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Suppression of murine retroviral disease by 2′,3′-didehydro-2′,3′-dideoxythymidine (D4T)

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Summary

The thymidine analog, 2′,3′-didehydro-2′,3′-dideoxythymidine (D4T), and 3′-azido-3′-deoxythymidine (AZT) were evaluated for activity against Friend virus complex (FV) in *Mus dunni* cells using a focal immunoenzyme assay. The 50% effective doses were, respectively, 1.2 and 0.1 μM for the two compounds; the 50% cytotoxic doses using trypan blue dye exclusion were 25.4 and >100 μM. Four FV inhibition experiments with D4T were run in F_1 hybrid mice containing the Rfv-$3^{r/s}$ genotype. This mouse strain allows the study of treatment effects on development of specific neutralizing antibodies and on splenomegaly, splenic and plasma virus titers, and splenic viral RNA. In the first experiment, D4T was given by oral gavage (p.o.) three times daily (t.i.d.) for 14 days beginning 4 h post-virus inoculation. All dosages used (187.5, 375, 750 mg/kg/day) significantly inhibited all viral parameters. Other experiments used D4T p.o. twice daily, with dosages of 46.9, 93.8, 187.5 and 375 mg/kg/day or four times daily with a dose of 375 mg/kg/day. No significant disease inhibition was seen using the twice daily treatment schedule, but efficacy was apparent using the four times daily treatment. The final experiment repeated the initial study, extending the t.i.d. treatments to 25 days and using dosages of 46.9, 93.8, 187.5 and 375 mg/kg/day. All but the lowest dose reduced each virus parameter. None of the D4T treatment regimens caused death in toxicity controls, although moderate host weight loss or less weight gain was seen, and variable hematocrit decreases occurred, particularly in mice receiving the highest drug dosage. Inhibition of natural killer (NK) cell activity also was seen in these same animals, but in infected mice, FV-induced decrease in NK cell

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activity was prevented by D4T treatment. Virus-specific neutralizing antibodies developed in all infected, treated animals. These data indicate D4T has potential as a possible candidate for anti-human immunodeficiency virus evaluations in the clinic.

Murine retroviral disease; 2′,3′-Didehydro-2′,3′-dideoxythymidine; Friend virus complex

Introduction

The thymidine nucleoside analog 2′,3′-didehydro-2′,3′-dideoxythymidine (D4T) has been reported to have potent in vitro inhibitory effects on human immunodeficiency virus (HIV) (Herdewijn et al., 1987; Lin et al., 1987; Mansuri et al., 1989) and on Moloney murine leukemia virus (Mansuri et al., 1990). The inhibition of these viruses by D4T was approximately equal to that of 3′-azido-3′-deoxythymidine (AZT), while the in vitro cytotoxicity using human hematopoietic progenitor cells was markedly less than AZT (Mansuri et al., 1989, 1990). These data suggest this compound potentially has a greater therapeutic index than AZT. In addition, the 5′-monophosphate metabolite of D4T does not accumulate as does that of AZT (August et al., 1988; Balzarini et al., 1989; Ho and Hitchcock, 1989; Perno et al., 1989), and the compound has exhibited significantly less murine toxicity than AZT (Mansuri et al., 1990).

These biochemical and toxicological differences between D4T and AZT, combined with D4T's potent in vitro retrovirus-inhibitory effects, have prompted the present study investigating the ability of this compound to inhibit the in vitro and in vivo murine retrovirus infections induced by the Friend virus complex (FV). The in vivo experiments utilized an F_1 hybrid mouse strain containing the Rfv-$3^{r/s}$ genotype that, unlike other commonly used mouse strains, has the ability to produce FV-specific antibodies which, as they increase, cause a reduction of viremia and numbers of infected cells (Chesebro and Wehrly, 1979; Chesebro et al., 1983; Morrison et al., 1986). Despite the presence of specific antibody, the animals develop splenomegaly which progresses to a terminal state. This animal infection model, which thus has several disease manifestations analogous to those seen in patients with acquired immunodeficiency syndrome (AIDS), has been utilized previously to demonstrate the FV disease-inhibitory effects of AZT (Morrey et al., 1990, 1991a). The present experiments also examined the influence of varied treatment schedules on the compound's FV disease-inhibitory effects.

Materials and Methods

Compound

The D4T used was provided by Dr. M. Mansuri, Bristol-Myers Squibb Company (Wallingford, CT). The drug was dissolved in sterile water. AZT, used for in vitro studies, was provided by Dr. Sandra Lehrman, Burroughs Wellcome Co. (Research Triangle Park, NC). Both compounds, when used in vitro, were dissolved in minimum essential medium (MEM) with 5% fetal bovine serum (FBS) with 0.1% $NaHCO_3$.

Virus

The Lilly-Steeves B-tropic strain of FV complex (Morrison et al., 1986) was obtained from Dr. Bruce Chesebro (NIH, NIAID, Rocky Mountain Laboratories, Hamilton, MT); the virus consisted of Friend murine leukemia virus (F-MuLV) and defective spleen focus-forming virus (SFFV). One FV stock, prepared as previously described (Morrey et al., 1990), was used in all studies.

Cells

Mus dunni cells (Chattopadhyay et al., 1981) were kindly provided by Dr. S.K. Chattopadhyay, Division of Cancer Biology and Diagnosis, National Cancer Institute, (Bethesda, MD). The cells were grown in MEM with 5% FBS.

Animals

Female B10.A and male A/WySn mice (Jackson Laboratories, Bar Harbor, ME) were mated to produce the (B10.A x A/WySn)F_1 mice. These F_1 hybrid mice were supplied as young adults weighing 18–23 g by Simonsen Laboratories (Gilroy, CA). Genetic confirmation of the mice was done by Dr. H.A. Hoffman of Animal Genetic Systems Co. (Rockville, MD).

In vitro antiviral procedure

Cells in 24-well flat-bottomed tissue culture plates were infected with 1500 focus-forming units (FFU) per well of FV suspended in MEM with 5% FBS and 8 μg/ml polybrene. The cells were washed 3 h later and then fed medium containing varying one-half $\log_{10}$ dilutions of AZT or D4T. The medium was changed with new medium containing drug on day 4. Each drug dose was tested in triplicate. The virus infectivity in the cells was evaluated using the focal immunoenzyme assay (FIEA) described previously (Sitbon et al., 1985; Chesebro and Wehrly, 1988; Morrey et al., 1991) with modifications as

described under in vivo viral parameters, below. Toxicity controls consisted of uninfected cells similarly treated with each drug concentration, run in triplicate. Viable cells in these controls were counted by trypan blue dye exclusion. A 50% cytotoxic dose (CD_{50}) and a 50% effective dose (ED_{50}) were determined using line of best fit on semi-log graph paper for each drug, and a resultant selectivity index (SI) determined as the CD_{50} divided by the ED_{50}.

In vivo viral parameters

Spleen weight, viral infectious centers (IC) and recoverable viral RNA in the spleen, and viremia were used as indicators of viral infection. The FIEA, employing monoclonal antibody (MAb) 48, was used to assay infected splenocytes and to quantitate FV in plasma. The background for the use of this MAb has been described previously (Morrey et al., 1991a). Serial dilutions of spleen cells or heparinized plasma were added to an 18-h monolayer of *M. dunni* cells seeded on 24-well tissue culture plates. After cultivation for a 5-day period, confluent monolayers were incubated with MAb48 for 30 min at 4°C, rinsed, and fixed with methanol for 5 min. After rinsing twice to remove methanol, the wells were incubated for 45 min at room temperature with peroxidase-conjugated goat antimouse immunoglobulin (Cappel, West Chester, PA). The substrate 3-amino-9-ethylcarbazole (Sigma, St. Louis, MO) in dimethylformamide was then added after rinsing with TNE (0.01 M Tris, pH 7.6, 0.5 M NaCl, 0.002 M EDTA) and incubated in the dark for 20 min at room temperature. Plates were rinsed with distilled water and foci counted and expressed as either $IC/10^6$ splenocytes or FFU/ml. Viral RNA in the spleen was determined by Northern dot blot analysis as previously described (Morrey et al., 1991b). Briefly, a standard concentration of spleen cells were lysed in a solution of vanadyl nucleoside as an RNase inhibitor and Nonidet P-40 at sufficiently low concentration to leave the nuclei intact. The nuclei were removed by ultracentrifugation, subjected to phenol/chloroform extraction, ethanol precipitation and suspension of the RNA, which was then denatured, blotted onto nitrocellulose (NC) paper and hybridized with DNA probe specific for F-MuLV long terminal repeat. The probe was labeled with [^{32}P]dCTP by random primer method. After exposure of X-ray film to the NC paper, the intensity of the dots containing the samples were compared to serially diluted F-MuLV single stranded RNA standards.

Antibody determination

The FV-specific neutralization test described by Earl et al. (1986) and modified as previously described (Morrey et al., 1990) was used. Inhibition of viral foci in *M. dunni* cells was used as the endpoint.

Natural killer (NK) cell activity

Splenic cells were assayed for their ability to lyse YAC-1 tumor cells in conventional chromium release assay (Warren et al., 1985) as an indicator of NK cell function. Ratios of effector:target cells of 100:1 and 50:1 were used. Cytotoxicity was expressed as: % chromium release = [(experimental counts per minute [cpm] – background cpm)/(maximum cpm – background cpm)] × 100.

In vivo experiment design

Four separate experiments were run to evaluate the in vivo FV-inhibitory efficacy of D4T. In all experiments, mice were inoculated intraperitoneally (i.p.) with 3.8×10^4 mouse 50% infectious doses (ID_{50}) of FV. In the first experiment, treatment with D4T at dosages of 750, 375, or 188.5 mg/kg/day began 4 h later, continuing three times daily (t.i.d.) for 14 days. The second and third experiments used dosages of 375, 187.5, 93.8, and 46.9 mg/kg/day, administered twice a day (b.i.d.) or four times a day (q.i.d.). These treatments were designed to be divided approximately equally over a 24-h period. The drug was administered by oral gavage (p.o.). Ten infected mice were treated with each dosage of D4T, and 20 infected mice received placebo as control. Three sham-infected mice were treated in parallel with each drug dosage to serve as toxicity controls. Three additional mice which were uninfected and untreated were held as normal controls. The toxicity and normal controls were weighed immediately prior to the initial treatment and then again 18 h after the final treatment. All virus-infected animals were killed 8 h after final treatment and their spleens and blood removed. The spleens were weighed and then processed for FV IC. The plasma was also assayed for virus titer. Any antiviral effects of heparin were eliminated by dilution in the viral assay.

A fourth experiment was run repeating the t.i.d. treatment schedule, but extending it to 25 days after virus inoculation. In this experiment, D4T doses were 375, 187.5, 93.8, and 46.9 mg/kg/day. The mice were killed 24 h after termination of treatment and spleens were weighed and assayed for viral IC and RNA. The blood was divided, with one-half assayed for plasma virus titer and anti-FV antibody; hematocrit values were determined with the other half. Toxicity control mice were also killed at this time, their spleens weighed and hematocrits determined. Splenocytes from both infected and toxicity control mice were also assayed for NK cell activity.

Nested analysis of variance (Sokal and Rohlf, 1981) was used for comparison of mean virus titers, spleen weights and immunologic data. Analysis was accomplished comparing the data obtained from the infected, treated animals to the infected, H_2O-treated controls run in the same experiment.

Results

In vitro anti-FV effects

The in vitro antiviral results are summarized in Table 1. Both compounds were inhibitory to FV as determined by reduced number of viral foci being detected; however, AZT was approximately 10-fold more potent in its antiviral effect. A count of viable cells treated with each drug dosage indicated D4T to be at least 4-fold more toxic than AZT in the *M. dunni* cells.

In vivo anti-FV effects

Treatment of the murine FV infection with D4T was significantly inhibitory to the infection when therapy was given t.i.d. or q.i.d. (Table 2). Using the latter treatment schedules, splenomegaly was inhibited and both splenic IC and viremia were reduced. The drug was reasonably well tolerated, although a moderate weight loss was often seen. No other adverse effects were noted in the toxicity control animals; spleens taken from these controls displayed no significant differences in size compared to the 73 mg weight of the normal control spleens. These initial positive findings using the t.i.d. treatment schedule prompted the experiment with the other two regimens. The dosages were reduced in the latter studies in view of the understanding that a dosage of 1000 mg/kg/day is lethally toxic to some mice on extended dosing (Mansuri et al., 1991) and in an effort to conserve the limited supply of the drug available to us. In a separately run q.i.d. study using 7- to 9-week-old mice, the anti-FV efficacy of D4T was insignificant (data not shown); a third experiment using the younger mice, as employed in the rest of this study, again yielded positive results similar to those seen in Table 2.

The effects of extended (25 day) D4T treatment on FV disease are summarized in Table 3. In this experiment, the drug was again effective, inhibiting splenomegaly, spleen and plasma virus titers, splenic viral RNA, and reducing the FV disease-associated hematocrit elevation usually resulting from increased circulating erythroblasts. Antibody response to the virus infection was not affected by D4T therapy. The toxicity controls all gained weight slightly (0.2–0.6 g compared to 1.9 g in normal controls). Hematocrit values in these toxicity control animals were significantly ($P < 0.05$) reduced from a mean of 44 in normal mice to a mean of 37.4 in mice treated with the high, 375 mg/kg/day, D4T dosage. The lower dosages did not cause significant changes in

TABLE 1

Inhibition of FV and cytotoxicity of AZT and D4T in *M. dunni* cells

Compound	50% Effective dose (μM)	50% Cytotoxic dose (μM)	Selectivity index
AZT	0.1	>100	>1000
D4T	1.2	25.4	21.2

TABLE 2

Effect of varying treatment schedules on the in vivo FV disease-inhibitory activity of D4T

Treatment schedule[b]	Treatment	Dosage (mg/kg/day)	Toxicity Controls		Infected, Treated[a]			
			Surv/total	Host wt. change[c] (g)	Surv/total	Mean spleen wt (mg ± S.E.)	Splenic FV IC (mean log/10^6 splenocytes ± S.E.)	Plasma FV (mean $\log_{10}$ FFU/ml ± S.E.)
b.i.d.	D4T	375	3/3	0.2	10/10	539±150	2.6±0.4	2.4±0.5
		187.5	3/3	0.0	10/10	485±127	2.7±0.4	1.7±0.2
		93.8	3/3	0.6	10/10	443±102	2.7±0.5	1.7±0.3
		46.9	3/3	0.2	10/10	445±106	3.2±0.4	1.9±0.3
	H_2O	0	–	–	20/20	476±102	3.1±0.4	2.1±0.3
t.i.d.	D4T	750	3/3	−0.5	10/10	294±1.4*	1.6±0.4*	1.4±0.2**
		375	3/3	−0.1	9/10	177±52**	0.6±0.4**	1.1±0.0**
		187.5	3/3	−0.4	10/10	280±61**	2.1±0.5	2.0±0.4*
	H_2O	0	–	–	20/20	695±157	2.9±0.4	2.8±0.5
q.i.d.	D4T	375	3/3	−0.3	10/10	356±115**	3.1±0.3*	2.9±0.3*
	H_2O	0	–	–	19/19	833±101	4.3±0.6	4.7±0.6

[a]Data determined 14 days after virus inoculation.
[b]All therapies p.o. beginning 4 h post-virus inoculation.
[c]Difference between initial weight prior to treatment and weight 18 h following final treatment. Normals gained 0.7 g (t.i.d. experiment) and 1.7 g (b.i.d., q.i.d. experiments).

TABLE 3

Effect of extended p.o. D4T treatment[a] on in vivo FV disease

Treatment	Dosage (mg/kg/day)	Infected, Treated[a]						
		Surv/total	Mean spleen wt (mg ± S.E.)	Mean hematocrit (±S.E.)	Splenic FV IC (mean $\log_{10}/10^6$ splenocytes ± S.E.)	Plasma FV (mean $\log_{10}$ FFU/ml±S.E.)	Mean FV RNA (ng $[10^{-4}]$ /splenocytes ± S.E.)	Mean anti-FV antibody ($\log_2$±S.E.)
D4T	375	10/10	287±184**	47.3±8.2	1.5±0.4**	1.3±0.2*	9.9±8.1	1.4±0.7
	187.5	10/10	601±193*	51.3±3.2	1.7±0.2**	<1.1**	5.5±3.1	1.6±0.6
	93.8	10/10	387±132**	45.4±2.1*	2.0±0.3*	<1.1**	2.1±0.3	1.7±0.6
	46.9	10/10	983±255	58.0±4.3	2.3±0.3	1.6±0.2	10.1±3.9	1.1±0.4
H_2O	0	20/20	1024±302	54.7±3.5	2.8±0.3	1.9±0.4	21.5±5.6	1.5±0.7

[a]t.i.d. × 25 days beginning 4 h post-virus inoculation.
*$P<0.05$; **$P<0.01$.

hematocrits. All D4T dosages reduced the NK cell activity in the toxicity control animals (Table 4). This reduction was seen using both effector:target cell ratios. Moderate but insignificant reductions in NK cell activity were seen at the lower D4T dosages. The placebo-treated, FV-infected mice exhibited a decline in NK cell activity at both effector:target ratios. The D4T treatment of the infected mice resulted in the prevention of depression of NK cell activity at

TABLE 4

Effect of extended p.o. D4T treatment[a] on natural killer cell activity in mice

Treatment	Dosage (mg/kg/day)	% Cr-51 release ± SE at effector:target ratios of			
		100:1		50:1	
		Toxicity	Infected	Toxicity	Infected
D4T	375	17.6 ± 1.8*	27.3 ± 2.1*	13.0 ± 1.7	19.5 ± 1.8*
	187.5	22.1 ± 1.7	25.6 ± 3.0	16.4 ± 1.2	15.5 ± 2.3
	93.8	21.4 ± 0.9	27.5 ± 3.7	15.7 ± 0.6	18.5 ± 3.0
	46.9	21.9 ± 1.4	17.2 ± 2.4	16.1 ± 1.1	11.6 ± 2.0
H_2O	0	27.4 ± 2.5	21.8 ± 2.0	20.3 ± 3.3	14.2 ± 1.9

[a]Treatment t.i.d. × 25 days.

*$P < 0.05$ (compared to H_2O controls in the same group).

all but the lowest D4T dosage level.

Discussion

These experiments indicate that the thymidine analog, D4T, is inhibitory to FV retrovirus infection, both in vitro and in an animal model. In a personal communication, Dr. Ismail Ghazzouli of Bristol-Myers Squibb has indicated that i.p. treatment of D4T was not effective against the Rauscher virus-induced infection of mice. In his study, D4T was used at dosages of 140 and 300 mg/kg/day, with the drug administered b.i.d. for 30 days beginning 3 h or 24 h post-virus inoculation. Only inhibition of splenomegaly was evaluated as a parameter in the Ghazzouli study.

Russell et al. (1989) have shown that D4T is rapidly absorbed in mice, with an oral availability of 98%. High levels of the drug were attained 5 min after p.o. administration, but a relatively short (17 min) serum half-life was seen. The mechanism of action of D4T against HIV involves enzymatic phosphorylation of the analog to a triphosphate, which then inhibits the reverse transcriptase of the virus and consequently viral replication (Ho and Hitchcock, 1989). D4T is apparently efficiently metabolized to the triphosphate, which persists in cells with a relatively long half-life of 190 min (Martin et al., 1989) despite the short half-life of the compound in serum as cited above. Martin et al. (1989) predicted that, based on these findings, D4T may be more effective in the clinic if given twice a day. Since Ghazzouli found poor in vivo murine retrovirus activity using such a treatment schedule, it was decided to initially utilize the oral t.i.d. treatments in the present experiments. Such a regimen, using an additional treatment per day, would provide more opportunities for the drug to maintain active levels in the blood. Carrying this premise to a q.i.d. treatment schedule also resulted in positive results.

It has previously been reported (Mansuri et al., 1989) that D4T administered

p.o. once daily for 30 days to mice was not lethally toxic in doses up to 500 mg/kg/day. At 1000 mg/kg/day, a 20% lethality occurred. Using the t.i.d. regimen in the present study, with treatment stopping after 14 days, was not lethal to mice at doses up to 750 mg/kg/day, although host weight loss occurred. Mansuri et al. (1989), in extensive histopathological evaluations, also observed that non-lethally toxic doses of D4T caused no discernible effects on erythrocyte, leukocyte or platelet parameters, but increases in erythrocytopenia and in mean corpuscular volume were seen at 1000 mg/kg/day in some mice.

Since D4T may be eventually considered as an alternative to AZT therapy for patients with AIDS, comparisons between the two drugs are meaningful. The in vitro FV-inhibitory activity of AZT was quite superior to D4T in this study; contrary to what has been reported by Mansuri et al. (1989, 1990) in other cell lines, D4T appeared more toxic than AZT to the *M. dunni* cells used in this study. This substantially lowered its in vitro SI. Such cell dependency in antiviral studies has been the subject of a recent review (Sidwell, 1986). Another variable possibly contributing to the contrasting in vitro toxicity of D4T is the differences in assay procedure; the present experiment utilized a media and drug replacement every other day, which was not done in the Mansuri et al. studies and would be expected to increase cytotoxic effects.

Comparisons may also be made regarding the in vivo activity of the two compounds. Although AZT was not run in parallel to D4T in this study, the protocols and the in vivo FV infection model were similar to what were used in an extensive study run with AZT which has been previously reported (Morrey et al., 1990). In those earlier studies, AZT was administered to FV-infected mice either i.p., t.i.d. for 24 days, or in drinking water for 22 days, beginning 4 h post-virus inoculation. In both AZT experiments, splenomegaly was inhibited and spleen and plasma FV titers were reduced with 4 dosages of AZT to a greater extent than was seen in the present study with any dose of D4T. It is important to note, however, that the reduction of FV IC in the spleen is a particularly challenging task for any antiviral drug (Morrey et al., 1991); it is therefore significant that D4T substantially reduced both this parameter and the splenic FV RNA. The various doses of AZT used in the treatment of FV-infected animals reduced toxicity control hematocrit values to a much greater degree than was seen using D4T. This observation correlated with the known induction of anemia and neutropenia in AZT-treated patients (Yarchoan et al., 1986; Richman et al., 1987). In our previously reported FV study with AZT, significant spleen enlargement was also observed at all AZT doses in the toxicity control mice, whereas D4T only reduced the spleen weights of toxicity control animals using the 375 mg/kg/day and higher dosages in the present study.

AZT therapy in the afore-mentioned Morrey et al. (1990) study resulted in a total lack of detectable neutralizing antibody in the FV-infected mice; it was suggested that the drug may have had a direct effect on immunoglobulin production in the mice. Treatment t.i.d. for 25 days with D4T, while

significantly lowering virus titers in spleens and plasma, did not appear to affect the development of FV-specific neutralizing antibodies, suggesting another advantage of the material over AZT. It has been previously shown that the production and persistence of these antibodies correlates with a reduction of infected cells and viremia (Morrey et al., 1990). One reason for the selection of the genetically defined mouse strain used in this study was to allow the FV antibody studies to be run, since other commonly used mouse strains, such as the BALB/c or Swiss Webster mouse, lack the Rfv-$3^{r/s}$ genotype and thus are incapable of producing significant levels of FV-specific antibody (Chesebro and Wehrly, 1979).

There is evidence that indicates NK cells may play a role in resistance to HIV infections (Creemers et al., 1985; Rook et al., 1985). We have previously reported that FV infection in the (B10.A × A/WySn)F_1 mouse caused a significant reduction in splenic NK cell activity (Morrey et al., 1990), an observation confirmed in the present study. Black et al. (1991) have found stimulation of NK cell activity in Rauscher murine leukemia virus-infected mice to be associated with the positive antiviral activity of a series of biological response modifiers. It was therefore of interest to determine the influence of D4T therapy on this potentially key immunologic factor. The moderate inhibition of NK cell activity by D4T in uninfected mice was not unexpected, since it is understood that AZT treatment causes similar, if not more profound effects (Black, P.L. and Ussery, M.A., personal communication). The apparent prevention of depression of NK cell activity in the FV-infected mice was most interesting; this may have been a result of the inhibition of the viral infection to the point that the usual depression of NK cell activity did not occur. The lack of depression in the infected animal due to direct D4T effects cannot be explained by our available data, but again correlates with the findings of Black et al. (1991) which showed that effects on NK cell activity in uninfected mice does not correlate well with effects in infected animals.

These data, added to the previously reported observations that D4T was much less toxic than AZT to human granulocyte macrophage and erythrocyte progenitor cells (Mansuri et al., 1989, 1990) and has different biochemical properties, further suggest the possible advantage of this compound as a potential anti-AIDS drug.

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