Growth Characteristics of Kilham Rat Virus and Its Effect on Cellular Macromolecular Synthesis

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Kilham rat virus (KRV) is adsorbed into the rat nephroma cell within 1 hr after infection. There follows a latent period of about 12 hr during which less than 1% of the input infectious virus can be accounted for. New infectious virions can be detected at about 12 hr and the maximal yield of virus is attained by 23 hr after infection. The increase in final virus yield is about 200-fold over that found in the latent period. During this 23-hr period of virus growth, the rate of protein synthesis remains 75 to 100% of that in the uninfected cell. Ribonucleic acid (RNA) synthesis during this period is maintained at 100 to 150% of that found in the control cells. The addition of the inhibitor of deoxyribonucleic acid (DNA) synthesis, 5-fluorodeoxyuridine (FUDR), up to 8 hr after infection completely suppresses virus production. After 8 hr, viral DNA production has started and FUDR inhibition progressively decreases until by 23 hr the addition of the inhibitor no longer causes a reduced virus yield. Viral DNA synthesis once initiated is required for the remainder of the 23-hr virus cycle. Viral DNA synthesis probably begins about 4 hr before the production of infectious virions. In the KRV-infected cells, DNA synthesis decreased sharply for 6 to 7 hr after infection in comparison to the uninfected cell. At 7 to 8 hr after infection, DNA synthesis in the infected cell increased and was maintained at a higher level than in the control cells for the rest of the virus growth period.

Kilham rat virus (KRV) is one of a group of small, icosahedral viruses containing single-stranded deoxyribonucleic acid (DNA) called the parvoviruses. Also included in the group are the H-1 virus, isolated from human transplantable tumors (17), the adeno-associated virus (I. Archetti and D. S. Bocciarelli, Proc. IV Congr. Ital. Microscopia Elettronica Soc. Padova, p. 25, 1963), and the minute virus of mice (4). KRV was originally isolated from a rat sarcoma (8) and has been found to cause a fatal neonatal disease, physical deformities, and mental retardation in newborn rats (7), mice (9), and hamsters (6). The virion contains a molecule of linear single-stranded DNA with a molecular weight of about 1.6×10^6 (13) and three major proteins (12).

There is little information available about the time course of infection of most of the parvoviruses as related to the synthesis of viral DNA, ribonucleic acid (RNA), and protein. KRV is a good example to study from the parvovirus group since it can be grown in a continuous cell line independent of helper virus. We first determined the period of time required for infected cells to produce new plaque-forming virus particles. During

this period of viral growth, the effect upon synthesis of DNA, RNA, and protein by the infected cell was compared with macromolecular synthesis during the same time periods in the uninfected cell. The inhibitor of DNA synthesis, 5-fluorodeoxyuridine (FUDR), was also used to determine the time course of viral DNA synthesis by measuring the effect of the drug on the final yield of infectious virions.

MATERIALS AND METHODS

Virus growth and purification. The conditions for growth and purification of KRV and the preparation of radioactive virus have previously been described (11). Monolayer cultures of the rat nephroma cell line containing approximately 2×10^6 cells in a 60-mm petri dish, were washed twice with Eagle medium and infected with 0.5 ml of KRV or 0.5 ml of Eagle medium (control or mock infection). The viral multiplicity was 4.7.

In studies on the growth characteristics of KRV, the virus was allowed to adsorb for 1 hr at 37°C in a humidified 5% CO₂ air mixture. The cultures were washed twice with 5.0 ml of Eagle medium, and then 4.0 ml of Eagle medium containing 5% fetal calf serum and 4 mM glutamine was added. At stated

times after infection, the cells were gently scraped from duplicate plates, centrifuged at $1,000 \times g$ and the supernatant fluid was assayed for extracellular virus. The cell pellet was frozen and thawed twice, resuspended in 1 ml of Eagle medium and treated with 1% receptor-destroying enzyme (Microbiological Associates Inc., Bethesda, Md.) for 16 hr at 37°C. The disrupted cell suspension was centrifuged at $2,000 \times g$ and the resulting supernatant fluid was assayed for virus (intracellular). Extraction of the cell pellet a second time yielded an insignificant amount of virus.

FUDR inhibition. In studies involving the use of FUDR, cells were prepared and infected or mock-infected as described above. The inhibitor, final concentration 1×10^{-6} M, was added at zero time or at the stated times to four duplicate-infected plates. At 30 hr after infection, all the plates were removed from the incubator and the cells were scraped from the plates. The cell suspension was centrifuged and the intracellular virus was extracted and titrated as above.

The presence of infectious virus was determined by adding 0.2 ml of virus dilutions to monolayer cultures (2×10^6 cells) of rat nephroma cells grown in 60-mm petri dishes (Falcon Plastics, Los Angeles, Calif.). The procedure for determination of plaque-forming units has been described previously (11).

Radiochemicals and assays for DNA, RNA, and protein synthesis. For determination of incorporation of labeled precursors into DNA, RNA, and protein, the following radioisotopes were used: ^3H -thymidine (20 Ci/mm), ^3H -uridine (20 Ci/mm), ^{14}C -reconstituted protein hydrolysates (0.1 mCi/ml) (New England Nuclear Corp., Boston, Mass.). The rat nephroma cells were grown and infected or mock-infected as described above. At various times after infection, the radioisotopes were added for 1 hr to separate, duplicate plates at a final concentration of $5 \mu\text{Ci}$ of ^3H -thymidine or ^3H -uridine per ml and $0.1 \mu\text{Ci}$ of ^{14}C -amino acid mix per ml. The cells were then gently removed from the plates, centrifuged at $1,000 \times g$ and the amount of the trichloroacetic acid-insoluble precipitate was determined by the method of Regan and Chu (10).

RESULTS

Growth characteristics of KRV. KRV was grown on the rat nephroma cell line in monolayer cultures. Duplicate plates were harvested for each time period. Both extracellular and intracellular virus titers were examined for each time period by assaying for plaque-forming units (PFU) on the rat nephroma cell line. At a multiplicity of 4.7 viral PFU per cell, according to the Poisson distribution, about 99% of the cells should be infected. Within 1 hr after infection, over 90% of the plaque-forming virus particles had been adsorbed from the medium. There followed a latent period of about 12 to 14 hr (Fig. 1) during which the number of extracellular and intracellular PFU remained constant. A rise in the titer of intracellular virus was observed about 12 to 14 hr after

FIG. 1. One step growth curve of Kilham rat virus in rat nephroma cell line. A viral multiplicity of 4.7 was used. Symbols: ●—●, Intracellular virus released from cells as described in the section on methods. ○—○, Extracellular virus spontaneously released into the medium. The infectious virus titer was determined by counting the number of plaques formed on rat nephroma cells by dilutions of the virus.

infection and continued until 23 to 24 hr after infection when a plateau was reached. There was increase in the final yield by at least a factor of 200 over the latent period.

Effect of KRV infection on total cellular RNA and protein synthesis. The incorporation of ^3H -uridine or ^{14}C -amino acid mix into acid-insoluble material was used to measure the total RNA or protein synthesis after infection of rat nephroma cells with KRV. Zero time values are those found after the 1-hr period of virus adsorption. As shown in Fig. 2, total RNA synthesis, after an initial depression, is maintained at a rate equal to or higher than that found in the control cells. The elevated RNA synthetic activity, particularly at 8 and 13 hr after infection, has been repeated on several occasions and is being investigated further.

The rate of protein synthesis in infected cells drops initially after infection but for the next 22 hr is maintained at a level 75 to 100% of that in the control cell.

Effect of FUDR on KRV replication. FUDR has been shown to inhibit thymidylate synthetase (3) and thereby blocks DNA synthesis. It is possible to determine the time course of viral DNA formation by adding FUDR at various times after infection and measuring the effect of the drug on the final yield of plaque-forming virus (14). In the rat nephroma cell line, FUDR (final concentration 1×10^{-6} M) causes a complete block in cellular DNA synthesis within 1 hr after addition. FUDR (final concentration 1×10^{-6} M) was added to duplicate plates of rat

FIG. 2. Incorporation of radioisotope precursors into DNA, RNA, and protein. Rat nephroma cells were infected by Kilham rat virus and at indicated intervals (0, 2, 4, 6, 8, 11, 13, 16, 24 hr) were exposed to ^3H -thymidine, ^3H -uridine, or ^{14}C -leucine for 1 hr. The acid-precipitable radioactivity was determined as described in Materials and Methods. Zero time values are those found after the 1-hr period of virus adsorption. Rate of incorporation by infected cells is expressed as percent of radioisotope incorporated by control cells at the same intervals. Symbols: ●—●, ^3H -thymidine; ○—○, ^3H -uridine, □—□, ^{14}C -amino acid mix.

nephroma cells at various times after infection, and all the infected cells were harvested at 30 hr and titrated. As seen in Fig. 3, when FUDR was added to virus-infected cultures at times up to 8 hr after infection, the formation of infectious virus was completely suppressed. When the inhibitor was added at times from 8 to 23 hr after infection, increasing amounts of virus were formed. By 23 hr after infection a maximal yield of virus was obtained and FUDR no longer exerted an inhibitory effect. The final PFU measured in FUDR-inhibited cultures represent the viral DNA synthesized prior to the time of FUDR addition and show the rate of KRV-DNA formation (14). Thus, KRV-DNA is made from about 8 to 23 hr after infection, and viral DNA synthesis is apparently required during the entire virus maturation cycle.

Cellular and viral DNA synthesis as measured by incorporation of ^3H -thymidine. A second method for determining the kinetics of synthesis of KRV and its effect on cellular DNA synthesis is by following the incorporation of tritium-labeled thymidine into acid-precipitable material at various intervals after infection. Control (mock-infected) rat nephroma cells and identical cultures infected with KRV were used. As shown in Fig. 4, DNA synthesis in the control cells increased rapidly for several hours after addition of fresh medium and then declined steadily for the rest of the observed 24-hr period. The cells appear to have been resting in late G2 or early S phase at the time of in-

FIG. 3. The rate of formation of viral DNA as determined by 5-fluorodeoxyuridine (FUDR) inhibition of viral synthesis. FUDR (1×10^{-6} M final concentration) was added to a rat nephroma cell line at various times after infection with KRV. The cells were harvested at 30 hr after infection, the intracellular virus was extracted as described in Materials and Methods. The infectious virus titer was determined by plaqueing the virus on rat nephroma cells. The results are expressed as percent of the plaque-forming units present at 30 hr after FUDR was added at the indicated times when compared to the amount of virus produced in 30 hr by an infected culture to which no FUDR was added.

fection. In rat nephroma cells infected with KRV, DNA synthesis decreased after infection when compared with the uninfected control cells until 6 to 7 hr after infection (Fig. 3 and 4). At 7 to 8 hr after infection, DNA synthesis in the infected cultures began to increase, and reached the level found in the control cells. DNA synthesis in virus-infected cells was then maintained at a level higher than that found in the control cells for the remainder of the 24-hr period observed (Fig. 2). The observed effect of KRV on cellular DNA synthesis was prevented by incubating the virus with KRV antiserum prior to infection but was not reversed by incubation with normal serum.

DISCUSSION

After infection of rat nephroma cells by KRV (virus multiplicity of 4), there is an eclipse or latent period of 12 to 14 hr followed by a period of increased production of infectious virions. Virus maturation was completed about 24 hr after infection. Brailovsky (9), by using a higher viral multiplicity of 10, found that KRV grown on primary rat cells has a shorter latent period (6 hr) but, as reported above, virus maturation was not completed until about 24 hr after infection.

FIG. 4. Comparison of DNA synthesis in uninfected rat nephroma cells with those infected with Kilham rat virus (KRV). The incorporation of ^3H -thymidine into acid-precipitable radioactivity was carried out for 1-hr periods as described in Fig. 2. Zero time values are those found after the 1-hr period of virus adsorption. Symbols: ---, Cells infected with KRV; —, uninfected cells.

Another parvovirus, H-1, grown in human newborn kidney at a virus multiplicity of 10 to 15 has a latent period of about 10 hr but does not complete maturation until 50 hr after infection (1). This variation in the length of the latent period and the time for virus maturation which exists among the parvoviruses is probably dependent on the virus multiplicity and the host cell used.

KRV infection had a rapid inhibitory effect on DNA synthesis in the rat nephroma cells. DNA synthesis decreased sharply for 6 to 7 hr after infection to about 50% of that in the uninfected cell. Since it is not known what percentage of the cells are involved in active viral production, it is possible that the DNA synthesis during this period of time is even more severely inhibited than apparent. At 7 to 8 hr after infection, DNA synthesis in the infected cell increased and was maintained at a higher level than in the control cells for the rest of the virus growth period. These results on the effect of KRV on total cellular DNA synthesis are in contrast to those reported for growth of KRV in rat embryo cells (15). In rat embryo cells using a viral multiplicity of 10, the DNA synthesis in the infected cell remains 70 to 95% of that in the infected cell until about 10 hr after infection when there is a sharp increase in DNA synthesis in the uninfected cell and a slight decline in DNA synthesis in the infected cell. These differences in results may be attributed to the type of cell infected, the virus multiplicity, or the

state of the cells at the time of infection. RNA and protein synthesis were also followed after KRV infection of rat nephroma cells. RNA synthesis was maintained at 100 to 150% of that found in the uninfected cells. Protein synthesis remained at 75 to 100% of that in the uninfected cells. These results are consistent with those reported for the synthesis of RNA and protein in rat embryo cells infected with KRV (15).

The kinetics of viral DNA synthesis was followed by using the inhibitor of DNA synthesis, FUDR. The production of virus in FUDR-inhibited cultures depends on the amount of viral DNA synthesized before the addition of inhibitor and thus shows the rate of viral DNA synthesis (14). In the studies reported here, the addition of FUDR up to 8 hr after infection completely suppresses virus production. After 8 hr, FUDR inhibition progressively decreases until, by 23 hr after addition of the inhibitor, it no longer causes a reduced virus yield. Thus, KRV-DNA is made from 8 to 23 hr after infection and appears to be required during the entire virus maturation cycle. Adenovirus, which serves as a helper for another parvovirus, AAV, has a replication cycle similar to that of KRV. Adenovirus DNA synthesis starts at 7 to 8 hr after infection and is required for the remainder of the 21-hr virus maturation cycle (5). Since intracellular KRV virions are made from about 12 to 23 hr after infection, the synthesis of viral DNA begins about 4 hr before the appearance of mature virus. About 12% of the final viral DNA has been synthesized by the time virus maturation is demonstrable.

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