A Study by Fluorescence Microscopy of the Replication of Inclusion Blennorrhoea Virus in HeLa Cell Monolayers

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(Received 3 October 1961)

SUMMARY

The infectious agent of inclusion blennorrhoea was seen by fluorescence microscopy as a particle of deoxyribonucleic acid (DNA) 0.2 μ diam. These infectious particles and particles inactivated at 4° and 56° were adsorbed by HeLa cell monolayers. The inactivated particles were visible for at least 6 hr., distributed at random on the cells, while the infectious particles rapidly disappeared. Multiple infections of the cells readily occurred and in cells so infected, a cluster of ribonucleic acid (RNA) particles was first observed at 3 hr. adjacent to the cell nucleus. Since the number of RNA particles, each of which contained a DNA core, was equal to the number of infectious particles adsorbed and since singly infected cells contained only one RNA particle, the infectious unit is almost certainly a single DNA particle. The RNA particle increased in size without dividing and the surrounding cell cytoplasm gradually dissolved to form the inclusion body. DNA particles which were protected from deoxyribonuclease by a layer containing RNA were first observed in the inclusions at 21 hr. and an average of one infective progeny was detected in each infected cell at 22–23 hr. indicating that infectivity was associated with the DNA particles. RNA, DNA and infectious DNA particles reached their maxima between 27–30 hr., 39–42 hr. and 34–38 hr. respectively and decreased thereafter. Infectious DNA particles were released from the inclusions. The total number of DNA particles/inclusion always exceeded the number of infectious DNA particles/inclusion. Glycogen was not detected in the inclusions suggesting that it may not be essential for the formation of virus.

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INTRODUCTION

The total number of particles/inclusion seen in Giemsa stained preparations of HeLa cells infected with inclusion blennorrhoea virus exceeds the number of infectious units/inclusion, suggesting that only a proportion of these particles are infectious (Furness & Fraser, 1962). Both ribonucleic acid (RNA and deoxyribonucleic acid (DNA) particles are seen in the inclusion found in human amnion and synovial cells infected with trachoma virus (Pollard, Starr, Tanami & Moore, 1960). In the present study, the formation of RNA and DNA particles in HeLa cells infected with inclusion blennorrhoea virus was observed by fluorescence microscopy and their relationship to infectivity determined.

METHODS

Infection of HeLa cell monolayers. The methods used to assay the LB1 strain of inclusion blennorrhoea virus in HeLa cell monolayers and to obtain one-step growth curves have been described (Furness, Graham & Reeve, 1960; Furness & Fraser, 1962). Monolayers required for staining were infected by the same method. The monolayers on coverslips in Leighton tubes were covered with 0.25 ml. tissue culture medium and inoculated with 0.1 ml. of a suitable virus suspension which was allowed to adsorb for 2 hr. at 30°. They were then freed from unadsorbed virus by washing with phosphate buffered saline (Dulbecco & Vogt, 1954), covered with 2 ml. medium and incubated at 37°. Sample monolayers were removed at intervals for staining and for the assay of intracellular infectious units. Some microscopic appearances were more easily studied in heavily infected monolayers inoculated with a virus suspension containing 2-4 infectious units/cell. When it was desired to infect cells with a single infectious unit, a diluted suspension which infected less than 5 % of the cells was used.

Acridine orange staining. The cell monolayers were washed in phosphate buffer (pH 6.8; G. T. Gurr Ltd., London) fixed in Carnoy's fluid for 10 min., rinsed in ethanol and washed in acetate buffer pH 3.0 (N-sodium acetate, 100 ml; N-HCl 99.5 ml.; water, 300.5 ml.). They were stained in 0.5 % (w/v) acridine orange (Imperial Chemical Industries stain R. 150) in acetate buffer (pH 3.0) for 30 min., washed for 30 min. in three changes of acetate buffer (pH 3.0) and sealed in acetate buffer (pH 3.0) with a mixture of beeswax and colophonium.

Fluorescence microscopy. A monocular microscope with apochromatic lenses was mounted horizontally and illuminated by a 250 watt high-pressure mercury vapour lamp of the Metro Vickers glass envelope type, the light being concentrated by a collecting lens and an Abbé condenser. The Wratten exciting filter No. 50 (Kodak Ltd.) was protected by a heat absorbing filter. Observations were made with a Watson oil immersion lens N.A. 1.37 and $\times 10$ ocular containing a minus blue barrier filter (Ilford Ltd.). With these filters, ribonucleic acid (RNA) appeared orange and deoxyribonucleic acid (DNA) green. The magnification was $\times 950$. Measurements were made with $\times 10$ and $\times 17$ micrometer oculars.

Specificity of acridine orange stain. To confirm the specificity of the acridine orange stain for both cell and viral RNA and DNA, uninfected and infected monolayers were stained and compared with replicate monolayers in which the RNA and DNA had been digested with ribonuclease (RNase) and deoxyribonuclease (DNase)

before staining. Aqueous stock solutions, 0.1 % (w/v), of crystalline RNase grade A (California Corporation for Biochemical Research, Los Angeles) were kept at -30° and diluted 1/10 in McIlvaine's buffer (pH 5.6) before use. DNase grade B (California Corporation for Biochemical Research, Los Angeles) 0.01 % (w/v) was prepared in veronal buffer as required. The monolayers were washed in phosphate buffer (pH 6.8), fixed in ethanol for 5 min., washed in veronal buffer, incubated in diluted enzyme for 1 hr. at 37° , washed in acetate buffer (pH 3.0), fixed in Carnoy's fluid for 10 min. and stained.

Iodine staining for glycogen. Monolayers were stained with 1 % (w/v) iodine in 2 % (w/v) aqueous solution of potassium iodide for 15 min., blotted dry, covered with immersion oil and examined at a magnification of $\times 240$ (Collier, 1961).

RESULTS

Identification of cell and viral RNA and DNA by fluorescence microscopy

Treatment with RNase and DNase abolishes the specific colour reactions of cell RNA and DNA with acridine orange (Armstrong, 1956; Armstrong & Niven, 1957). The specificity of our staining and the activity of our enzyme preparations were confirmed by this method. RNase 0.01 % (w/v) digested the orange-staining material within the inclusions in 40 min. and within the cells in 60 min.

Cell DNA was digested by 0.01 % (w/v) DNase in 20 min. at 37° , but the viral DNA was not affected by incubation with 0.1 % (w/v) DNase for 2 hr. at 37° . However, when the preparations were treated with 0.01 % (w/v) RNase for 60 min. at 37° , subsequent treatment with 0.01 % (w/v) DNase for 60 min. at 37° abolished the staining reaction of the viral DNA in the inclusion, indicating that the viral DNA was protected from the enzyme activity of DNase by a layer containing RNA. The specific colour reactions of both cell and viral DNA and RNA were unaffected in controls incubated in buffer only.

Staining of virus suspension. Virus was released from the inclusions, 72 hr. after infection of the cells, by ultrasonic vibrations (Furness & Fraser, 1962). Smears were prepared on coverslips, air dried, fixed in Carnoy's fluid for 10 min. and stained with acridine orange. Large numbers of DNA particles were seen similar in size to those in inclusions. No RNA particles were identifiable.

Adsorption of inactivated and infective virus by HeLa cells. Since virus suspensions contained both infectious and non-infectious particles, the adsorption of both infective and inactivated virus to HeLa cell monolayers was investigated. Inclusion blennorrhoea virus was completely inactivated in 10–15 min. at 56° ; at 4° , 99 % became non-infective in 14 days (Fig. 1). To ascertain whether the temperature used for inactivation affected adsorption of the particles, monolayers were inoculated with 0.1 ml. of a virus suspension reduced in infectivity by holding for 14 days at 4° and with the same suspension heated for 10–15 min. at 56° .

After adsorption of the inactivated virus for 2 hr. at 30° , the cells were well washed, covered with 2 ml. medium and incubated at 37° . At intervals monolayers were stained and examined. There was no significant difference in the results after inactivation at the two temperatures (Table 1); the virus was adsorbed, remained visible for over 6 hr. and then disappeared. To compare the rate of adsorption and disappearance of infective and inactivated virus a suspension was prepared from

cells 40 hr. after infection, when the inclusions contained the greatest number of infectious units and release of progeny had not begun (Furness & Fraser, 1962); part was inactivated by heating for 15 min. at 56°. Immediately after adsorption (Table 1) and 6 hr. later, there were fewer infectious particles than inactivated DNA particles. It was impossible to decide whether the particles were within the cells.

Fig. 1. The loss of infectivity for HeLa cells of inclusion blennorrhoea virus suspended in cell culture medium at 4°.

Table 1. *DNA particles adsorbed to HeLa cell monolayers inoculated with infective and inactivated inclusion blennorrhoea virus*

Inoculum	After adsorption, incubated at 37° for (hr.)			
	0	3	6	9
	Relative no. particles/field			
Inactivated at 4°	++++	++++	++++	+
Inactivated at 4° followed by 56° for 15 min.	++++	++++	++++	++
Inactivated at 56° for 15 min.	++++	++++	++++	++
Infective virus	++	+	—	—
++++	Max. no. particles/field.			
++	Significant reduction in no. particles/field.			
+	An occasional particle/field.			

Formation of RNA, DNA and infectious DNA particles in infected HeLa cells. The viral RNA stained a different shade of orange from that in the cell cytoplasm, so that even small viral RNA particles could be distinguished from the cell cytoplasm. In heavily infected monolayers inoculated with 2–4 infectious units/cell, a single cluster of RNA particles about 0.5 μ diam. was seen adjacent to the cell nucleus at 3 hr. Monolayers were inoculated with dilutions of virus suspensions and the RNA particles in the inclusions counted after 9 hr. incubation at 37°, when the particles were readily identified. The number of RNA particles was proportional to the

dilution of the inoculum (Table 2). In cells estimated to have adsorbed 1·8 infectious particles from the inoculum there was an average of 1·4 RNA particles/inclusion suggesting that each infectious particle was the precursor of one RNA

Fig. 2. The formation of RNA, DNA and infectious units in HeLa cell monolayers infected with inclusion blennorrhoea virus. Ordinate: The mean number of intracellular infectious units per 100 infected cells plotted on a logarithmic scale.

Table 2. *The relationship of the infectious units in the inoculum to the number of RNA particles/inclusion*

(a) Dilution of inoculum	(b) No. of inclusions examined	(c) RNA particles in inclusions	(d) Average no. of RNA particles/ inclusion (c/b)
Expt. no. T. 92			
0	50	71	1·4*
1/10	50	51	1·0
Expt. no. T. 96			
0	100	1365	13·65
1/2	100	558	5·58
1/4	100	281	2·81
1/8	100	192	1·92

* From titration of original suspension, estimated number of infectious units adsorbed from undiluted inoculum 1·8/cell, so that at 1/10 dilution about 20% cells were infected with one infectious particle.

particle which was not infectious (Fig. 2). This was confirmed by examining every 2 hr. monolayers in which less than 5% of the cells were infected with a single infectious particle. With a single exception, the inclusions contained only one RNA

particle which increased in size without any evidence of binary fission, reaching a maximum between 27–30 hr. Thereafter the amount of RNA decreased. DNA particles, about $0.2\ \mu$ diam. were first observed in the inclusions at 21 hr. but treatment of the earliest RNA particles with RNase revealed a DNA core which increased in proportion to the RNA during the development of the inclusion. The relation between RNA and DNA particles and infectious units found is summarized in Fig. 2. The virus was non-infective 4 hr. after adsorption to the cell. An average of one infective virus progeny/cell was detected at 22–23 hr. suggesting that infectivity was associated with DNA. The number of DNA particles reached a maximum between 39–42 hr. and infectious particles between 34–38 hr. An accurate total count of the DNA particles/inclusion was impossible, but their number exceeded the number of infectious units/inclusion. After reaching a maximum both infectivity and DNA particles decreased, suggesting release from the inclusion. In monolayers in which about 5 % of the cells were infected, secondary RNA inclusions were seen in previously uninfected cells 69–72 hr. after the initial infection confirming that the infective virus was released and adsorbed to uninfected cells (Furness & Fraser, 1962).

The formation of inclusion bodies. When first detected the RNA particles were surrounded by cell cytoplasm. By 10–15 hr. the cytoplasm immediately around the particles had dissolved leaving the particles often arranged round the periphery of the clear area which enlarged to form the typical cytoplasmic inclusion body adjacent to the cell nucleus. At all stages inclusion bodies unaccountably varied greatly in size.

Glycogen. Although glycogen has been detected in inclusion bodies in HeLa cells infected with this strain of inclusion blennorrhoea virus (Furness, Graham, Reeve & Collier, 1960) it was not found in the present work within 72 hr. of infection.

DISCUSSION

The stages in the replication of inclusion blennorrhoea virus in HeLa cells infected with several infectious particles are given schematically in Fig. 3*a–c*. (*a*) After the adsorption of both infective and inactivated virus, DNA particles, $0.2\ \mu$ diam. are distributed at random on the HeLa cells. It is probable that the particles lie within the cells which are known to be phagocytic but this was not demonstrable. Most of the infectious particles disappear immediately after adsorption, i.e. during the adsorption period and at this stage there is no evidence of any gross cellular abnormality. (*b*) At 3 hr. a single cluster of RNA particles $0.5\ \mu$ diam. appears in the cytoplasm near the nucleus of each cell. (*c*) These RNA particles do not divide but enlarge, arrange themselves in a ring similar to that seen in Giemsa stained preparations of chick embryo cell cultures infected with trachoma virus (Gordon, Quan & Trimmer, 1960) and then seem to coalesce. At the same time, the cell cytoplasm gradually dissolves leaving the particles within a vacuole which increases in size to form the distinctive cytoplasmic inclusion body. (*d*) By 21 hr. DNA particles are also seen within the inclusion body and are presumably released from within the RNA which reaches its maximum between 27–30 hr. The appearance of DNA particles roughly coincides with the detection of infective virus (*e*) Only an occasional RNA particle $0.5\ \mu$ diam. and many DNA particles are found in the inclusion bodies at 66–72 hr. From these results we tentatively conclude that the

infectious unit is a single DNA particle which is the precursor of the one RNA particle seen in the cytoplasm of cells infected with a single infectious unit.

Treatment of the particles with RNase and DNase shows that the RNA particle contains a DNA core, and the DNA particle is covered with a substance which contains RNA but not enough to be visible by its fluorescence. Although the possibility that the enzyme preparations contained effective enzymes other than

Fig. 3. Schematic representation of the stages in the replication of inclusion blennorrhoea virus in HeLa cells infected with several infectious DNA particles. (a) After adsorption a few DNA particles 0.2μ diam. may be seen on the HeLa cell. (b) 3 hr. infectious DNA particles have disappeared and a cluster of RNA particles 0.5μ diam. appears near the cell nucleus. (c) 18 hr. RNA particles increase in size and finally coalesce. A vacuole is formed around particles 10–15 hr. (d) 27 hr. RNA particle reaches maximum. Many DNA particles which first appear at 21 hr. seen within the inclusion. (e) 66–72 hr. Large inclusion body contains many DNA particles and an occasional RNA particle about 0.5μ diam.

the two nucleases cannot be excluded, one possible interpretation of the results is that the RNA particle is an enlarged DNA particle, in which the RNA has increased sufficiently to be detectable.

Similar stages in the growth of the related organisms of the psittacosis-lymphogranuloma group were first described for psittacosis virus by Bedson, and his co-workers (Bedson, 1933; Bedson & Bland, 1932, 1934; Bland & Canti, 1935) and their reports have been amply confirmed by other authors. It was generally believed that

the virus remained at least partially infective throughout the growth cycle; though Girardi, Allen & Sigel (1952) concluded that psittacosis virus underwent a non-infective phase. Our observations are consistent with this conclusion.

Other animal viruses so far investigated in this way have proved to lose their infectivity and their original structure during the eclipse phase, their nucleic acids being dispersed in the host cell. Inclusion blennorrhoea virus qualifies for classification as a typical animal virus in that it has a non-infective phase, during the first 3 hr. of which it also became undetectable by the available techniques. Nevertheless, the effective part of the virus material from each particle appears to migrate to a single site near the nucleus, suggesting that during the eclipse, the infective material continues to exist as a discrete particle. By itself, this difference does not, in our view, at present constitute a very cogent reason for excluding the agent of inclusion blennorrhoea from the class of animal viruses, particularly since, for many of the animal viruses, the dispersal of viral nucleic acids during the eclipse phase of replication has been assumed by analogy with those for which dispersal has been proved.

We wish to thank Miss Margaret Ham for her excellent technical assistance. The work of one of us (G.W.C.) was supported by a U.S. Public Health Service Grant administered by the Medical Research Council Working Party on Non-Specific Urethritis.

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