Effect of Novobiocin on Mycoplasma Virus L2 Replication

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L2 is a temperate mycoplasma virus containing 11.8 kilobase pairs of negatively superhelical double-stranded DNA. We observed L2 DNA with less superhelicity in novobiocin-treated cells than that in untreated cells. However, although no change in viral DNA superhelicity could be found in novobiocin-treated novobiocin-resistant cells, L2 production decreased in these novobiocin-treated cells.

Mycoplasma virus L2 is an enveloped virus containing 11.8 kilobase pairs of double-stranded circular DNA (molecular weight, 7.8×10^6) (8, 11). The DNA is negatively superhelical, with a superhelix density greater than that of ϕ X174 replicative-form I DNA (RFI) but less than that of PM2 phage DNA (11). L2 virions are roughly spherical particles, about 50 to 125 nm in diameter, which appear to be DNA-protein condensations, each bounded by a lipid-protein membrane (10).

Infection of *Acholeplasma laidlawii* cells by L2 is noncytotoxic (13). After a 90- to 120-min latent period there is a release of progeny virus lasting 4 to 6 h. Infected cells continue to grow, and virus maturation and release involve budding from the host cell membrane (9, 12, 13). Productive infection is followed by the establishment of a lysogenic state (13, 14), with L2 DNA integrated into the cell genome (4).

The L2 infection cycle allows studies of the role of DNA superhelicity in DNA replication and maturation of an enveloped virus. Hence, the effect of novobiocin on intracellular L2 DNA replication was investigated. Novobiocin inhibits bacterial DNA replication; transcription is inhibited to a lesser extent (15). The effect is due to novobiocin inhibition of DNA gyrase (6), an enzyme that catalyzes the ATP-dependent introduction of negative superhelical turns into circular double-stranded DNA. Gyrase is needed for processes (involving cell, plasmid, and phage DNAs) that require negatively superhelical DNA, such as DNA replication, transcription, repair, and recombination (reviewed in reference 1).

We report here studies using novobiocin to investigate the role of DNA superhelicity in mycoplasma virus L2 DNA replication and maturation. The effect on L2 growth of 2-h treatments with different novobiocin concentrations 2.5 h postinfection is shown in Fig. 1. Virus yield decreased logarithmically with increasing novobiocin concentrations up to 5 μ g/ml. At the maximum concentration used in these studies (10 μ g of novobiocin per ml) L2 yield was about 5% that of untreated infected cells.

The effect of novobiocin on L2 growth was examined during the viral one-step growth cycle (Fig. 2). At various times postinfection, a sample of infected cells was treated with 1 μ g of novobiocin per ml. Untreated infected cells showed a characteristic L2 growth curve (see reference 13). Novobiocin treatment inhibited viral growth at all times during infection.

The kinetics of the novobiocin effect on L2 yield were examined by the addition of 1, 5, or 10 μ g of novobiocin per

ml to infected JA1 cells 2 h postinfection (Fig. 3a). Increasing novobiocin concentrations decreased the rate of progeny virus production, with complete inhibition at 10 μ g of novobiocin per ml.

A study of the effect of novobiocin on the kinetics of L2 yield was repeated with JA1 Nov^r cells. Spontaneous Nov^r variants were selected on plates containing 100 μ g of novobiocin per ml and cloned. Growth of JA1 cells is inhibited threefold in 1 μ g of novobiocin per ml and 200-fold in 10 μ g of novobiocin per ml. However, no inhibition of JA1 Nov^r cell growth was observed, even at the highest novobiocin concentration used (100 μ g/ml).

Although JA1 Nov^r cells are resistant to 100 μ g of novobiocin per ml, 1 to 10 μ g of novobiocin per ml inhibited L2 viral growth in JA1 Nov^r cells (Fig. 3b), but L2 inhibition was not as extensive as in JA1 cells (Fig. 3a). To correct for differences in L2 yield between JA1 and JA1 Nov^r cells, the yield at 5 h postinfection in novobiocin-treated cells must be compared to that for untreated cells (Fig. 3). In both JA1 and JA1 Nov^r cells, 1 μ g of novobiocin per ml reduced L2 yield to 48% of that in untreated cells. However, L2 yield in the presence of 5 μ g of novobiocin per ml was reduced to 5% of that in untreated JA1 cells and 21% of that in untreated JA1 Nov^r cells. Similarly, 10 μ g of novobiocin per ml reduced the L2 yield to 1% of that in untreated JA1 cells and to 7% of that in untreated JA1 Nov^r cells. These data show that although L2 growth is inhibited by novobiocin in both JA1 and JA1 Nov^r cells, there is significantly less inhibition in JA1 Nov^r cells.

The effect of novobiocin on viral DNA synthesis was examined. At 1.5 h postinfection, culture samples received different amounts of novobiocin, and [³H]deoxythymidine was added to label DNA synthesized in the presence of novobiocin. After 4 h, cells were harvested and lysed, and nascent DNA was analyzed by velocity sedimentation in sucrose gradients (Fig. 4). Under these conditions, L2 RFI molecules (circular superhelical double-stranded DNA) were in fraction 17 and replicative-form II DNA (RFII) molecules (a relaxed form of RFI) were in fraction 10. Material sedimenting faster than fraction 20 was cellular DNA.

The amount of DNA synthesized at each novobiocin concentration was measured as the total counts per minute in each gradient and is given in the legend to Fig. 4. The decrease in DNA synthesis with increasing novobiocin concentrations followed a logarithmic relationship, similar to that between virus yield and novobiocin concentration (Fig. 1). Hence, novobiocin inhibition of virus production reflects inhibition of DNA synthesis.

The sucrose gradients (Fig. 4) showed that at novobiocin concentrations up to 1 μ g/ml, both viral and cellular DNA replication are inhibited to the same extent. At 2.5 μ g of

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FIG. 1. Novobiocin-L2 virus yield dose-response curve. A log-phase culture of JA1 cells was infected with L2 at a multiplicity of infection of 5. Growth, media, and assay of L2 as PFU were as described previously (11, 13). After 30 min of adsorption, cells were harvested by centrifugation and resuspended in fresh warm medium to remove unadsorbed L2. At 2 h after resuspension and incubation, samples were removed and mixed with different concentrations of novobiocin. PFUs were assayed after a further 2 h of incubation. The PFU data (○) are shown relative to the virus yield of infected cells not treated with novobiocin. The same dose response curve is followed by the relative amount of DNA synthesized in L2-infected cells (●), given in Fig. 4, as a function of novobiocin concentration.

novobiocin per ml, the data were obscured by a spread of the cellular DNA peak.

To investigate the degree of superhelicity of L2 DNA synthesized in the presence of novobiocin, infected cell lysates were analyzed by CsCl-ethidium bromide equilibrium centrifugation. This analysis of purified L2 [³H]DNA produced two peaks (Fig. 5a). The less dense peak in fractions 16 and 17 should be linear and nicked circular DNA molecules and the more dense peak in fractions 23 and 24 should be circular superhelical DNA molecules, since covalently closed molecules (due to topological constraints) bind less ethidium than the corresponding linear and nicked circular forms and, hence, have a greater density. In agreement with this, JA1 cell DNA, which is sheared to linear molecules in lysates, formed a band at about fraction 16 (Fig. 5c). (Both L2 viral DNA and JA1 cellular DNA are 32% guanine plus cytosine [5].)

DNA from untreated infected JA1 cells formed two peaks, one at fractions 16 and 17 and one at fractions 23 and 24 (Fig. 5b). However, DNA from infected JA1 cells treated with 2.5 µg of novobiocin per ml contained these two peaks plus an additional, more dense peak at fraction 27 (Fig. 5d). This more dense peak must represent intracellular viral DNA which bound less ethidium bromide than normal superhelical L2 DNA (in fractions 23 and 24) and, therefore, must have less negative superhelicity than normal L2 DNA. DNA from infected JA1 Nov^r cells treated with 2.5 µg of novobiocin per

FIG. 2. Effect of novobiocin on L2 growth. JA1 cells were infected as described in the legend to Fig. 1. After resuspension, the culture was diluted 10⁴-fold in warm medium, divided into two parts, and incubated at 37°C. One part (●) was assayed for PFU at several times to give a one-step growth curve. The other part (○) was used for novobiocin treatment. At 0.5, 2, and 3 h postinfection, samples were removed, and novobiocin was added (final concentration, 1 µg/ml). Each treated sample was incubated for another 2 h and assayed for PFU.

FIG. 3. Kinetics of novobiocin inhibition of L2 growth. Cells were infected and incubated as described in the legend to Fig. 1. At 2 h postinfection, the culture was divided into four parts, each receiving either 0 (●), 1 (○), 5 (△), or 10 (□) µg of novobiocin per ml, and incubation was continued. Virus titers at each time point are expressed relative to the initial titer of infectious particles. (a) JA1 cells. (b) JA1 Nov^r cells.

FIG. 4. Velocity sedimentation of infected cell lysates. JA1 cells were infected and incubated as described in the legend to Fig. 1. At 1.5 h postinfection, the culture was divided into four parts, each 5-ml sample receiving either 0 (●), 0.5 (○), 1.0 (Δ), or 2.5 (□) μg of novobiocin per ml. Each also received 30 μCi of [^3H]deoxythymidine per ml and was incubated for a further 4 h. At 5.5 h postinfection, all samples were lysed with 0.4% Sarkosyl NL97 and analyzed by sucrose gradient centrifugation. The lysis and 5 to 20% (wt/vol) sucrose gradient centrifugation conditions have been described previously (2), as have the procedures for measurement of acid-precipitable radioactivity (7). For each gradient, the percent acid-precipitable counts per minute in each fraction is plotted. The total in each gradient was 739,578 cpm for no novobiocin, 605,146 cpm for 0.5 μg of novobiocin per ml, 323,192 cpm for 1.0 μg of novobiocin per ml, and 105,678 cpm for 2.5 μg of novobiocin per ml. The peak in fraction 17 is L2 RFI DNA, and the material in fraction 10 is L2 RFII DNA, as determined from a parallel gradient containing purified L2 [^3H]DNA. Material sedimenting faster than fraction 20 is cellular DNA. The data scatter in fractions 27 and 28 is due to mixing with the cushion during unloading of the gradients.

ml contained material only in two peaks, in fractions 16 and 17 and fractions 23 and 24 (Fig. 5e). Therefore, a reduction in negative superhelicity in intracellular L2 DNA due to novobiocin treatment was observed in JA1 cells, but not in JA1 Nov^r cells.

These studies indicate that mycoplasmas have a novobiocin-sensitive DNA gyrase activity which is involved in L2 viral DNA replication. In JA1 cells, but not in JA1 Nov^r cells, L2 DNA with less negative superhelicity is synthesized after novobiocin addition (Fig. 5). However, novobiocin does cause some inhibition in L2 production in JA1 Nov^r cells (Fig. 3b), although L2 DNA synthesized in novobiocin-treated JA1 Nov^r cells appears to have normal negative superhelicity (Fig. 5e). This suggests that L2 replication also involves another novobiocin-sensitive process in addition to

FIG. 5. CsCl-ethidium bromide equilibrium centrifugation of L2 DNA synthesized during novobiocin treatment. Unless otherwise stated, cells were infected with L2, treated with novobiocin, and labeled with 30 μCi of [^3H]deoxythymidine per ml as described for Fig. 4. Samples were then lysed with 0.4% Sarkosyl NL97 (2) and analyzed by CsCl-ethidium bromide equilibrium centrifugation. The lysate was mixed with CsCl to a refractive index of 1.388 in TES buffer (0.01 M Tris, 0.001 M EDTA, 0.1 M NaCl, pH 8.0) and ethidium bromide (final concentration, 100 $\mu\text{g}/\text{ml}$) and centrifuged as described previously (2). (a) Purified L2 [^3H]DNA. (b) [^3H]DNA from infected JA1 cells which received no novobiocin. (c) JA1 [^3H]DNA. (d) [^3H]DNA from infected JA1 cells treated with 2.5 μg of novobiocin per ml. (e) [^3H]DNA from infected JA1 Nov^r cells treated with 2.5 μg of novobiocin per ml. The Nov^r cells were labeled with 50 μCi of [^3H]deoxythymidine per ml.

DNA supercoiling. A similar situation has been reported for adenovirus replication, which seems to involve two novobiocin-sensitive steps, one being DNA gyrase-dependent DNA synthesis and the other being DNA maturation or encapsidation or both (3). Since the L2 chromosome is packaged in the virion as a superhelical molecule (11), L2 assembly and budding may also involve a novobiocin-sensitive process. However, we cannot exclude the possibility that novobiocin inhibition of L2 virus in JA1 Nov^r cells is due to a small decrease in superhelicity which has not been resolved in these studies or to decreased transcription of viral DNA under these conditions.

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