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Inhibition of Cytomegalovirus Replication by Smooth-Muscle Relaxing Agents[1] (42581)

T. ALBRECHT*,[2] C. H. LEE,* D. J. SPEELMAN,*,[3] AND O. S. STEINSLAND†

*Departments of *Microbiology and †Pharmacology, The University of Texas Medical Branch, Galveston, Texas 77550*

Abstract. The effect of the smooth-muscle relaxing agents, papaverine, sodium nitroprusside, and verapamil, on the replication of human cytomegalovirus (CMV) was investigated. At a concentration of 100 μM, infectious yields of CMV were reduced by 1.23 to 5.72 $\log_{10}$ by these drugs (papaverine, 5.72 $\log_{10}$; nitroprusside, 1.85 $\log_{10}$; verapamil, 1.23 $\log_{10}$). The ED_{50} for papaverine was found to be somewhat less than 1 μM, a concentration which appears to be within the range achieved clinically. Papaverine did not irreversibly modify treated cells to a virus-resistant state since treatment of cells with papaverine from 24 hr before until immediately prior to CMV infection did not significantly reduce CMV yields. Replication of CMV was most sensitive to inhibition when papaverine was added at or before 6 hr after CMV infection. Addition of papaverine at later times resulted in a substantial reduction of the inhibitory effect on virus yields, suggesting that the phase of CMV replication sensitive to papaverine inhibition occurred early in the replication cycle. These results, particularly in light of the potency of papaverine, indicate that some smooth-muscle relaxing agents have significant antiviral activity toward the replication of CMV.

Cytomegalovirus (CMV) is known to be responsible for serious congenital infections and for life-threatening infections in persons of all ages whose immune systems have been compromised. Recently, the association of severe CMV infections with loss of immune responsiveness has been emphasized by recognition of the frequent complication of CMV disease in individuals with acquired immune deficiency syndrome (AIDS). Although several drugs have been demonstrated to influence infections by other herpesviruses, except for 9-(1,3-dihydroxy-2-propoxymethyl) guanine (DHPG) (1, 2), these drugs are, for the most part, ineffective against life-threatening CMV disease (3). It may be reasonable, however, to explore approaches other than conventional therapies which have involved nucleoside analogs or interferons since CMVs seem to have unique effects on their host cells (4–6). Such a novel approach to the development of therapeutic treatment for CMV infections was suggested by our recent findings which revealed that characteristic cellular responses to CMV infection, including rounding, "contraction," "relaxation," and enlargement, were blocked by several smooth-muscle relaxing agents. Here we describe the inhibition of the replication of CMV by three smooth-muscle relaxing agents (papaverine, sodium nitroprusside, verapamil). Papaverine was the most potent of these drugs, producing a 95.4% reduction of CMV yields with a dose of 6.3 μM, suggesting that this drug might have potential clinical significance for the treatment of CMV infection. The finding that these drugs also inhibit CMV replication suggests that there may be a relationship between inhibition of these cellular responses and interference with CMV replication. The remarkable potency of papaverine, however, could not be correlated completely with inhibition of early cellular responses.

Materials and Methods. *Materials.* Verapamil was obtained from Knoll Pharmaceutical Co. (Whippany, NJ). Sodium nitroprusside and papaverine (except as noted below) were obtained from the University of Texas Medical Branch Hospital Pharmacy (Galveston, TX). Sodium nitroprusside was supplied by Abbott Laboratories (North Chicago, IL). Papaverine was supplied by Eli Lilly and Co. (Indianapolis, IN). DHPG from a single lot supplied by the Syntex Corp. (Palo Alto, CA)

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[2] To whom all correspondence and reprint requests should be addressed.

[3] Present address: Bristol-Myers Co., P.O. Box 4755, Syracuse, NY 13221.

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was distributed by Dr. Leslye D. Johnson (Hepatitis Program Officer, Development and Applications Branch, Microbiology and Infectious Diseases Program, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD) to us and to other investigators examining the potency of antiviral drugs for CMV. Papaverine from a single lot supplied by Eli Lilly was similarly distributed.

Assays for inhibition of infectious CMV yields. Human embryo skin–muscle (SM) cells (7) between the 5th and 25th passage were cultured in Leighton tubes, as described previously in detail (4). When the SM cells had attained confluence, they were inoculated with CMV (strain AD169) at a calculated multiplicity of about five plaque-forming units (PFU) per cell. After 1 hr for adsorption, the inocula were removed, the cells were washed twice with maintenance medium (Eagle's MEM with Earle's salts, 0.150% $NaHCO_3$, and 5% γ-irradiated fetal calf serum), and each tube was inoculated with 1 ml of maintenance medium, with or without one of the drugs. All drug solutions were made immediately prior to use and the maintenance medium, with or without the drug, was replaced every 24 or 48 hr. Cell cultures were maintained at 37°C in the absence of antibiotics. At selected times after infection, virus replication was stopped by quick-freezing and duplicate or triplicate Leighton-tube cultures were stored at −90°C, until they were assayed for infectivity (8).

Assays for the rate of cell DNA synthesis. The rate of cell DNA synthesis was determined by measuring the incorporation of [*methyl*-^{3}H]thymidine (ICN Biomedicals, Inc., Irvine, CA) into cell DNA. CMV and cell DNA were separated by isopycnic centrifugation in CsCl. The methods have been described in detail previously (8).

Results. Infectivities assayed at various times during the single-step replication of CMV demonstrate that the smooth-muscle relaxing agents, verapamil, nitroprusside, and papaverine, inhibited CMV multiplication (Fig. 1). Papaverine was the most potent of the three drugs; at a concentration of 30 μg/ml (80 μM) the CMV yield was inhibited by 5.21 $\log_{10}$ at 120 hr postinfection (PI). Nitroprusside at a concentration of 30 μg/ml (115 μM) reduced the expected infectious yield by 2.85 $\log_{10}$. Verapamil, at 30 μg/ml (61 μM),

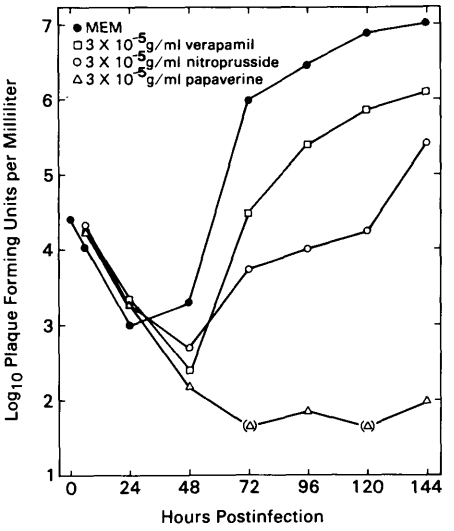

FIG. 1. Inhibition of CMV replication by papaverine (△; 30 μg/ml; 80 μM), sodium nitroprusside (○; 30 μg/ml; 115 μM), or verapamil (□; 30 μg/ml; 61 μM). Medium with or without (●) drug was changed daily. Symbols in parentheses indicate maximum surviving virus infectivity, since within the limits of the plaque assay, surviving virus infectivity was not detected at these drug concentrations and intervals of exposure.

reduced the yield by 1.03 $\log_{10}$. Quantitatively similar results were obtained in three additional experiments. The antiviral effect of the smooth-muscle relaxing agents was not dependent on the type of human fibroblast used as substrate. For example, papaverine (10 μg/ml) reduced CMV yields in cells derived from embryonic thyroid, lung, and SM to about the same degree (4.16, 4.44, and 4.01 $\log_{10}$, respectively). Apparently, the inhibitory effect of these drugs was not due to cellular toxicity since cell viability was not affected over a 120-hr period when measured by trypan blue exclusion (data not shown). Moreover, the doubling time for the papaverine-treated cells after papaverine was removed was very similar to the doubling time for cells not treated with papaverine.

The inhibitory effect of these smooth-muscle relaxing drugs on CMV replication was concentration-dependent (Fig. 2). Papaverine was the most potent inhibitor, producing significant inhibition at all concentrations tested. Log_{10} reductions in yields of CMV obtained from cells treated with papaverine at concen-

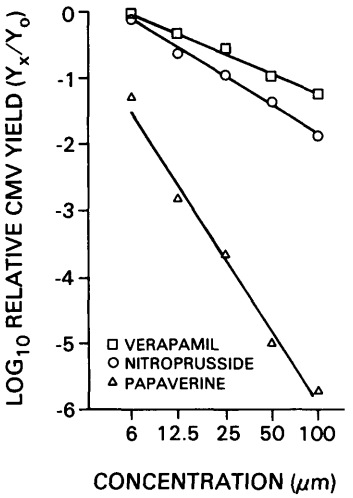

FIG. 2. Inhibition of CMV replication as a function of the concentration of smooth-muscle relaxing agents (△, papaverine; ○, sodium nitroprusside; □, verapamil). The experimental conditions were essentially the same as those described in the legend to Fig. 1 except that the maintenance medium with or without drug was changed every 48 hr instead of every 24 hr. All data were obtained from cell cultures harvested at 96 hr PI. Y_x, CMV infectivity in drug-treated, CMV-infected cell cultures; Y_0, CMV infectivity in the absence of drug in CMV-infected cell cultures. Infectivities (for each datum point) were the averages of the values obtained by assay of three independent cell cultures.

trations of 6.3, 12.5, 25, 50, and 100 μM were 1.34, 2.82, 3.70, 5.08, and 5.72, respectively. Sodium nitroprusside and verapamil were much less potent, reducing CMV yields by 1.85 and 1.23 $\log_{10}$, respectively, at a concentration of 100 μM. The data for the concentration effect obtained in several experiments were transformed to a logarithmic scale and then fit with a simple linear regression model. The slope was -1.095 ± 0.078 μM^{-1} for papaverine, -0.425 ± 0.087 μM^{-1} or nitroprusside, -0.332 ± 0.095 μM^{-1} for verapamil, and -1.000 ± 0.390 μM^{-1} for DHPG (data not shown).

The inhibitory effect of papaverine on CMV replication was examined further by determining the time at which addition of papaverine was most effective in inhibiting CMV yields. As shown in Table I, papaverine was not effective when added 24 hr prior to infection and removed at the time of virus infection. Thus, it appears that papaverine treatment did not irreversibly alter the cells to a virus-resistant state. Papaverine was most effective, demonstrating about a 3.4 $\log_{10}$ reduction of CMV yields, when added before (0 hr) or during (6 hr) the rounding and contraction (early) phase of the cellular response to CMV infection which occurs from 5 to 12 hr after infection (PI) (3, 5, 6). When added at 12 or 24 hr PI, that is, when cells were about to enter or were in the relaxation phase, the effectiveness of papaverine was reduced to a 2.7 $\log_{10}$ inhibition of CMV yields. Addition of papaverine at 48 hr, after the cells had begun to enlarge, produced only a 1.42 $\log_{10}$ reduction in infectivity. Thus, CMV replication was most sensitive to inhibition when papaverine was added before initiation of the late-phase cellular responses of relaxation and enlargement. Addition of papaverine after cytomegaly had progressed resulted in a 96-fold decrease in the effectiveness of papaverine relative to addition of the drug from 0 to 6 hr PI.

Discussion. As noted above, the smooth-muscle relaxing agents (papaverine, sodium nitroprusside, verapamil) inhibited morphologic cellular responses to CMV infection (6, 9). The present finding that the smooth-muscle relaxing agents also inhibit CMV replication suggests that these cellular responses are involved in virus replication, or alternatively that CMV-induced physiologic responses susceptible to inhibition by these smooth-muscle relaxing agents are involved in both morphologic cellular responses and CMV replication. In support of these views is the observation that both CMV replication and late cellular responses (6) are most sensitive to inhibition by these smooth-muscle relaxing agents when they are added before or during the contractile phase. Furthermore, with respect to sodium nitroprusside and verapamil, there is reasonably good agreement between the concentration of drug required to produce inhibition of early cellular responses and CMV replication. Thus, at first glance it would seem reasonable to propose that there is a direct correlation between inhibition of the early cellular response and CMV replication.

On the other hand, in the case of papaverine, CMV replication appeared to be more sensitive to inhibition by this drug than did the early cellular responses. CMV yields were reduced by factors of -1 to -6 $\log_{10}$ relative to

TABLE I. EFFECT OF THE TIME OF ADDITION ON THE ANTIVIRAL ACTIVITY OF PAPAVERINE ON CMV REPLICATION

Time of addition[a] (hr PI)	Virus yield (PFU/ml)[b]	Percentage of control[c]	Log_{10} reduction[c]
No papaverine	2.1×10^7		
−24 to 0	1.7×10^7	81	0.079
−12 to 0	1.8×10^7	86	0.079
−2 to 0	2.0×10^7	95	0.021
−24 to 96	8.9×10^3	0.042	3.37
−12 to 96	8.9×10^3	0.042	3.37
−2 to 96	8.6×10^3	0.041	3.39
0 to 96	8.1×10^3	0.039	3.41
2 to 96	8.0×10^3	0.038	3.42
4 to 96	8.3×10^3	0.040	3.40
6 to 96	7.9×10^3	0.038	3.66
12 to 96	4.1×10^4	0.20	2.71
24 to 96	3.8×10^4	0.18	2.74
48 to 96	7.9×10^5	3.8	1.42

[a] Concentrated papaverine (300 μg/ml) was added to maintenance medium at the times indicated to make a final concentration of 10 μg/ml (27 μ*M*). For the control infected cell cultures, the appropriate dilution of the diluent for papaverine was made in the maintenance medium. The cells were infected with CMV at a multiplicity of infection of 4 PFU/cell. The medium was changed at 48 hr PI.

[b] Virus yields were measured at 96 hr PI by plaque assay.

[c] Relative to CMV yields in the absence of papaverine.

untreated controls at concentrations from 6.3 to 100 μ*M* papaverine. In contrast, early-phase cellular responses were only inhibited at the highest concentrations. A 14-fold higher concentration of papaverine was needed to inhibit rounding and contraction by 0.85 log_{10} than was required to inhibit CMV replication to a higher level (1.34 log_{10}).

Although visual quantification of cellular responses possibly is not as quantitatively accurate as measurements of virus yields, replication of CMV seems to be significantly more sensitive to papaverine inhibition than CMV-induced rounding and contraction. The marked difference in the potency of papaverine and the other smooth-muscle relaxing agents in causing inhibition of virus replication suggests that the former drug may have an additional effect beyond that which inhibits rounding and contraction of CMV-infected cells.

The mechanism through which papaverine and the other drugs inhibited CMV-infected cells from contracting and from producing high infectious yields is not known; however, several possibilities may be considered. First, it is tempting to speculate that the mechanism of action underlying the relaxing effect of these drugs on smooth muscle (10–13) may prevent at least the initial cell rounding. Thus, the greater potency of papaverine relative to nitroprusside may have resulted from increased levels of both cyclic AMP (cAMP) and cyclic GMP (cGMP) (12, 13) rather than from cGMP alone (11). Second, it is possible that in addition to the cyclic nucleotide phosphodiesterase effect (12, 13), papaverine may have other effects, which have not yet been identified. Finally, it is possible that a critical physiologic event(s) (e.g., the rise in intracellular free $[Ca^{2+}]$) (14) may be important to both early cellular responses and CMV replication, but at quantitatively different levels.

The potency of papaverine in inhibiting CMV yields may be of clinical interest. The concentrations tested are in range of those used in patients (15) and thus this drug may prove to have some role in the clinical management of CMV infections. Based on data of the type presented in Fig. 2, the median inhibitory dose (ED_{50}) for papaverine appears to be somewhat less than 1 μ*M* against CMV strain AD169 and the ED_{90} about 1.6 μ*M*, whereas the average for the median inhibitory doses published for DHPG (16–19) is 4.5 μ*M* against the same strain of CMV (although the ED_{90}

in our assays was about 2.0 μM). Thus, papaverine appears to be as potent as this nucleoside analog in preventing CMV replication *in vitro*. Furthermore, papaverine usage may be less likely to be hampered by toxic complications (20). Papaverine inhibited cellular DNA synthesis in mock-infected cells (72–96 hr PI) by 53% at a concentration of 10 μg/ml (27 μM). Thus, in the case of papaverine there appears to be about a 26-fold difference in the concentrations inhibiting cellular DNA synthesis (53% inhibition at 10 μg/ml) and CMV replication (50% inhibition at <0.38 μg/ml). Since the papaverine effect appears to be synergistically enhanced by cotreatment with DHPG [$\log_{10}$ yield reductions of 1.40 for papaverine (3 μM), 1.24 for DHPG (3 μM), and 3.98 for the combination (3 μM +3 μM)] toxic complications by high concentrations of the individual agents may be reduced further.

The variety of virus pathogens susceptible to papaverine is not known, but will certainly extend beyond CMV. Dr. Peter Medveczky (Department of Pharmacology, The University of Massachusetts Medical Center, Worcester, MA 01605; personal communication) has found that the infectious agent associated with AIDS, LAV (HTLV III), is susceptible to papaverine inhibition, although papaverine was not as potent an inhibitor of this agent as it is of CMV. Furthermore, Yoshikawa and Yamanouchi recently reported that measles virus was sensitive to papaverine inhibition, which they attributed to increased intracellular [cAMP] (21). Considering the diversity of the viruses sensitive to papaverine, it is possible that other viruses will be identified as susceptible to papaverine inhibition.

In any case, the antiviral effect of smooth-muscle relaxing agents, particularly in view of the potency of papaverine, may be of value in improving our understanding of the replication of CMV and other viruses and possibly contribute to controlling these problematic infections.

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Note added in proof. Dr. P. Medveczky (Department of Pharmacology, The University of Massachusetts Medical Center, Worcester, MA 01605) and Dr. J. Drach (Department of Pharmacology, The University of Michigan, Ann Arbor, MI 48109), using papaverine from one of the lots employed in this study and either dot-blot hybridization techniques to measure yields of CMV DNA or the plaque assay to measure infectious yields, found the ED_{50} for papaverine to be 1.5 or 1.0 μM, respectively.

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