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Potentiating effect of ribavirin on the anti-herpes activity of acyclovir

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Summary

The combined antiviral effects of acyclovir (ACV) and ribavirin (Rbv) on herpes simplex virus type 1 (HSV-1) and pseudorabies virus (PRV) in cell cultures and on experimental HSV-1 keratitis in rabbits were studied. The antiviral activity in vitro was based on cytopathogenicity inhibition and yield reduction. The combination of the two drugs exhibited synergy as evaluated graphically (isobolograms). Rbv also potentiated the antiviral effect of ACV in vivo, in the experimental HSV-1 keratitis model in rabbits. This was evident from both the severity of corneal lesions and virus shedding in the tear film. The potentiating effect of Rbv on the anti-HSV-1 activity of ACV in vitro was reversed by guanosine.

Acyclovir; Ribavirin; Combination chemotherapy; HSV-1; PRV; Keratitis

Introduction

Acyclovir (ACV) is a potent and selective anti-herpes agent, that is widely applied in clinical practice. A potential problem in viral chemotherapy with ACV is the emergence of drug-resistant strains (Crumpacker et al., 1982; Smith et al., 1980). This justifies the search for effective combinations of ACV with other virus inhibitors. ACV is selectively phosphorylated by herpes simplex virus (HSV)-encoded thymidine kinase (Elion et al., 1977; Fyfe et al., 1978). Cellular enzymes (Miller and Miller, 1980, 1982; Öberg, 1983) then catalyze the conversion of ACV-MP its triphosphate form (ACV-TP). ACV-TP inhibits viral DNA synthesis

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(Furman et al., 1979), which probably results from inactivation of the HSV-1-encoded DNA polymerase (Furman et al., 1984).

Since dGTP can competitively prevent the initial binding of ACV-TP to the susceptible enzyme (Furman et al., 1984; St. Clair et al., 1984), and since the level of dGTP increases following treatment of HSV-1-infected cells with ACV (Furman et al., 1982), dGTP may have the potential to protect the HSV-1 DNA polymerase from inactivation. Furthermore, an agent that reduces the rate of synthesis of dGTP would decrease the intracellular dGTP pool. Such an agent could facilitate inactivation of the HSV DNA polymerase by ACV-TP. Ribavirin (Rbv), as its 5′-monophosphate (Rbv-MP) inhibits IMP-dehydrogenase (Miller et al., 1977; Streeter et al., 1973) and causes depletion of the intracellular guanine nucleotides pool (Lowe et al., 1977; Miller et al., 1977). Based on this concept, one might expect an enhancement of the anti-herpesvirus activity of ACV, if the levels of the natural competing substrate (dGTP) are reduced by Rbv. Recent investigations carried out by De Clercq and his group (Baba et al., 1987; Balzarini et al., 1989; Balzarini et al., 1990) have demonstrated that Rbv potentiates the anti-retrovirus activity of several purine 2′,3′-dideoxynucleosides. In the present study, we have investigated the anti-herpesvirus activity of ACV combined with Rbv in vitro and in vivo.

Materials and Methods

Antiviral compounds

Acyclovir was a generous gift from Dr. G. Elion (Burroughs Wellcome Co., Research Triangle Park, N.C.); ribavirin (1-β-D-ribofuranosil-1,2,4-triazole-3-carboxamide) was provided by Dr. M. Lidak (Institute of Organic Synthesis, Latvian Acad. Sci., Riga).

Viruses, cells and media

HSV-1 strain DA was obtained from Dr. S. Dundarov, Institute of Infections and Parasitic Diseases, Med. Acad., Sofia. The virus was cultivated in diploid cultures of human embryonic skin-muscle fibroblasts (HESMF). The cells were grown and maintained in minimal Eagle's medium containing 10% calf serum. Pseudorabies virus (PRV), strain Bucharest, was grown in primary chick embryo fibroblasts (CEF). The cell culture medium consisted of medium 199, 10% tryptose phosphate broth supplemented with 10% calf serum (growth medium) or 5% calf serum (maintenance medium). Secondary CEF cultures were grown as microcultures in 96-well microplates. The cell culture medium was medium 199, 10% triphosphate broth supplemented with 5% calf serum.

Cytopathic effect (CPE)-inhibition assay

Activity of ACV and Rbv alone, and in combination, against HSV-1 and PRV were determined by measurement of viral CPE reduction in 96-well microplates

(Libro, Flow Laboratories). Confluent cell monolayers were incubated with 100 $CCID_{50}$ of virus for 1 h at 37°C and immediately thereafter exposed to twofold dilutions of each compound. Three wells per dilution were tested. After complete cell destruction in virus controls, which occurred generally at 2–3 days after viral inoculation, CPE was scored on a 0–4 basis with 4 representing total cell destruction. These data were used to draw dose-response curves for each compound. From these graphs the concentration inhibiting CPE by 50% (ED_{50}) was determined. In combination chemotherapy experiments, the concentrations selected were below the ED_{50} for each inhibitor. Dose-response curves were drawn for ACV in combination with varying concentrations of Rbv and for Rbv in combination with varying concentrations of ACV. The type of interaction of both inhibitors was determined by isobologram plotting after determining fractional inhibitory concentrations (FIC), as described by Biron and Elion (1982).

In tests which were run in parallel with the antiviral assays in confluent cell monolayers (which had not been infected) the same concentrations of the compounds and their combinations were examined microscopically for their effect on normal cell morphology. No cytotoxicity was observed.

Yield reduction assay

Investigations were carried out on confluent cell monolayers in tubes. ACV and Rbv alone and in combination were added after 1 h virus adsorption (virus inoculation dose 100 $CCID_{50}$/0.1 ml). The cells were then incubated for 48 h at 37°C. After freezing and thawing, the virus titers of the samples (pools from 4 test tubes) were determined by titration in microplates, and are expressed as $CCID_{50}$/0.1 ml. Cytotoxicity was monitored by trypan blue dye exclusion.

To evaluate combination effectiveness, a mathematical technique was used (Smith et al., 1982). Therefore, the n-fold titer reduction was calculated by dividing the virus titer of the 'no-drug' control by the virus titer in the presence of drug or drug combination. By definition, titer reduction of the 'no-drug' control is 1. The quotient of the n-fold virus titer reduction by the drug combination and the product of the virus titer reductions by the individual drugs, $Q = n_{(1+2)}/n_1 \times n_2$, indicates the type of interaction between the drugs. If $Q > 1$, $= 1$ or < 1; the interaction is synergistic, additive or antagonistic, respectively.

Reversal studies by guanosine

Guanosine (Guo) was obtained from Serva (Heidelberg, Germany). The studies were performed by the yield reduction assay in HESMF cells infected with the HSV-1 strain DA. Treatment with ACV and/or Rbv was done in parallel without and with Guo at a molar ratio to Rbv of 10:1. Controls containing virus and medium only and virus, medium and Guo were run simultaneously. The results are means of three independent experiments.

Ocular HSV-1 infection in rabbits

Scarified eyes of Chinchil rabbits (2.1–2.8 kg) were bilaterally inoculated with $10^{4.4}$ $CCID_{50}$ HSV-1 (DA strain) per eye by dropping 40 μl of the virus suspension into the lower cul-de-sac and gently rubbing the eye lid over the cornea for 30 sec.

Drug treatment

The rabbits were divided into 4 groups (3 rabbits/group). Group 1 received 50 μl 0.02% ACV in 1.4 polyvinylalcohol (PVA), applied five times a day at 2-h intervals during 7 consecutive days, beginning 72 h after virus inoculation (daily dose: 50 μg); group 2 were treated with 1% Rbv in 1.4% PVA (50 μl) hourly, twelve times daily (daily dose: 6 mg). Treatment began 4 h after virus inoculation and continued for 10 days in accordance with the schedule of Sidwell et al. (1973); group 3 received combined therapy with ACV and Rbv applied in the same manner as for each drug alone; group 4 were treated with placebo (1.4% PVA) in the same way. Rabbits whose eyes were scarified, but not exposed to virus, were also treated with ACV and/or Rbv as toxicity control. No toxicity was noted.

Measurement of virus in tear film

Virus shedding was monitored by culture of the preocular tear film of all groups. The samples were taken before the first daily treatment with cotton-tipped swabs, wetted in phosphate buffer, by passing over the upper and lower conjunctival cul-de-sac, lightly rolled over the corneal epithelium and retained in the nasal fornix for 5 to 10 sec for maximum tear film absorption (Trousdale et al., 1980). Swabs were eluted in vials with 1 ml cell culture medium at 4°C for 1 h and then virus titrations were done in microplates.

Determination of the severity of the corneal lesions

On day 3 post inoculation, all eyes were stained with 2% fluorescein solution and subsequently washed out with physiologic solution and then the corneal lesions were examined. The severity of corneal lesions was scored as described by Maudgal et al. (1980) from grade 0 to 5. The significance of the results was assessed by Student's *t*-test. P values > 0.05 were considered significant.

Results

Antiviral effectiveness of ACV-Rbv combination in vitro

The activity of the combination ACV and Rbv was evaluated against HSV-1 in HESMF cells and against PRV in CEF cells using a CPE inhibition method. The

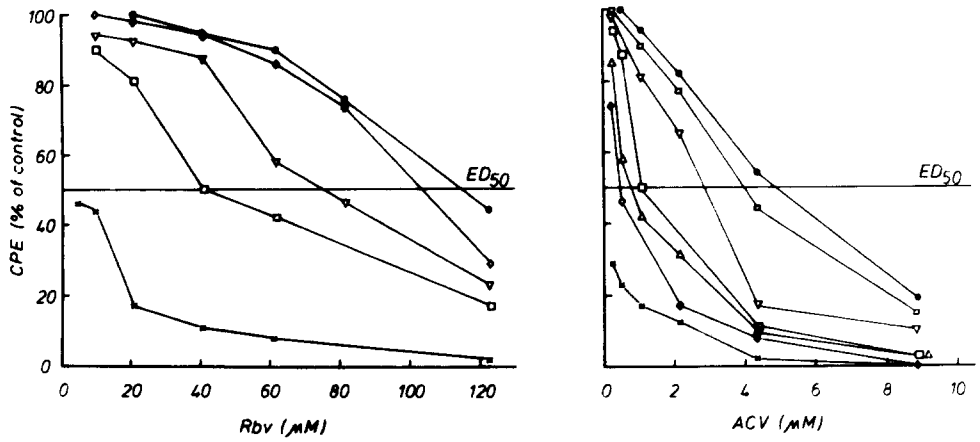

Fig. 1. Dose-response curves for the inhibitory effects of ACV and Rbv on the cytopathogenicity of HSV-1 (strain DA) in HESMF cells. (Left) ACV concentrations, in combination with Rbv: zero (●—●); 4.4 μM (×—×); 1.1 μM (❑—❑); 0.55 μM (▽—▽); 0.27 μM (◇—◇). (Right) Rbv concentrations, in combination with ACV: zero (●—●); 123 μM (×—×); 82 μM (◇—◇); 61 μM (Δ—Δ); 41 μM (❑—❑); 20 μM (▽—▽); 10 μM (▱—▱).

dose-response curves obtained are presented in Fig. 1 and Fig. 2. Combination of the two drugs resulted in a greater degree of inhibition of CPE than did either drug alone, without producing any toxicity to the confluent host cells. Analysis of these data by dose isobolograms showed synergistic activity against both HSV-1 and PRV (Fig. 3). The FIC indexes were less than 1, thus indicating a synergistic interaction.

Similar results were obtained using the yield reduction method. The results

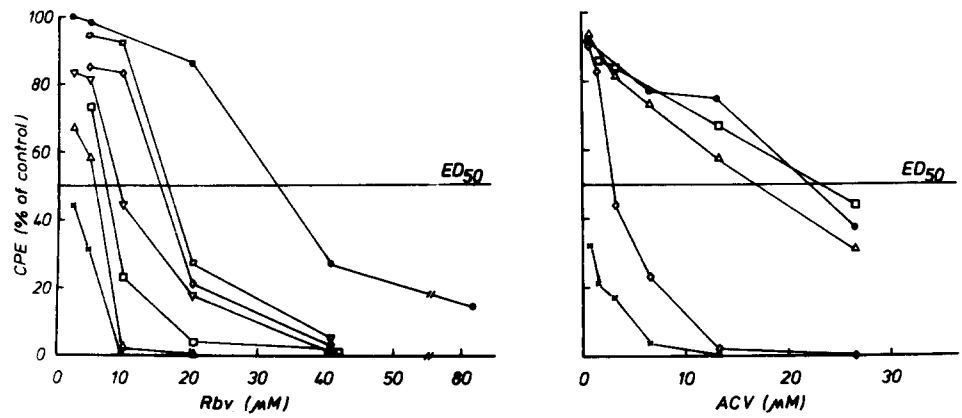

Fig. 2. Dose-response curves for the inhibitory effects of ACV and Rbv on the cytopathogenicity of PRV (strain Bucharest) in secondary CEF cells. (Left) ACV concentrations, in combination with Rbv: zero (●—●); 26.7 μM (×—×); 13.3 μM (Δ—Δ); 6.7 μM (❑—❑); 3.3 μM (▽—▽); 1.7 μM (◇—◇); 0.8 μM (▱—▱). (Right) Rbv concentrations, in combination with ACV: zero (●—●); 20 μM (×—×); 10 μM (◇—◇); 5 μM (Δ—Δ); 2.6 μM (❑—❑).

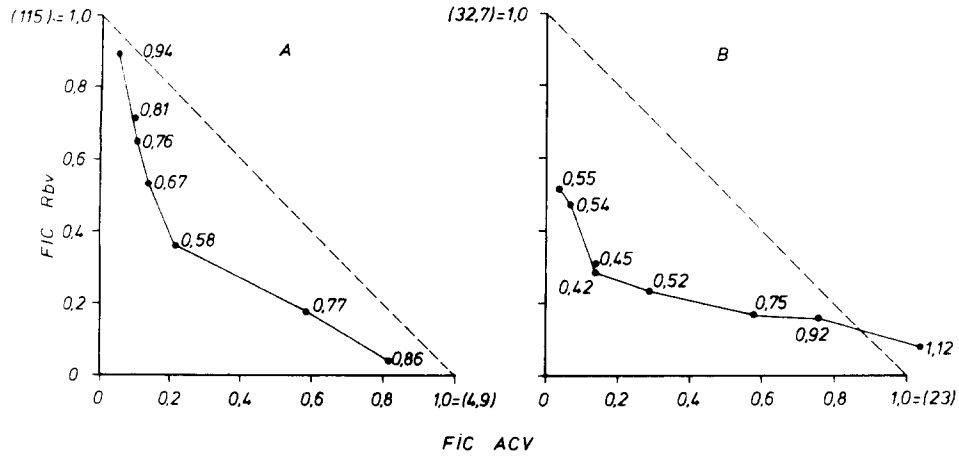

Fig. 3. Synergistic activity of ACV combined with Rbv against HSV-1 (strain DA) (A) and PRV (strain Bucharest) (B). The numbers refer to the FIC indexes. The numbers within parentheses next to the FIC of 1.0 refer to the ED_{50} (μg per ml).

obtained for different concentrations of ACV and of Rbv used in combination are shown in Table 1. The degree of virus yield reduction by the combinations was considerably higher than the virus yield reduction by the individual compounds. Reduction in virus titer of HSV-1 by 2.2 μM ACV or 123 μM Rbv was found 316- and 14.8-fold, respectively. When the drugs were combined, complete virus reduction (> 10^6-fold) was achieved, thus indicating marked synergism. Also, marked synergism was obtained with ACV and Rbv against PRV. In all cases, the Q values were substantially higher than 1 (Table 1).

Reversal of combined inhibitory effects of ACV and Rbv by guanosine

The experiments were done with HSV-1 infected HESMF cells. Guo was added together with the drugs. Inhibition of HSV-1 replication by ACV and Rbv was reversed upon addition of Guo (Fig. 4). In the presence of 810 μM Guo (10-fold higher concentration than that of Rbv), virus yield at 48 h post-infection was 2.1×10^6 $CCID_{50}$/0.1 ml, despite the presence of ACV (2.2 μM) and Rbv (82 μM), which in the absence of Guo reduce virus yield by 6.3 and 2.3 log, respectively.

Efficacy of combined ACV and Rbv treatment on experimental HSV-1 keratitis in rabbits

To induce keratitis in rabbits, the HSV-1 strain DA was selected. The severity of corneal lesions and the titer of virus shed in tear film cultures during ACV, Rbv and combination therapy are represented in Fig. 5. Topical application of Rbv resulted in slight reduction of corneal lesions as compared to placebo-treated rabbits. A 1-degree decrease in lesion severity was observed between days 7 and 9. Improvement after ACV treatment was clear, but on day 10 post infection per-

TABLE 1

Effects of ACV and Rbv used alone and in combination on virus yield reduction of HSV-1 in HESMF cells and of PRV in CEF cells

Compounds	Concentration (μM)	Virus	Virus yield ($CCID_{50}/0.1 \times 10^4$)	*n*-fold virus titer reduction	Q^a
Control		HSV-1	316		
ACV	2.2		1	316	
Rbv	123		21.4	14.8	
ACV+Rbv			0	3.16×10^6	676
ACV	1.1		10	31.6	
Rbv	82		31.6	10	
ACV+Rbv			0.17	1859	5.9
Control		PRV	316		
ACV	6.7		3.16	100	
Rbv	20.5		17	18.6	
ACV+Rbv			0	3.16×10^6	1699
Control			468		
ACV	4.4		46.8	10	
Rbv	16.3		21	22.3	
ACV+Rbv			0.003	1.56×10^5	699
ACV	4.4		46.8	10	
Rbv	4.1		100	4.68	
ACV+Rbv			0.21	2228	47.6
ACV	2.2		316	1.5	
Rbv	4.1		100	4.68	
ACV+Rbv			3.16	148	21

[a] Q = the quotient of the *n*-fold virus titer reduction of the drug combination and the product of the virus titer reductions by the individual drugs.

sistence of single punctate lesions in some of the eyes was observed. Following combination therapy with ACV and Rbv separate punctate lesions were noted only between days 4 and 7 and they disappeared on the 8th day after infection. Mean reductions in the keratitis scores were as follows: Rbv, 29.7%; ACV, 68.6%; ACV + Rbv, 80.9% (Table 2).

For all groups the degree of corneal lesions correlated with the amount of virus in the tear film. From day 4 until the end of the experiment no significant difference was observed between the Rbv- and placebo-treated rabbits with respect to tear film virus titer. In the ACV-treated rabbits, the virus titer was reduced by 1–1.5 $\log_{10}$-fold between the days 5 and 8. In the tear film of the rabbits treated with ACV+Rbv the reduction in virus titer was 2–2.5 $\log_{10}$. After the 8th day of combination chemotherapy virus shedding no longer occurred. In the control group, as well as the ACV treatment group and Rbv treatment group, virus continued to be shed.

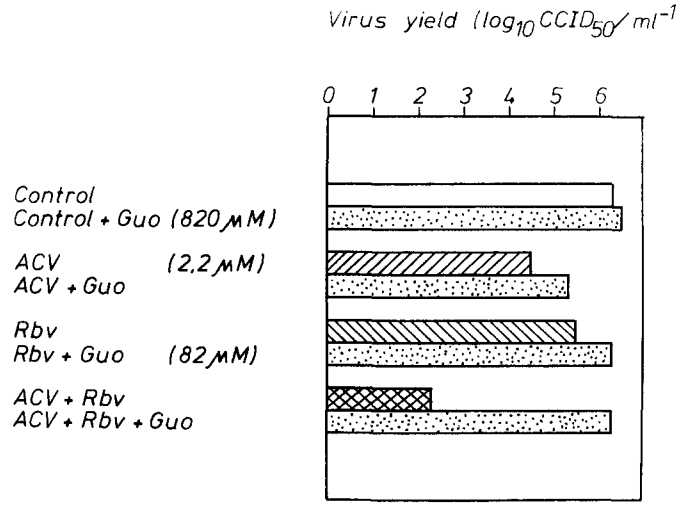

Fig. 4. Reversal of the combined inhibitory effects of ACV and Rbv on HSV-1 (strain DA) by guanosine. The results represent means of three independent experiments.

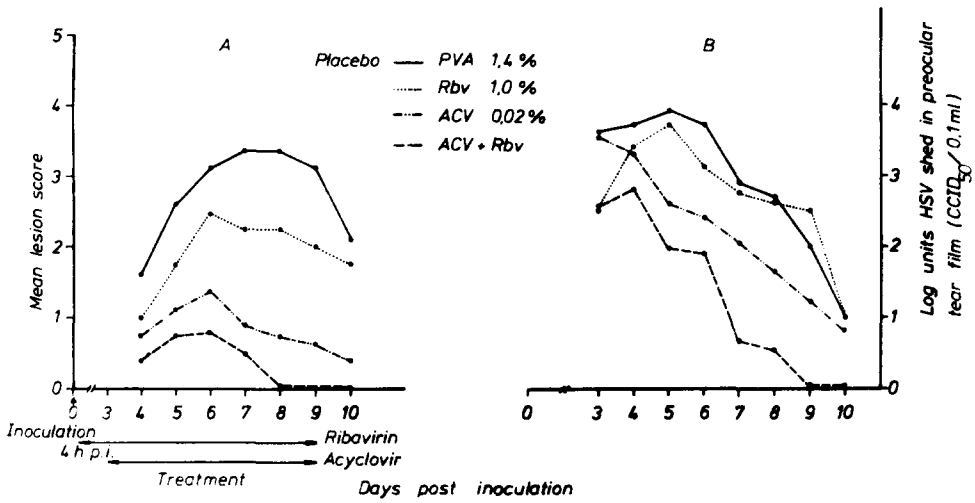

Fig. 5. Corneal epithelium lesion severity and tear film virus titers in HSV-1 (strain DA)-infected rabbits during ACV, Rbv and ACV+Rbv combination therapy. Eye drops were administered as described in Materials and Methods.

Discussion

ACV is an inhibitor of the DNA polymerase of herpes viruses and suppresses virus DNA replication (Furman et al., 1979, 1984). As a consequence of the inhibition of viral DNA polymerase by ACV-TP, dGTP pool levels increase in HSV-infected cells (Furman et al., 1982). Ribavirin 5′-monophosphate is a potent inhibitor of IMP dehydrogenase (Miller et al., 1977; Streeter et al., 1973). This leads to a

TABLE 2

Effects of ACV, Rbv and ACV+Rbv combination on experimental HSV-1 keratitis in rabbits

		Days post inoculation							Average
		4	5	6	7	8	9	10	
ACV	D.M.D.[a]	0.85	1.5	1.73	2.47	2.62	2.48	1.73	1.91
	Reduction[b]%	53.1	57.7	55.8	73.3	77.7	80	82.4	68.6
Rbv	D.M.D.	0.6	0.85	0.6	1.12	1.12	1.1	0.35	0.82
	Reduction %	37.5	32.7	19.3	33.2	33.2	35.5	16.7	29.7
ACV+Rbv	D.M.D.	1.2	1.85	2.27	2.87	3.37	–	–	2.3
	Reduction %	75	71.1	73.2	85.2	100	–	–	80.9

[a]Daily mean differences in keratitis score between control (untreated) and treated eyes.
[b]Daily reduction of keratitis score expressed in percent.

marked reduction in the pool size of dGTP (Lowe et al., 1977; Müler et al., 1977). Since dGTP competes with ACV-TP for the initial binding to DNA polymerase, a reduction in dGTP level should result in an increased antiviral effectiveness of ACV. A similar explanation was proposed by Baba et al. (1987) to account for the enhancement of the anti-HIV activity of 2′,3′-dideoxyguanosine by ribavirin. These authors postulated that the decrease in the intracellular dGTP pool would make its competitor ddGTP more effective in interacting with the HIV target enzyme, reverse transcriptase.

In the present study we have established that the combination of ACV and Rbv shows increased activity against HSV-1 and PRV replication in cell culture. A potentiated antiviral effect was also observed in vivo, in the experimental HSV-1 keratitis model in rabbits. The enhanced antiviral activity of the combination ACV-Rbv was evident from the decreased severity and duration of the ocular infection as well as the reduction in virus shedding in the tear film. The antiviral activity of the ACV-Rbv combination was reversed by guanosine. As guanosine is converted within the cells to dGTP, our observations suggest that probably the reduction in dGTP levels accounts for the potentiating effect of Rbv on the antiviral activity of ACV.

The synergistic action noted with the combination of ACV and Rbv against HSV, together with the fact that HSV may not develop resistance to Rbv (Allen and Fingel, 1977; Huffman et al., 1977), point to the clinical potential of this combination in the therapy of herpesvirus infections.

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