No. 1, Volume 10 of the *Journal of General Microbiology* was issued on 12 February 1954.

No. 2, Volume 10 of the *Journal of General Microbiology* was issued on 9 April 1954.

NOSSAL, G. J. V. & DE BURGH, P. M. (1954). *J. gen. Microbiol.* **10**, 345-352.

Ectromelia Virus Multiplication in Mouse Liver

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SUMMARY: The multiplication of ectromelia virus in mouse liver cells has been studied using intravenous inocula of 10^6 to 10^{10} LD₅₀ doses. About 30% of this virus is found in the liver in 5 min., and most of this disappears as infective virus in the next hour. After a lag period, the length of which depends on the number of LD₅₀ doses/cell which initiated the infection, the virus titre increases again to the 5 min. level. Subsequently, steps of multiplication, each separated by a lag period, occur within each cell. The efficiency of virus multiplication over the range tested is independent of the infecting dose.

Since the classical experiments of Ellis & Delbrück (1939), who demonstrated that bacterial virus multiplication occurred in stages, it has been found that many animal viruses follow similar patterns. Briefly, these stages are: attachment of virus on to host cell; disappearance of part or all of infectivity; a phase during which no new infective virus can be demonstrated; a period of intracellular rise in infective titre followed by release of this virus which may infect further cells and repeat the cycle. Such growth curves are conveniently studied in the fertile hen's egg, and findings similar to the above description have been made using influenza virus (Henle, Henle & Rosenberg, 1947), vaccinia (Briody & Stannard, 1951), Newcastle Disease virus (Gordon, Birkeland & Dodd, 1952), meningopneumonitis virus (Girardi, Allen & Sigel, 1952), and other viruses. The study of multiplication of animal viruses in their natural hosts is made difficult by many factors which tend to obscure the events in any one cell. Some of the important factors are: (a) the difficulty of determining how many cells are infected by the inoculum; (b) the heterogeneous cell populations of the organs most commonly studied (lung and brain); (c) the infective titre which at any time represents the net result of virus formation and removal, by lymph or blood, or destruction, or entry into further susceptible cells. Fenner (1949) pointed out that the multiplication of ectromelia virus in the mouse was an excellent model for the study of host-virus relationships.

The following studies were undertaken in an attempt to lessen some of the above difficulties and thus to demonstrate a one-step growth curve of ectromelia virus in mouse liver. A preliminary account of this work has been published (Nossal & de Burgh, 1953).

MATERIALS AND METHODS

Mice. Albino Swiss mice, 6-10 weeks old, were used for the growth-curve experiments; similar mice or heterogenetic coloured mice of the Walter and Eliza Hall Institute (Melbourne) strain were used for the bio-assays of infectivity.

Virus. The 'Moscow' strain of ectromelia virus was used throughout. Stock suspensions of virus were made by grinding infected livers in a mortar previously chilled to -15° , and homogenizing with nutrient broth, the ice crystals formed acting as an abrasive. These suspensions were stored in sterile test tubes plugged with cotton-wool at -15° ; they retained their titre unchanged for over 2 months. When stocks of very high titre were required they were prepared as follows. Mice were inoculated intravenously with large doses of virus, and were sacrificed late in the infection, but before they became moribund; the livers were removed and homogenized in 10% sucrose solution at $0-4^{\circ}$, in a pestle and mortar, to make a 10-15% suspension of liver. Then the suspension was subjected to either of the following procedures to concentrate the virus. (1) The suspension was centrifuged 1 to 3 times at 4000 *g* for 8-12 min. to remove cell debris, red blood cells, nuclei and mitochondria, and the supernatant fluid centrifuged at 23,000 *g* for 12-20 min. to deposit the virus. Sometimes the suspensions were layered over 30% sucrose solution to achieve better separation. (2) The suspension was centrifuged once at 1500 *g* for 10 min. and the supernatant centrifuged at 23,000 *g* for 12 min. to give a pellet which contained most of the virus.

Method (1) has the virtue of yielding suspensions of virus much freer from contaminating cell debris, but it requires more manipulations, and as no refrigerated centrifuge was available, the preparations got rather warm with a resultant decrease in titre. Method (2) results in a bulky pellet which also contains most of the mitochondria. This can be homogenized in a chilled (-15°) mortar and rendered sufficiently disperse to allow the intravenous injection of 0.1 ml. By either method, the final suspension represents the virus from up to 2 g. liver in 1 ml.

Growth curves. These were studied by one or both of two techniques:

(1) Large numbers of mice were inoculated intravenously at times known within 5 min. for each mouse. With very concentrated stock suspensions, a proportion of mice died immediately. Mice were taken in groups of 3, 4 or 5 at intervals and killed by decapitation. The livers or large samples thereof were removed, inspected, weighed and ground with nutrient broth in the manner described, stored at -15° , and titrated within 2 weeks.

(2) Parts of the growth cycle can conveniently be studied in one mouse. The mouse is infected and anaesthetized just before material is taken to obtain the first point on the curve. Of many anaesthetics tried, the most satisfactory was urethane (about 0.15 ml. of 25% (w/v) solution/20 g. mouse, given intraperitoneally). This is quick to act (1 min.), not lethal in effective doses, and wears off very slowly (>12 hr.). At no stage was there any evidence that the infective process was altered in any way by the anaesthetic. An incision $\frac{3}{4}$ -1 in. long was made in the abdominal wall in the mid-line, and a lobe of the liver brought through the wound to the surface. Then either a small piece (c. 20 mg.) was cut from the edge of the liver and bleeding immediately stopped by application of a small piece of gelfoam (Upjohn and Co.) soaked in a solution of thrombin (200 i.u./ml.) or a whole lobe of liver was removed. The latter gives more satisfactory control of bleeding and larger pieces of liver are obtained.

By these methods, two to six samples can be obtained from one mouse, covering a period as long as 8 hr.

Histology. All mice were given 0.5 ml. 20% (w/v) glucose in water by intra-peritoneal injection at 90 and 45 min. before killing, to ensure that all cells capable of synthesizing and storing glycogen had enough substrate to do so. Small pieces of liver were fixed in Bouin-Allen or 80% ethanol, and paraffin sections (8 μ .) cut. They were stained with haematoxylin and eosin or with the periodate-Schiff stain for glycogen.

Cell counting. The number of glycogen-deficient cells in twenty-two fields at a magnification of 168 were counted. This represented the total number of deficient cells in 22×450 , i.e. 9900 liver cells, there being approximately 450 cells/field. This was taken as the number of glycogen-deficient cells/10,000. When a relatively large proportion of liver cells was deficient, fewer cells were counted, but never less than ten fields.

Direct measurements of the number of liver cells were made after the method of Brues, Drury & Brues (1936).

Virus titration. The livers were ground in chilled mortars with nutrient broth as described above, and bioassays of infectivity were made, using the Reed & Muench (1938) method of calculating the mouse LD 50. Serial twofold ($0.3 \log_{10}$) steps were used, with three mice per step; the standard error was calculated by the Pizzi (1950) formula, and was usually about 0.2 log unit. Occasionally, preliminary titrations using a modification of the Ipsen (1944) method were used.

RESULTS

It was noted by de Burgh (1950) that one of the first demonstrable changes in liver cells infected with ectromelia virus was loss of ability to store glycogen. This phenomenon can be used to study the extent and spread of infection in the liver. When a count is made of the fraction of liver cells which show glycogen deficiency, when this change is well established (18 hr. or more after infection), and this number is multiplied by 1.4×10^8 (the number of liver cells was found to be 1.4×10^8 /g. liver with a standard deviation of 0.25×10^8), Table 1 can be constructed.

Table 1. *Relationship between the amount of virus injected and the number of cells unable to form glycogen*

Log ₁₀ LD 50 doses injected	Log ₁₀ no. of liver cells glycogen deficient	No. of deficient cells/100 LD 50 doses injected
6.1	5.85	60
6.5	6.25	56
6.8	6.45	45
7.1	6.85	56
7.5	7.25	56
7.8	7.45	45
8.1	7.65	35
9.0	8.10	12
9.2	8.10	8
10.0	8.15	1.4

Further, when we inject an inoculum and measure the infectivity of the liver 5 min. later, we obtain the relationship shown in Table 2. From Table 2 it appears clear that the proportion of virus in an infecting dose which is adsorbed to the liver in 5 min. is fairly constant over a large range of values, and is about one-third.

Table 2. *Proportion of injected virus found in the liver 5 min. after injection*

Log LD 50 doses injected	Log 5 min. titre	Percentage of inoculum found in liver at 5 min.
6.0	5.8	63
7.9	7.4	31
8.0	7.6	40
9.2	8.8	40
10.0	9.3	20

Fig. 1. Virus growth curves. The solid lines are virus growth curves at three different levels and each point represents a pool of (usually) three mice. Each pair of points joined by a dotted line represents an individual mouse.

From Table 1, it can be seen that with inocula over a range of 10^6 – 10^8 LD 50, the ratio of the number of deficient cells to the inoculum varies from about one to three to one to two, so in this range two LD 50 produce two to three glycogen-deficient cells. With inocula of 10^9 LD 50 and over, practically all liver cells are infected at zero time, as judged by glycogen deficiency, and Table 2 shows that the average infection per cell is more than one LD 50.

Fig. 1 shows the result of three experiments demonstrating the growth cycles

of ectromelia virus multiplication in the liver, using seed inocula of 10^{10} , 10^9 and 10^6 LD₅₀. In the experiment with the highest dose, 0.2 ml. of virus concentrate proved immediately fatal. We therefore gave two injections of 0.1 ml. separated by 5 min., and took the first titration point 7.5 min. after the first injection. At this time, some of the first injection was already disappearing. An addition of 0.3 log unit to the titre obtained would therefore seem to be a truer indication of the amount of virus reaching the liver. An initial liver titre of $10^{9.3}$ LD₅₀ represents 14 LD₅₀/cell, and this figure is used

Fig. 2. Rate of increase of virus. The logarithm of the increase in titre, i.e. log titre at various times minus log 5 min. titre, is plotted against time in hours, for various dose levels.

in later discussion, although the titre actually found, giving 7 LD₅₀/cell, appears in Fig. 1. Fig. 2 is a compendium of all our results over a large range of inocula, and plots the amount of virus multiplication that has taken place against time.

The growth curve in Fig. 1 obtained with an inoculum of 10^6 LD₅₀ seems at first sight to show features very similar to those demonstrated in other systems, namely attachment of virus to liver, maximum at 3 min., disappearance of most of this during the next 8 hr., and then stepwise increase in infectivity separated by lag periods, becoming exponential after the third rise. A consideration of the other curves, however, shows that the explanation is not so simple. In these, practically all the cells are infected at zero time, i.e. there is practically no population of fresh susceptible cells to be infected

by the first release of newly formed virus. Yet the increase in virus continues and is still stepwise. We must therefore conclude that the increase of virus titre in any one cell is stepwise.

On closer study these further facts become apparent:

(a) With a multiplicity of infection of 2.8 LD 50/cell, the first rise in infectivity occurs earlier than with less than 1 LD 50/cell, and with 14 LD 50/cell, it occurs earlier still.

(b) The titre after the first rise is quite close to the 5 min. value, and this was true in all our experiments. At the end of this rise, there is one LD 50 for every one present at 5 min.

(c) After this first rise, the titre remains constant for a variable period of time, depending on the initial dose, and then an increase of some 20 to 40-fold takes place.

(d) This rise is followed by another lag period. In the weakest dose curve there is actually a drop in infectivity, presumably due to the invasion of fresh susceptible cells and disappearance of virus in them. This lag is followed by another rise in titre.

(e) The highest titre is independent of the initial dose (within the range being discussed) and is about 10^{11} LD 50/g. liver.

(f) The efficiency of virus multiplication, expressed as the titre at any time divided by the 5 min. titre, is fairly constant over the range tested, i.e. there is little auto-interference.

DISCUSSION

Two differences can be seen between this host-virus system and others.

(a) Fourteen infectious units (LD 50's) can multiply in one cell and produce the same final titre as does one LD 50. (b) The increases in any one cell follow a stepwise pattern. Both these findings strongly suggest that in this system, the fundamental virus-synthesizing centres must be sought at a subcellular level.

In this connexion, the findings of Watson (1952) are of great interest. She studied the multiplication of mumps virus in the embryonated egg, using specific staining with fluorescein-labelled immune serum and found that the staining seen was such as to suggest multiple foci of infection within each cell. Ackermann & Kurtz (1952) and Schwerdt & Pardee (1952) suggested that such virus-synthesizing foci may be mitochondria. Preliminary experiments with ectromelia virus have shown that infectivity of the order of one-tenth of the total, and no detectable complement-fixing antigen, is present on mitochondria, but this does not necessarily mean that they are not the site of virus production.

The finding that the titre after the first increase corresponds to the original infecting titre suggests two possibilities which will be considered in more detail. First, the eclipse period could represent a breaking up of virus into smaller parts which are non-infective. A proportion only of infectious units, say 1 in 30, yields units effective in initiating multiplication, which starts in the eclipse period, so that at the end of the first rise, with a thirty-fold multiplication of effective units, the 5 min. titre is regained. If the above

process took place with an original titre of 10^8 , that is about one LD₅₀/cell, and if only one in thirty of the infecting units multiplied, only one cell in thirty would show evidence of infection. That this is not found is shown in Table 1 where the number of glycogen-deficient cells is found to be about one-third to one-half of the injected dose, and Table 2 shows a similar ratio between titre at 5 min. and injected dose, indicating 1 to 1.5 glycogen deficient cells/infectious unit in the liver at 5 min. Unless there is multiplication in some cells, and not in others (although all show the same histological changes), it is difficult to regard the first decrease and increase in titre as simple multiplication.

Another possibility is that the first decrease and increase in titre does not represent multiplication but is a preliminary step to establishing control over some of the synthetic functions of the host. This would involve either a non-infectious host-virus complex without actual breakdown of the virus, infectivity returning as the host-virus complex dissociated, or there could be a temporary break-up of the virus particle. This preliminary step should condition one or more of the cells' synthetic centres, so that after a lag period, multiplication of a stepwise nature could take place. Each centre appears to be exhausted by the production of some thirty particles, which then initiate similar processes on further centres.

This host-virus system differs from others studied at comparable dosage levels, but the differences may not be so great as appears at first sight. Continuing virus production after the initial increase, with or without obvious steps, may be a function of the type of host cell, rather than of the virus and may be more obvious in liver than in other cells. The preliminary period without overall multiplication could exist in other systems but, if it occupied a smaller proportion of the time between infection and death of the cell, it would not be obvious.

This work was carried out under a grant from the National Health and Medical Research Council, Canberra.

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(Received 20 July 1953)