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Effect of cyclosporin A on an experimental chronic viral infection of the central nervous system

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Summary

The effects of cyclosporin A (CsA) on neuropathological lesions induced by a chronic viral infection have been tested in the experimental model of the mouse hepatitis virus 3 (MHV3) infection. Daily injections of CsA (50 mg/kg) inhibited the expression of the MHV3-induced ependymitis, meningitis, hydrocephalus and vasculitis. The effect was preserved even if CsA treatment was initiated 15 days after virus infection but was lost if CsA treatment was given later on or for a shorter period of time. Viral titers in brains of chronically infected mice were not affected by CsA treatment. During the first week following MHV3 infection, CsA treatment increased both the percentage of acute death (31 vs. 10%) and the viral titers in brain and liver of infected mice. In this model, the timing of CsA treatment appeared critical for the balance between its beneficial effect on CNS lesions and the risk of increased acute mortality.

Introduction

Cyclosporin A (CsA) is a very potent immunomodulating agent whose beneficial effect on graft tolerance has been demonstrated both in experimental animals and in transplant patients. CsA appears also active against several autoimmune diseases such as uveitis or type I diabetes, whereas the consequences of CsA treatment on viral disease and post-viral immune responses have been less extensively studied (Johnson, 1985). In view of the potential therapeutic importance of CsA in human post-infectious and chronic viral encephalitis, a series of experiments was designed to appraise the therapeutic effects of CsA on central nervous system (CNS) lesions induced by an experimental chronic viral infection.

Intraperitoneal injection of mouse hepatitis virus type 3 (MHV3), a member of the coronavirus group, into adult C_3H mice, results either in early death due to acute hepatitis or in the development of a chronic disease with neurologic manifestations and virus persistence in surviving animals (Virelizier, 1975; Tardieu, 1986). Chronic neurologic disease results from a meningitis, ependymitis and encephalitis, beginning 3–4 weeks after

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infection followed by a permanent communicating hydrocephalus. Later (after the 6th week post-infection), a chronic thrombotic vasculitis develops affecting meningeal and parenchymal vessels at the brain stem level (Tardieu, 1982). This model allowed to study the effect of CsA both on the acute phase, depending on the intensity of the viral replication, and on the chronic intra-CNS lesions induced by a chronic viral infection.

Materials and methods

Mice and virus

Inbred mouse strains C_3H/He Orl were purchased from the Centre de sélection et d'élevage d'animaux de laboratoire, CNRS, Orléans-La Source, France.

MHV3 was originally obtained from J.M. Dupuy, Bicêtre, France. It was plaque purified and grown on L-929 cells as previously described (Tardieu, 1986).

Ten-week-old animals were infected intraperitoneally (i.p.) with 10^2 pfu of MHV3.

Viral titration

For viral titration, animals were anesthetized with ether and perfused with phosphate-buffered saline (PBS) for 5 min through the left ventricle. The liver and brain were removed and frozen at −70°C in 2 ml of PBS solution, thawed, sonicated and assayed for the presence of virus by plaque titration on L-929 cells (Sturman, 1972).

Neuropathological studies

For neuropathological studies, animals were perfused with paraformaldehyde (10%, w/w) using the same procedure as for PBS perfusion. Organs were removed and processed as previously described (Tardieu, 1986).

For each brain, two independent examiners evaluated the intensity of meningitis, hydrocephalus and vasculitis (notation: 0, +, ++, +++) on coronal serial, 7 μm thickness sections at the brain stem and the lateral hemispheres level.

Anti-MHV3 antibody and α-interferon titration

Blood samples were obtained in anesthetized animals by retro-orbital puncture or by direct cardiac puncture at the time of death.

Anti-MHV3 antibodies were titered either by enzyme-linked immunoabsorbent assay (ELISA) or by neutralization. For neutralization assays, 10^2 pfu of MHV3 were incubated for 45 min at 37°C with serial dilutions of sera obtained from infected or non-infected mice and subsequently titered by plaque assay. The highest dilution of serum reducing viral titer by more than 80% was used as the neutralizing titer.

Interferon was assayed by a vesicular stomatitis virus (VSV) plaque reduction assay on L-929 cells. Each sample of serum was individually tested. The absence of MHV3 infectivity was checked in each sample.

Cyclosporin A injection

Cyclosporin A was obtained from Sandoz Laboratories, diluted (10 mg/ml) in olive oil (at 60°C) and injected subcutaneously every day (1 mg per animal, i.e. 50 mg/kg).

Cyclosporine A levels were determined by high-performance liquid chromatography (HPLC) in whole blood obtained by retro-orbital puncture performed just before the next CsA injection. Briefly, this titration technique involved the preparation of blood samples by liquid–liquid extraction followed by isocratic reversed-phase liquid chromatography at 75°C and ultraviolet (UV) detection at 210 nm. Quantification was performed relatively to cyclosporine D as an internal standard. This method permitted reliable measurements (standard deviation < 15%) between 10 and 2000 ng/ml. The determinations were kindly performed by L. Vernillet and J.F. Le Bigot, Laboratoire Sandoz, France.

Study design

126 animals were infected with MHV3 and CsA treatment proceeded as follows. Group A: continuous daily subcutaneous injection of olive oil (diluted vehicle of CsA); group B: continuous daily CsA treatment started at the same time as MHV3 infection; group C: continuous daily CsA treatment started either 15 or 35 days after MHV3 infection; group D: discontinuous treatment: daily CsA treatment given for 15 days, started at day 15 or 35 post-infection (p.i.). In each group several parameters were recorded: animal deaths, viral titers in liver and brain each day during the first

week p.i. to determine viral replication and then at 5 and 12 weeks p.i. to determine viral persistence, anti-MHV3 antibody titers every 2 weeks during 12 weeks, neuropathological lesions determined 5 and 12 weeks p.i.

As control groups, 41 mice were kept non-infected but were injected daily with CsA or olive oil.

Statistical analysis

(a) Statistical analysis of mouse survival. Statistical analysis of mouse survival was performed with the Log Rank test (Mantel, 1959). For each group, the number of deaths was recorded each day during 12 weeks p.i. This number of observed deaths was compared to the number of expected deaths if the mortality would be identical in the different groups (null hypothesis) and for one group the relative death rate was the ratio between the number of observed and expected deaths. In the different groups, the death rates were compared with the χ^2 test to determine the statistical significance of the differences.

(b) Statistical analysis of the viral titers. The comparison of the means of viral titers, determined in liver or brain of infected mice, between CsA-treated and untreated groups was performed using the Student *t*-test.

(c) Statistical analysis of neuropathological lesions. For each group of mice, we determined the number of infected brains with no neuropathological lesion, one of the lesions usually observed after MHV3 infection (i.e. meningitis, hydrocephalus, vasculitis), two associated lesions or three associated lesions. Neuropathological lesions were ranked unrespective to the intensity of the lesions. The comparison between the different groups of mice was performed using the χ^2 test to determine the statistical significance of the observed differences.

Results

CsA blood levels in CsA-treated MHV3-infected mice

CsA blood levels were repeatedly tested in the same group of 15 infected animals at the end of the 2nd, 4th, 6th and 11th week of treatment. CsA blood levels varied from 1300 to 3500 ng/ml (mean ± 1 SD: 2381 ± 1153 ng/ml) which is consistent with an adequate immunosuppressive effect.

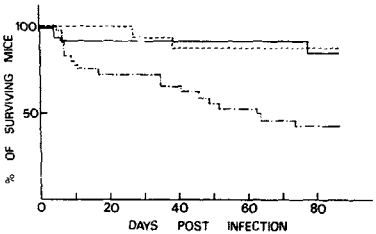

Fig. 1. Survival curves of CsA-treated or untreated MHV3-infected mice. 126 C_3H mice were infected i.p. with 10^2 pfu/mouse of MHV3, treated either with CsA (— · — · —) or olive oil (———) in which CsA is usually diluted. Treatment was started the same day as virus injection and given daily up to the end of the experiment. As a control 15 mice were kept uninfected but were treated with CsA (- - - - -).

Effect of CsA treatment on survival of infected animals

Intraperitoneal MHV3 infection induced an acute hepatitis during the first 10 days p.i. leading to the death of a small percentage of mice (10%) (Fig. 1; Table 1: group I-A). The surviving infected mice developed a chronic CNS infection and 8% of chronically infected animals died after the 10th week p.i. (Fig. 1; Table 2: group I-A).

TABLE 1

EFFECT OF CsA ON SURVIVAL OF MHV3-INFECTED MICE FROM DAY 3 TO 14 POST-INFECTION

Group	Type of treatment	Number of mice	% of death	Relative death rate [a]
Infected (I)				
A	Olive oil	80	10	0.88
B	CsA	46	31	2.40 [b]
Uninfected (NI)				
A	Olive oil	25	0	0
B	CsA	15	0	0

[a] Determined by Peto's Log Rank test.

[b] The comparison of death rates by χ^2 test between groups I-A and I-B was statistically significant at $P < 0.01$.

TABLE 2

EFFECT OF CsA ON SURVIVAL OF MHV3-INFECTED MICE FROM WEEK 2 TO 12 POST-INFECTION

Group	Treatment			Number of mice	% of death	Relative death rate [a]
	Type	Initiation (week p.i.)	Duration (weeks)			
Infected (I)						
A	Olive oil	0	12	25	8	0.66
B	CsA	0	12	29	34	3.16 [b]
C	CsA	2	10	14	21	1.58
C′	CsA	5	7	9	11	0.78
D	CsA	0 or 2 or 5	5	24	0	0
Uninfected (NI)						
A	Olive oil	0	25	25	10	0.74
B	CsA	0	15	15	13	1.05

[a] Determined by Peto's Log Rank test.

[b] The comparison of death rates by χ^2 test between groups I-A and I-B (or I-C or I-C′ or I-D) was statistically significant ($P < 0.001$).

When CsA treatment was started at the same time as the MHV3 infection and given daily during 12 weeks, the global percentage of death increased to 65% (Fig. 1). 31% of these deaths occurred during the acute phase of MHV3 infection (3rd to 14th day p.i., Table 1: group I-B) and 34% all along the chronic CNS infection phase (2nd to 12th week p.i., Table 2: group I-B). In the control group of uninfected but CsA-treated mice the rate of death was only 13% and occurred respectively at the 27th and 36th day p.i. (Fig. 1; Tables 1 and 2: group NI-B).

If CsA treatment was started at the 2nd or the 5th week p.i., i.e. after the acute phase of the MHV3 infection, the percentages of death became 21% and 11% respectively (Table 2: groups I-C and I-C′), and compared to the 8% of late death in the infected, untreated mice (Table 2: group I-A) did not reach statistical significance any more.

If CsA treatment was given only for 2 weeks none of the tested animals died even if treatment was started at the same time as the MHV3 infection (Table 2: group I-D).

Effect of CsA treatment on the biological markers of the viral infection

(a) Effect of CsA treatment on viral titers in liver and brain of MHV3-infected mice. We determined the viral titers in liver and brain of nine CsA-treated and ten untreated mice at the 4th day p.i., a time when the viral replication reaches its highest level, as previously described (Tardieu, 1986). The mean of viral titers in liver or brain obtained from three different experiments was moderately but significantly ($P < 0.05$) higher in CsA-treated mice compared to control mice (Table 3).

Viral titers were determined every day during the first week p.i. in four CsA-treated and four untreated mice. In MHV3-infected but untreated mice, viral titers in liver and brain increased from the first day p.i. to reach their highest level the 4th day p.i. and then progressively decreased to a low value ($< 10^2$ pfu/g) the 7th day p.i., confirming

TABLE 3

VIRAL TITER IN LIVER AND BRAIN OF MHV3-INFECTED MICE AT DAY 4 POST-INFECTION

	Brain ($\log_{10}$pfu/g)	Liver ($\log_{10}$pfu/g)
Olive oil [a]	3.7 ± 1	3.5 ± 0.1
CsA [b]	4.8 ± 1 [c]	4.8 ± 1.2 [c]

[a] In the olive oil treatment group, each value is the mean ± standard deviation of nine viral titers in liver or brain determined at day 4 p.i. from three different experiments.

[b] In the CsA treatment group, each value is the mean ± standard deviation of ten viral titers in liver or brain determined at day 4 p.i. from three different experiments.

[c] The difference observed in viral titers in organs of untreated and CsA-treated animals was significant at $P < 0.05$ as tested by Student t-test.

previously described results (Tardieu, 1986). In CsA-treated mice the kinetics of the viral titers followed the same curve as in the untreated mice but the highest titer obtained the 4th day p.i. was moderately higher (data not shown).

When tested at 5 and 12 weeks p.i., viral titers in brain and liver of untreated or CsA-treated infected mice were similar, i.e just reaching the detection level of 10^2 pfu/g of tissue in both groups of mice.

(b) Effect of CsA treatment on serum α-interferon titers of MHV3-infected mice. Alpha-interferon titers determined 24 h after MHV3 infection were similar in the individually tested serum samples of eight untreated and eight CsA-treated mice, even if CsA treatment was started 7 days before MHV3 infection (interferon titer between 300 and 800 IU/ml).

(c) Effect of CsA treatment on MHV3 antibody titer in infected mice. In a pool of three sera as well as in two individually tested sera from MHV3-infected animals, anti-MHV3 antibodies rose during the first 5 weeks p.i. to reach a titer varying from 1/1600 to 1/6400, as judged by the ELISA technique. This titer persisted up to the 12th week p.i. Neutralizing antibodies were present in the same sera at a titer of 1/400 to 1/800. Five different sera from infected, CsA-treated mice were similarly tested by ELISA between the 4th and the 12th week p.i. and identical anti-MHV3 antibody titers were observed. The same was true in the sera of three mice whose CsA treatment begun 15 days after MHV3 infection.

Effect of CsA treatment on neuropathological lesions induced by MHV3

Neuropathological studies were performed on 58 chronically infected mice: 46 were sacrificed at the 5th or the 12th week p.i., 12 brains of 16 spontaneously dead animals were removed just after death. Neuropathological observations obtained at the 5th or 12th week p.i. were similar and therefore results were pooled.

CsA markedly decreased the neuropathological consequences of MHV3 chronic CNS infection. As shown in Figs. 2 and 3 the effect was observed on the three types of MHV3-induced lesions: meningitis, hydrocephalus and vasculitis.

When CsA treatment was begun at the same time as MHV3 inoculation and injected daily up to the end of the experiment (Table 4: group I-B) only 7% of mice expressed the three typical MHV3-induced neuropathological lesions compared to 64% in the olive oil treatment group (Table 4: group I-A) (difference statistically significant at $P < 0.01$).

This effect of CsA was observed even if the treatment was begun 15 days after MHV3 inocula-

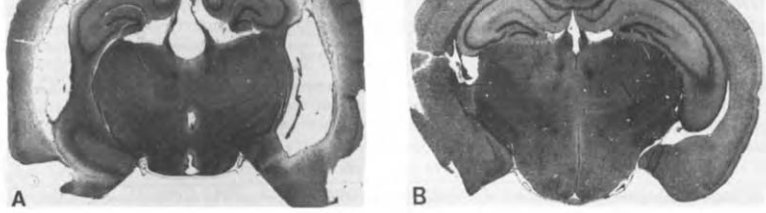

Fig. 2. Lateral hemispheres of MHV3-infected C_3H mice (week 12 p.i.). *A*: Brain from a control infected animal with a quadriventricular hydrocephalus and white matter deterioration presumably due to high pressure and CSF infiltration. *B*: Brain from a CsA-treated, infected animal with minimal hydrocephalus. CsA treatment was started the same day as MHV3 infection and given daily for 12 weeks. (×13)

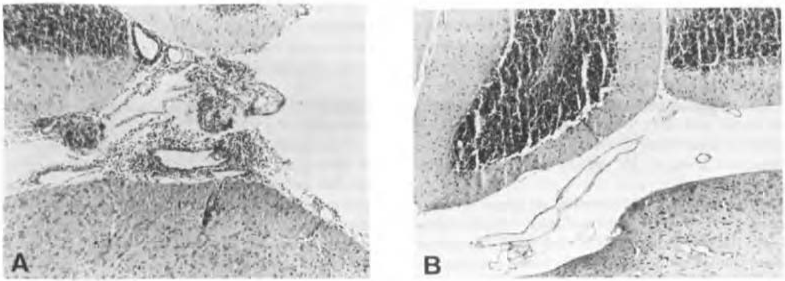

Fig. 3. Brain stem of MHV3-infected C_3H mice (week 12 p.i.). *A*: Intense vasculitis of parenchymal and meningeal vessels around the brain stem of a control infected animal. *B*: Absence of vasculitis around the brain stem of a CsA-treated infected mouse. CsA treatment was started the same day as MHV3 infection and given daily for 12 weeks. (×43)

tion (Table 4, group I-C: 8% of mice expressing three lesions; $P < 0.05$) but disappeared if the treatment was begun later (Table 4, group I-C′: 34% of mice expressing three lesions; $P > 0.10$) or for a shorter period of time (Table 4, group I-D: 40% of mice expressing three lesions; $P > 0.10$).

CsA treatment did not decrease significantly the number of mice completely free of neuropathological lesions because, as shown in Table 4, even in group I-B (last column), 20% of mice still expressed one type of neuropathological lesion, usually at a less intense degree (grade +) than observed in CsA-untreated group I-A or in CsA-treated group I-D (grade + + +). When CsA was started the 5th week p.i. (group I-C′) instead of the 2nd week p.i. (group I-C), hydrocephalus was observed in three of the six mice at an intermediate degree of intensity (+ +) although meningitis and vasculitis observed in four of the six mice remained at a low degree of intensity (+).

Twelve animals died spontaneously and their brains were obtained just after death for neuropathological study. In the two animals belonging to the CsA-untreated group (group I-A) meningitis, hydrocephalus and vasculitis were intense. In

TABLE 4

EFFECT OF CsA ON MHV3-INDUCED NEUROPATHOLOGICAL LESIONS

Infected group (I)	Treatment			Number of mice	% of mice expressing [a]			
	Type	Initiation (week p.i.)	Duration (weeks)		No lesion	Three types of lesions	Two types of lesions	One type of lesion
A	Olive oil	0	12	11	36	64 [b]	0	0
B	CsA	0	12	15	66	7	7	20
C	CsA	2	10	12	33	8	17	42
C′	CsA	5	7	6	34	34	16	16
D	CsA	0 or 2 or 5	2	10	40	40	10	10

[a] As judged by neuropathological studies at 5 or 12 weeks p.i. or just after death, performed by two independent examiners. The recorded MHV3-induced neuropathological lesions were: hydrocephalus, meningitis and vasculitis. Lesions were analyzed on coronal serial sections at the lateral hemispheres and the brain stem level. The neuropathological lesions were counted unrespective of the intensity of the lesion.

[b] The comparison of the number of mice expressing three types of lesions (hydrocephalus + meningitis + vasculitis) by χ^2 between groups I-A and I-B (or I-C) is statistically significant at $P < 0.01$ (and $P < 0.05$), respectively.

the nine animals belonging to the CsA-treated group I-B or I-C no lesions (seven mice) or a slight degree of meningitis or vasculitis without hydrocephalus were observed. In one mouse belonging to the CsA-treated group I-C′ an intense hydrocephalus was found without vasculitis and with a very slight degree of meningitis.

Discussion

Cyclosporin A appears to modify both the acute viral disease and the consequences of the chronic CNS infection induced by a neurotropic virus, the MHV3.

During the acute phase of the infection, viral titers in liver and brain of CsA-treated mice were higher than those observed in untreated but infected mice, as was the percentage of acute death among CsA-treated infected mice. These differences, although statistically significant, were not as dramatic as in other experimental models testing CsA effect on acute viral diseases. Thus, when BALB/c mice were infected with herpes virus type 2, the acute mortality increased from 20% in control infected animals to 90–100% in CsA-treated mice (Armending, 1982a, b). A similar increase in acute mortality after CsA treatment was observed in mice suffering from myocarditis after coxsackie B3 infection (O'Connell, 1986) or from interstitial pneumonia after vesicular stomatitis virus infection (Charan, 1986). On the other hand, CsA treatment did not modify acute mortality and/or virus titer in organs of mice infected either by influenzae (Schiltknecht, 1985), mouse cytomegalovirus (Gui, 1982) or lymphochoriomeningitis virus (Loliger, 1985). Gathering these different experimental data, variations in the effect of CsA on an acute viral infection might depend upon (a) the mechanism of the virus-induced lesions: CsA appears to decrease lesions induced by an acute post-viral immune response (Loliger, 1985) but to increase acute cytopathogenic lesions due to the viral replication itself (Chaaran, 1986; O'Connell, 1986); (b) the dose of CsA used: CsA titers in blood, however, were usually not available in previous experimental reports; (c) the timing of CsA treatment: death rate increased significantly only if CsA treatment was started at the same time as the virus injection, as we observed in the present report. These different factors have to be carefully analyzed to evaluate the benefits and risks of a CsA treatment during human inflammatory diseases potentially induced by a viral infection.

In our model, the increased viral replication in CsA-treated animals did not depend on a decreased production of α-interferon since α-interferon titers were similar in blood of CsA-treated and control infected mice, a result in agreement with previous reports (Armending, 1982a, b; Gui, 1982). It might then depend on the effect of CsA either on post-viral cytotoxic activities essentially mediated by T cell and NK cells (Armending, 1982a, b; Chaaran, 1986) or directly on the viral interaction with CNS cells (Gui, 1982).

During the chronic phase of the infection, the mortality increased significantly among animals receiving long-term CsA treatment started the same day as virus inoculation (34 vs. 8% in the infected but untreated group). In the brain of these animals which died during the chronic phase, none of the three MHV3-induced neuropathological lesions was observed. Viral titers in brains were unmodified as compared to control infected mice as were anti-MHV3 circulating antibody titers. On the other hand, long-term CsA or olive oil treatment of uninfected animals induced a mortality rate of 13 and 8%, respectively. Thus, the increase in mortality rate among CsA-treated infected mice appears to depend on different additive factors, i.e. the chronic viral infection and the chronic treatment with olive oil alone or mixed with CsA.

The neuropathological consequences of MHV3 infection (meningitis, hydrocephalus and vasculitis) were absent in 93% of CsA-treated mice compared to 36% of untreated mice. During the chronic CNS infection induced by MHV3, the meningitis and the later appearing vasculitis reflect the intracerebral hyperimmune response induced by viral inoculation. This post-viral immune response is in part under T cell control as judged by its suppression by cyclophosphamide (Tardieu, 1982) or CsA (present report). Physiopathologic mechanisms leading to development of hydrocephalus are still unclear: the high affinity of MHV3 for ependymal cells (Tardieu, 1986) suggests a poten-

tial direct effect of the virus on ependymal cells. Moreover, cyclophosphamide in spite of its inhibitory effect on meningitis had no effect on hydrocephalus (Tardieu, 1982) suggesting that meningitis is not the main cause of hydrocephalus. The efficacity of CsA to suppress hydrocephalus development in MHV3-infected mice is particularly striking when CsA treatment is started at the same time as virus injection. This suggests that ependymitis, which participates in hydrocephalus formation may depend for its induction not only on the direct effect of the virus on ependymal cells but also on an early cytotoxic immune response against virus-infected cells. Outside the CNS, a previous report showed that CsA treatment can reduce the chronic inflammation of lungs induced by influenza A virus with a concomitant alteration of the delayed-type hypersensitivity against the virus (Schiltknecht, 1985). In the CNS an effect of CsA on chronic immune response has been suggested by the inhibitory effect of CsA on the expression of experimental allergic encephalitis (EAE) (Armending, 1982a, b) and by the inhibitory effect of CsA on inflammation and demyelination in the spinal cord of mice persistently infected with Theiler murine encephalomyelitis virus (Rodriguez, 1986). In this last experimental model, the decreased demyelination correlated with a decreased proliferation of T lymphocytes to virus and myelin antigens.

Finally, when CsA injections were started 15 days p.i., i.e. after the acute phase of the viral disease, most of the effects of treatment were preserved. When CsA treatment begun after the 5th week p.i., i.e. when the inflammation is already established, the intensities of meningitis and vasculitis were decreased but not abolished whereas hydrocephalus was not or slightly modified. Similar results on the importance of the timing to start CsA treatment have been observed in the Theiler virus infection model (Rodriguez, 1986).

To preserve CsA efficiency on intra-CNS inflammatory lesions but to maintain a low risk of morbidity and mortality due to viral replication, the best time to initiate the treatment seems to be the early post-infection period (in our model the end of the 2nd week post-infection). This is important for the clinical usefulness of CsA against post-viral inflammatory diseases and may have implications towards the treatment of human post-infectious encephalitis.

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