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Inhibition of replication of human respiratory syncytial virus by 6-diazo-5-oxo-L-norleucine

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Abstract

The effect of 6-diazo-5-oxo-L-norleucine (L-DON), a glutamine analog, on RSV replication was studied. At a concentration of 0.01 mM L-DON, 99% of RSV replication in treated CV-1 cells was inhibited. At this concentration of L-DON, the level of cellular protein synthesis was identical to untreated control cells. Trypan blue staining revealed that all the cells remained viable even at concentrations of L-DON as high as 10 mM. In addition, L-DON added as late as 24 h post infection can effectively suppress viral replication. Analysis of viral mRNA levels by Northern blot revealed that secondary transcription and subsequent steps in the virus life cycle were inhibited. Immunoprecipitation of viral proteins from drug treated or untreated cultures showed that synthesis of all viral proteins was drastically reduced by L-DON, with a slightly greater inhibition of viral glycoproteins. Furthermore, immunofluorescent staining showed that drug treated cells expressed both F and N proteins and that F was inserted into the membrane as the native F protein.

Keywords: Human respiratory syncytial virus; L-DON; Protein synthesis; Transcription

1. Introduction

Human respiratory syncytial virus (RSV) is an enveloped, cytoplasmic virus, containing a single-stranded, negative sense RNA of approximately 15 kb (Lambert et al., 1980; Huang and Wertz, 1982; Huang and Wertz, 1983). The genome encodes 10 mRNAs (Collins and Wertz, 1983; Collins et al., 1984) which translate into 10 distinct polypeptides (Huang et al., 1985). RSV is currently classified in the family of paramyxoviridae and is the leading cause of lower respiratory disease in infants and young

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children (McIntosh and Chanock, 1990). There is neither an effective vaccine nor a potent antiviral agent currently available. An RSV vaccine composed of formalin-inactivated virus is not only ineffective in protecting against infection but also enhances the severity of disease during the course of subsequent natural infection (Kim et al., 1976). It has been shown that ribavirin may reduce the severity of illness and the degree of virus shedding during acute RSV infection (Hall et al., 1983; McIntosh et al., 1984). However, ribavirin was shown to have certain side effects. It was shown to be teratogenic or embryolethal in hamsters, rats and rabbits. In subhuman primates, however, ribavirin was not teratogenic (Fernandez et al., 1986). Nevertheless, the teratogenicity induced in the species mentioned and the potential risk involved for pregnant women cannot be overlooked. Additionally, the sophisticated aerosol procedures and high cost of ribavirin administration have limited its usage. This has prompted the search for other antiviral agents which may be more effective and safe than ribavirin.

6-Diazo-5-oxo-L-norleucine (L-DON) is a glutamine analogue which specifically inhibits γ–glutamyl transpeptidase (Pittillo and Hunt, 1967) and also inhibits nucleic acid production by interfering with the amino acid transfer reaction during purine and pyrimidine synthesis (King et al., 1978). L-DON has recently been used in phase II human trials as an antisarcoma agent. Patients treated with L-DON did not experience any severe adverse side effects even at high doses (Earhart et al., 1990). This indicates that L-DON has potential use as an anti-viral agent in humans. In this study, we investigated the effects of L-DON on RSV replication in CV-1 cells. Our data show that in the presence of L-DON, release of extracellular virus particles was drastically reduced, and intracellularly, synthesis of all viral proteins was greatly inhibited. Furthermore, the site of inhibition is not at the level of primary transcription.

2. Materials and methods

2.1. Cells and cell culture

The CV-1 (ATCC: CCL-70) cells were obtained from American type culture collection (Rockville, MD, USA) and were cultured in Earle's Minimum Essential Medium (MEM), supplemented with 10% fetal calf serum containing 2 mM glutamine, 100 units/ml of penicillin and 100 μg/ml of streptomycin.

2.2. Compound

6-diazo-5-oxo-L-norleucine (L-DON) was purchased from Sigma Chemical (St. Louis, MO, USA). A 100 mM stock solution was prepared in MEM and 0.5 mL aliquots were stored at -20°C. Appropriate dilutions were prepared immediately prior to use.

2.3. Virus

Subtype A, strain A2 of RSV (obtained from Dr. Edward Dubovi, University of North Carolina, Chapel Hill) was propagated in CV-1 cells. The infectious virus titers

were determined by plaque assay on CV-1 cells as described (Huang and Wertz, 1982). Briefly, confluent CV-1 monolayers, cultured in 24 well tissue culture plates at 2×10^5 cells/well, were inoculated with serial 10-fold dilutions of virus samples. After 2 h of virus adsorption, the cells were washed with MEM and overlaid with MEM in 0.5% agarose supplemented with 1% fetal calf serum. Plaques were visualized by staining with 2% crystal violet in phosphate buffered saline (PBS) pH 7.2.

2.4. Isotopic labeling of infected cells

CV-1 cells were infected with RSV at an M.O.I. of 3. After 2 h of adsorption, the cells were either treated with L-DON or left untreated. At 46 h post infection, the cells were depleted of cysteine and methionine for 20 minutes at 37°C (Huang et al., 1985) and then labeled with Trans ^{35}S-Label at 50 μCi/ml (specific activity > 1000 Ci/mM) (ICN Biochemicals, Irvin, CA, USA) for 2 h in the absence of L-DON. The cells were either lysed directly in SDS-PAGE sample buffer or lysed in 1% NP-40 in TKM (50 mM Tris-HCl, 100 mM KCl, 5 mM $MgCl_2$ pH 7.3) and cytoplasmic extracts were used for immunoprecipitation.

2.5. Immunoprecipitation of infected cell lysates

Trans ^{35}S-labeled virus or mock infected cell lysates were mixed with hyperimmune guinea pig antisera (Biowhittaker, MD, USA) and incubated overnight at 4°C. The complexes were adsorbed onto *Staphylococcus aureus* cells by being mixed with 40 μl of pansorbin (Calbiochem-Behing, CA, USA). The adsorbed complexes were pelleted in an eppendorf centrifuge, resuspended in SDS-PAGE sample buffer and boiled for 4 min. Samples were analyzed by SDS-PAGE.

2.6. Indirect immunofluorescence assay

Since it was reported that in Vero cells infected with Human parainfluenza virus type II (HPIV-2), L-DON selectively inhibited viral glycoprotein synthesis (Nishio et al., 1990), we chose to study the effect of L-DON on the RSV fusion protein (F), which is a glycoprotein and the RSV nucleocapsid protein (N), which is not a glycoprotein. Monoclonal antibodies, C797 (anti-N), produced initially against the Long strain (subtype A) of RSV was generously provided by Dr. Erling Norby (Karolinska Institute, Sweden). Monoclonal antibodies against F (fusion) protein were generously provided by Dr. Jose A Melero (Centro Nacionalde Microbiologia, Majadahonda, Spain). The antibodies were diluted 1:50 prior to use as the primary antibody. FITC-conjugated rabbit anti-mouse IgG (Bartels, Baxter Co. Seattle, USA) was used as the secondary antibody. After washing with phosphate buffered saline (PBS), the slides were mounted and examined with a Zeiss ultraviolet light fluorescence microscope.

2.7. Preparation of hybridization probes by in vitro transcription

RSV N gene cloned into pT7/T3α–18 vector and RSV F gene cloned into pGEM3 vector were digested with *Pst*1 and *Hind*III, respectively, to linearize the DNA for

transcription. Antisense probes were synthesized in a 20 μl reaction containing 40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT (Dithiothreital), unlabeled rNTPs (1 mM each of ATP, GTP, UTP), 30 μCi of ^{35}S-CTP (Amersham), 0.5 μg of linearized DNA template. The reaction mixture was equilibrated at 40°C for 5 min after which 40 units of T7 (F gene) or 40 units of T3 (N gene) RNA polymerase were added and incubated at 40°C for 1 h. The transcripts were isolated by digestion of the DNA template with 10 units of DNase 1, followed by phenol/chloroform (1:1) extraction and ethanol precipitation.

2.8. Slot blot hybridization

Preparation of total RNA from cells was as described (Huang and Wertz, 1982). RSV-infected, L-DON treated or untreated or mock-infected cells were washed 3 × with RSB (10 mM Tris, 10 mM NaCl, 1.5 mM $MgCl_2$, pH 8.5; diluted 1:3 in dH_2O). Cells were collected and NP-40 was added to 0.5% to lyse the cells. Nuclei were removed by brief centrifugation. RNA was isolated from the cytoplasmic lysates by phenol extraction and ethanol precipitation. Purified total RNA was then immobilized onto immobilon-N (Millipore) membranes and hybridized to ^{35}S-labeled probes specific for the fusion (F) gene or the nucleocapsid (N) gene for 18 h at 42°C.

3. Results

3.1. Effect of L-DON on RSV replication in CV-1 cells

CV-1 cells were infected with RSV at an M.O.I. of 3. After 2 h adsorption at 36°C, infected cells were washed three times with MEM and then incubated with MEM containing various concentrations of L-DON. Incubation continued for 48 h. At this time the virus-infected, untreated cells showed 100% cytopathic effect (CPE). The culture fluids were harvested and virus infectivity titers were assayed by plaque formation. Table 1 showed the dose-dependent inhibition of RSV replication by L-DON. At a concentration of 0.01 mM of L-DON, 99% of viral replication was inhibited. Although

Table 1
Effects of L-DON on Virus Growth in CV-1 Cells

Dose of L-DON (mM)	Virus yield (p.f.u./ml)	Inhibition (%)
0.0	6.48 ± 0.03	0.00%
0.001	6.40 ± 0.02	15.00%
0.01	3.48 ± 0.04	99.9%
0.1	2.0 ± 0.03	> 99.99%

Effects of L-DON on RSV replication. CV-1 cells were infected with RSV at an M.O.I. of 3 and allowed to adsorb at 36°C for 2 h. Cells were then washed and incubated with MEM containing various concentrations of L-DON. After incubation for 48 h, culture supernatants were harvested and virus titers were determined by plaque forming assay in CV-1 cells. Assays were done in triplicate in 24-well plates and log virus titers are shown.

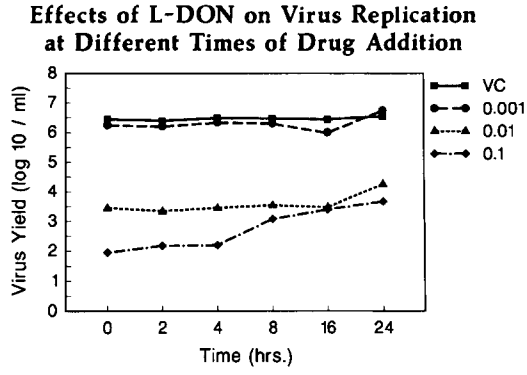

Fig. 1. Effects of L-DON on RSV replication after addition of drug at different time points post infection. CV-1 cells were infected with RSV and allowed to adsorb for 2 h, as described in Table 1. Cells were then washed and incubated with MEM and various concentrations of L-DON were added at the time points indicated. At 48 h post infection, the culture supernatants were harvested and assayed as in Table 1. Virus titers are the average of triplicate wells with a standard deviation of ± 0.05.

an infectivity titer of 3×10^3 pfu/ml was detected in the medium at 0.01 mM of L-DON, no CPE was observed after 48 h of incubation, whereas the untreated, infected cells exhibited complete CPE. To assess the toxicity of L-DON to CV-1 cells, the cells were treated with 0.001 mM, 0.01 mM, 0.1 mM, 1 mM and 10 mM of L-DON for 48 h and protein synthesis was measured by incorporation of ^{35}S-methionine and cysteine. At 0.001 mM, 0.01 mM, 0.1 mM and 1 mM L-DON, the level of radioactivity incorporated by cells was identical to incorporated radioactivity for untreated control cells (data not shown). When 10 mM L-DON was used to treat cells, the level of incorporated activity was slightly lower as compared to untreated control cells. However, when cells that had been treated with any concentration of L-DON, even 10 mM, were stained with trypan blue, all cells were viable (data not shown).

3.2. Effects of L-DON on virus replication at different times of drug addition

RSV-infected CV-1 cells were treated with different concentrations of L-DON at various time points post infection. The medium were harvested at 48 h post infection and viral titers were determined by plaque assay. Virus-infected cells treated with 0.001 mM L-DON exhibited only a slight inhibition of viral replication at the different time points tested (Fig. 1). However, at concentrations of 0.01 mM or 0.1 mM L-DON added as late as 24 h post infection (P.I.), RSV replication was still effectively inhibited. These results suggest that L-DON can suppress RSV replication even at the times that a significant quantitiy of virus was being released into the medium.

3.3. Effect of L-DON on viral protein synthesis

L-DON is known to interfere with glycoprotein synthesis or the formation of functional glycoproteins (Pratt and Green, 1976; Sabina and Telasco, 1979; Goldstein

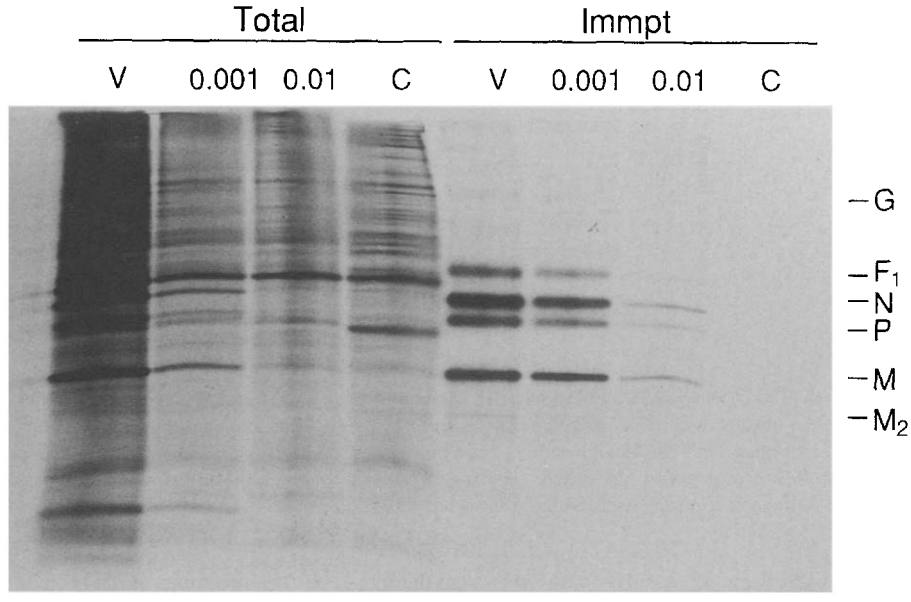

Fig. 2. Effect of L-DON on viral protein synthesis. CV-1 cells were infected with RSV at an M.O.I. of 3. After 2 h of adsorption, the cells were either treated with L-DON or left untreated. At 46 h P.I., RSV-infected and L-DON treated or untreated and mock-infected cells were depleted of cysteine and methionine for 20 min and then labeled for 2 h with Trans ^{35}S-Label (50 μCi/ml; specific activity >1000 Ci/mM). Radiolabeled cells were analyzed directly or after immunoprecipitation with hyperimmune RSV antisera by SDS-PAGE and autoradiography. RSV-infected cell (V); L-DON added in mM (0.001) and (0.01); mock-infected cell (C).

and Guskey, 1984; Nishio et al., 1990). To determine which virus-specific proteins were inhibited by L-DON in virus-infected CV-1 cells, cells were infected with RSV as described above and various concentrations of drug were added after virus adsorption. After incubation for 46 h, the cells were labeled for 2 h with Trans ^{35}S label and subsequently lysed in SDS-PAGE sample buffer and analyzed. Fig. 2 showed that host protein synthesis was not inhibited by RSV infection at the drug concentrations used and that viral protein synthesis was markedly decreased at 0.01 mM L-DON. To better determine which virus specific proteins were inhibited by L-DON, immunoprecipitation was carried out using hyperimmune RSV antisera. The results, shown in Fig. 2, indicated that the G, F, N, P, M and M2 proteins of RSV were significantly inhibited. Densitometric analysis of the bands corresponding to the immunoprecipitated viral proteins confirms the inhibition of viral protein synthesis. Treatment with 0.001 mM L-DON reduced levels of the F protein to 43.5% of control levels. Treatment with 0.01 mM L-DON reduced levels of F protein to 2.7% of control levels. Treatment of cells with 0.001 mM L-DON reduced levels of N, P and M to 56.6%, 47.9% and 67.5% of control levels, respectively. At 0.01 mM L-DON, levels of N, P and M were reduced to 8.0%, 9.1% and 13.1% of control levels, respectively. These data suggest that all viral proteins are inhibited by L-DON at comparable levels and that the inhibition is dose-dependent. Even though the F glycoprotein was inhibited slightly more than the

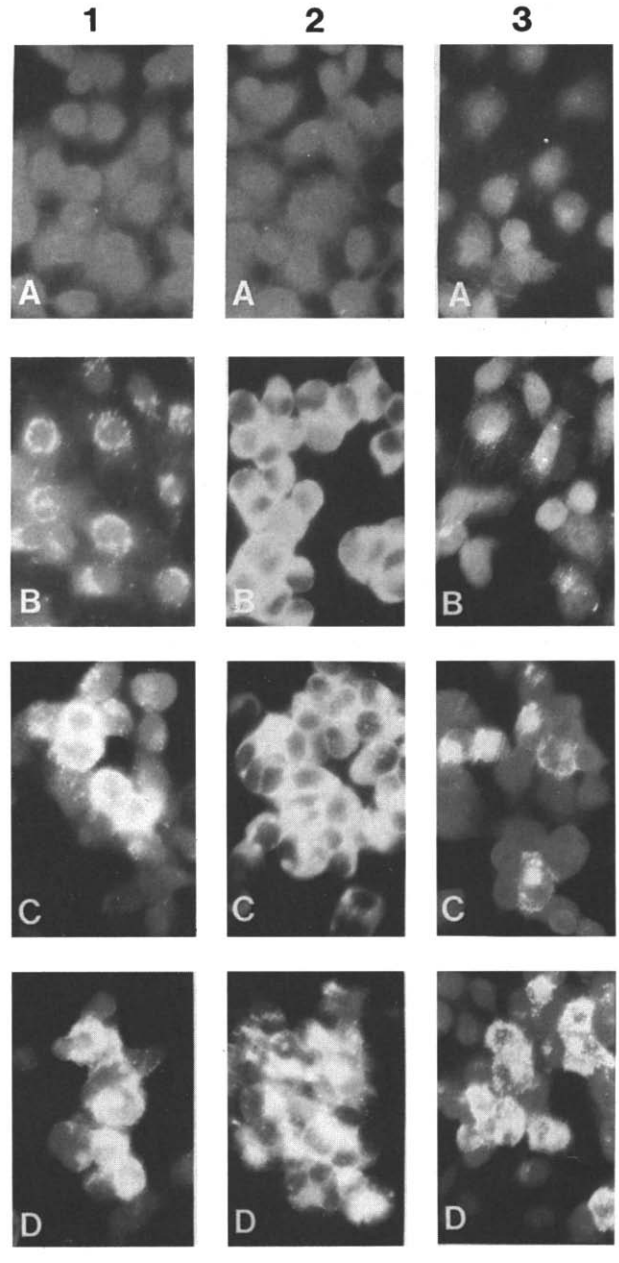

Fig. 3. Immunofluorescence staining showing effects of L-DON on protein synthesis. CV-1 cells were uninfected (A) or infected with RSV and treated with 0.01 mM L-DON (B) or 0.001 mM L-DON (C) or no drug (D). Cells were stained with monoclonal antibodies (at 1:50 dilution) directed against the F protein (panels 1 and 2) and the N protein (panel 3) at 24 h P.I. Panel 1 was fixed with formaldehyde only. Panels 2 and 3 were fixed with formaldehyde and treated with 0.05% Triton X-100. The fields shown are representative of all cells stained.

other viral proteins at 0.01 mM L-DON, synthesis of F was not completely inhibited. Given the previously discussed finding that cellular protein synthesis was not reduced by treatment with L-DON at concentrations up to 0.1 mM, the inhibition of viral protein synthesis observed was not due to a general inhibition of the cellular protein synthesis machinery.

It was demonstarted that in Vero cells infected with HPIV-2, L-DON selectively and completely inhibited viral glycoprotein synthesis (Nishio et al., 1990). However, in the case of RSV, this phenomena was not observed in the immunoprecipitation analysis. It is therefore, desireable to examine the distribution of the viral proteins in infected cells. To do this, virus-infected cells were treated or untreated with L-DON at 0.001 mM and 0.01 mM. After 24 h incubation with the drug, the cells were collected and either fixed with formaldehyde only or fixed with formaldehyde and treated with Triton-X-100 at 0.05%. The cells were stained with mAbs directed against the F (Fig. 3, panels 1 and 2) and N (Fig. 3, panel 3) proteins of RSV. Protein-specific fluorescence was observed in all of the RSV infected cultures whether it was treated or untreated with L-DON. This finding is consistent with the results from the immunoprecipitation experiment. There appeared to be no significant selective inhibition of glycoproteins as determined by viewing the relative intensity of staining. To determine whether the F proteins synthesized were integrated into the membrane, the cells were fixed with formaldehyde and stained with a monoclonal antibody directed against F protein. Fig. 3, panel 1 shows that the F protein was expressed on the surface of the cells in the presence of L-DON.

3.4. Effects of L-DON on viral mRNA synthesis

The observation that L-DON can also inhibit nucleic acid synthesis led to the question of whether inhibition of viral mRNA synthesis may contribute to the observed decrease in synthesis of viral proteins. In order to determine whether L-DON treatment of RSV-infected cells resulted in a decrease in levels of viral mRNA, total RNA was isolated from RSV-infected cells which were treated or untreated with L-DON, and from uninfected, untreated control cells as described previously (Huang and Wertz, 1982). Total RNA (3 μg) was immobilized onto Immobilon-N membranes and hybridized to ^{35}S-labeled antisense riboprobes specific for the F and N genes. In CV-1 cells infected with an M.O.I. of 3, progeny virus were first released at about 12 h P.I. and mRNA synthesis peaked at about 16–18 h P.I. By 24 h P.I., significant titers of virus were produced and levels of virus titers peaked at about 48 h P.I. (our unpublished observations). Based on these observations, we chose to analyze the viral mRNA levels at 8 h, 16 h and 24 h P.I. As determined by densitometric analysis of the bands shown in Fig. 4, at 8 h P.I. the viral mRNA synthesis in L-DON treated or untreated cells was similar indicating that the inhibition is not at the level of primary transcription. At 16 h P.I. viral mRNA synthesis was drastically reduced when virus infected cells were treated with 0.01 mM of L-DON, whereas, viral mRNA synthesized in cells treated with 0.001 mM of L-DON is similar to untreated, virus-infected cells.

At 24 h P.I. levels of both F and N mRNA continued to increase in untreated, infected control cells. However, levels of viral mRNA from cells treated with either 0.01 mM or 0.001 mM L-DON were lower or remained the same at 24 h P.I., indicating the

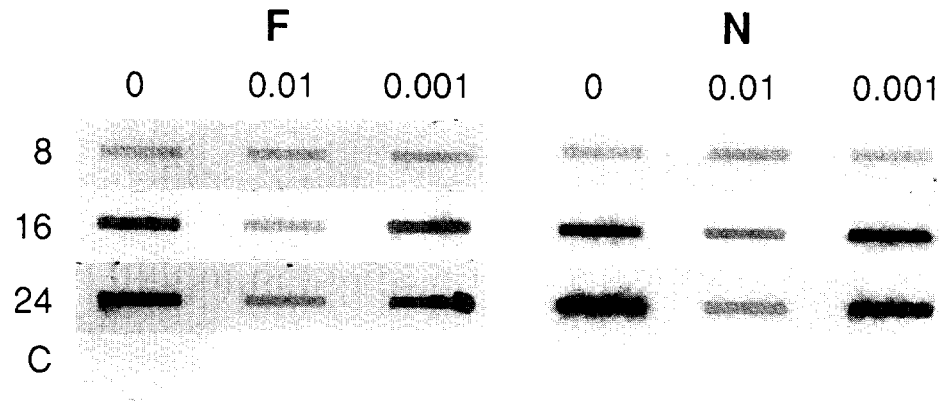

Fig. 4. Effects of L-DON on mRNA synthesis. Total RNA was extracted at 8, 16, 24 h post infection from CV-1 cells which had been infected with RSV and either untreated, treated with 0.01 mM L-DON or 0.001 mM L-DON. 3 μg of RNA was immobilized onto Immobilon-N membranes. Blots were hybridized to ^{35}S-labeled riboprobes recognizing the mRNA of either the fusion protein (F) or the nucleocapsid protein (N). Uninfected and untreated CV-1 cells were used as a control (C). Hybridization was carried out for 18 h at 42°C.

ability of L-DON, even at low concentrations, to suppress the ongoing synthesis of viral mRNA observed in untreated control cells.

4. Discussion

L-DON is a potent inhibitor of RSV replication. At a concentration of 0.01 mM added after virus attachment, L-DON inhibited 99.9% of RSV replication as determined by plaque assay. Our results show that RSV is far more sensitive to L-DON than HPIV-2, mumps virus, and vesicular stomatitis virus (VSV) as reported by Nishio et al., 1990. It is also evident that L-DON is not cell-type specific since it has similar effects in RSV-infected Hep-2 cells (data not shown). At a concentration of 0.01 mM L-DON, RSV-infected cells contained 3×10^3 pfu/ml in the medium, however, the cells showed no detectable CPE. This result is consistent with the previously reported finding that 0.12 mM of L-DON inhibited plaque formation of VSV in Hep-2 cells (Goldstein and Guskey, 1984).

Surprisingly, when L-DON was added as late as 24 h P.I. (the last time point in the assay), virus replication was still strongly suppressed. This suggests that L-DON can effectively reduce virus shedding in an already infected culture. This has significant implications for treatment of RSV-infected patients.

L-DON inhibits γ-glutamyl transpeptidase and it was shown to specifically inhibit glycosylation of viral glycoproteins in HPIV-2 infected Vero cells (Nishio et al., 1990). However, in RSV-infected, L-DON-treated CV-1 cells, synthesis of all viral proteins was suppressed and inhibition of viral glycoproteins was only slightly more pronounced. In addition, in the presence of L-DON, both glycoproteins F1 and G comigrate with

native, glycosylated F1 and G when analyzed by SDS-PAGE, indicating that RSV glycoproteins synthesized in L-DON-treated cells are glycosylated (Fig. 2). Although F was expressed at lower levels, it was inserted into the cell membrane as in untreated cells (Fig. 3, panel 1). Taken together, these results suggest that the glycoprotein synthesis is quantitatively reduced instead of qualitatively altered as shown for HPIV-2 (Nishio et al., 1990). It can not be ruled out that the unglycosylated glycoproteins became unstable and were undetectable by the methods employed in this study. By comparing these features of L-DON action against RSV with those reported for HPIV-2 (Nishio et al., 1990), it becomes evident that the mechanism of action may be different even though both viruses are in the same family.

Another function of L-DON is that it inhibits nucleic acid production by interfering with amino transfer reactions during purine and pyrimidine synthesis (King et al., 1978). Viral mRNA synthesis was analyzed by Northern blot and densitometric analysis showed that at 8 h P.I. the levels of F and N mRNA synthesized were similar in L-DON treated and untreated virus-infected cells. Since one RSV growth cycle is 12 h for the experimental conditions of this study, this result indicates that primary transcription was not affected. At 16 h P.I., however, the levels of F and N mRNA synthesized were markedly decreased in virus-infected cells treated with L-DON as compared to untreated, virus-infected cells. This suggests that secondary transcription and subsequent steps in viral replication were inhibited by L-DON. Interestingly, at 24 h P.I., levels of F and N mRNA continued to increase in untreated controls but this increase was suppressed by even low concentrations of L-DON. These results are similar to the reported effect of L-DON on HPIV-2 mRNA synthesis (Nishio et al., 1990). The inhibition of purine and pyrimidine synthesis by L-DON may be responsible for the observed decrease in mRNA synthesis, however, we cannot rule out an alternative possibility that since L-DON decreases protein synthesis, a decrease in levels of the viral polymerase may contribute to the inhibition in the levels of viral mRNA. Given the two known mechanisms of the function of L-DON, we did not observe similar inhibition of viral glycoprotein synthesis as was observed in the HPIV-2 studies (Nishio et al., 1990). However, the inhibition of mRNA synthesis was similar to that observed with HPIV-2 and L-DON treatment. This discrepency between RSV and HPIV-2 remains to be clarified.

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