % of the virus was inactivated in 6 hr. at 37° (Table 1). In the two experiments, the virus was assayed under different conditions which may explain this inconsistency.

To verify that diminution of the volume of supernatant fluid did not affect the accuracy of titrations, the results of four assays were analysed statistically (Furness *et al.* 1960). The random distribution of the inclusions and the accuracy of the titrations were not altered, the standard error being within ± 10 %.

The multiplication of inclusion blennorrhoea virus in HeLa cells. Three one-step growth curves for inclusion blennorrhoea virus are summarized in Fig. 2. In these experiments respectively 1.2, 1.8 and 4.8 % of the cells in the monolayers were infected. The results therefore reflect the behaviour of singly infected cells. After adsorption at 30° for 2 hr., at least 88 % of the adsorbed virus was irrecoverable, the remainder being released as infective virus on disintegrating the cells. Thereafter, the temperature was raised to 37° to allow normal maturation of the virus. Less

than 1 % of the adsorbed virus was infective 4 hr. later. With this virus-cell system, the period during which virtually no virus was recoverable is termed the eclipse stage and is considered to end when each infected cell contains an average of one

Fig. 2. One-step growth curves of 3 experiments with inclusion blennorrhoea virus in HeLa cell monolayers at 37°.

Fig. 3. Effect of pre-incubation for 24 hr. at 30° on one-step growth curve of inclusion blennorrhoea virus in HeLa cell monolayers at 37°. For convenience, this experiment was done in two parts (● and ○).

infectious unit; in these experiments it lasted 22–23 hr. Once replication commenced, the intracellular infectious units increased exponentially until a peak was reached at 34–38 hr. with a yield of 35–60 infectious units/inclusion. From 42–48 hr. until the experiments were ended, a quantity of virus equivalent to 1–2 % of the intracellular virus was present in the supernatant medium at each assay. Concomitantly, the number of intracellular infectious units progressively decreased.

From 18 to 52 hr. sample monolayers were stained by Giemsa. Although the visible particles in the inclusions could not be counted accurately, they obviously exceeded the numbers estimated by infectivity titrations.

The eclipse phase of inclusion blennorrhoea virus at 30°. Inclusions could not be seen in infected monolayers after 24 and 48 hr. incubation at 30° suggesting that the maturation of inclusion blennorrhoea virus was affected. To ascertain whether the virus replicated during incubation at 30°, the technique for obtaining one-step growth curves was modified after adsorption at 30°, by continuing incubation of the washed monolayers at this temperature for a further 24 hr. Thereafter the temperature was raised to 37° and the intracellular infectious units assayed periodically. The number of infected cells did not decrease as a result of incubation at 30° indicating that the intracellular virus remained viable. The curve itself (Fig. 3) ran parallel to those obtained previously and the yield of sixty infectious units/inclusion was not affected (Fig. 2). After 24 hr. at 30°, the incubation period required for the production of infectious units at 37° was reduced by about 8 hr. (Fig. 3). Thus inclusion blennorrhoea virus in eclipse continued to mature at 30° at only one-third of the rate at 37°.

DISCUSSION

The replication of inclusion blennorrhoea virus in HeLa cells was studied by a technique similar to that reported previously for vaccinia virus (Furness & Youngner, 1959*a, b*) and poliovirus (Furness, 1961) in which multiplication in singly infected cells is synchronized by adsorbing virus at temperatures which retard the rate of replication. The rate of maturation of inclusion blennorrhoea virus at 30° was one-third that at 37°; it was therefore negligible during the 2 hr. adsorption period and growth of the virus was virtually synchronized.

Our results indicated that this agent behaved like other animal viruses in that it had a stage during which infective virus could not be demonstrated. Meningo-pneumonitis virus of the psittacosis group was grown in tissue cultures of allantoic membranes (Girardi, Allen & Sigel, 1952) and L cells (Higashi, Notake & Fukada, 1959) with similar results. After adsorption to the cells, inclusion blennorrhoea virus and meningo-pneumonitis virus were not detected for 20–23 hr. Thereafter they increased exponentially until a peak was reached at 34–38 hr. Both viruses were heat labile at 37° and 35°, respectively. Heat inactivation of inclusion blennorrhoea virus during adsorption was obviated by our techniques. No evidence, however, was presented to exclude this possibility during adsorption of meningo-pneumonitis virus so it is possible that the assays of this virus underestimated the total number of infectious units. Nor was the release of meningo-pneumonitis virus from infected cells reported. Infective inclusion blennorrhoea virus was found in the supernatant medium suggesting spontaneous release from the inclusions. Since inclusion blennorrhoea virus is labile at 37° the unexpectedly small amount of infective virus found in the supernatant medium could be accounted for by heat inactivation and adsorption to the monolayer.

There was no evidence of cell to cell spread of virus as occurred in similar experiments with vaccinia virus in tissue culture (Furness & Youngner, 1959*a, b*).

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