

Protection from spontaneous lymphoma development in SJL/J(v⁺) mice neonatally injected with dualtropic SJL-151 virus

(dualtropic retrovirus/murine B-cell lymphoma)

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ABSTRACT In previous studies dualtropic type C retroviruses were isolated from spontaneous B-cell lymphomas that appear with a high incidence in SJL/J(v⁺) mice. In this report the possible *in vivo* pathogenic effect of one cloned dualtropic isolate, designated SJL-151, was investigated. SJL/J(v⁺) and CBA/J mice, neonatally injected with SJL-151 alone or in combination with SJL-ecotropic virus, were initially studied for virus recovery 4-8 weeks after infection by spleen cell cocultivation with mouse SC-1 and mink ML indicator cells. Whereas ecotropic virus was easily detected in treated mice, dualtropic virus was recovered only from the spleen cells of animals coinfecting with SJL-ecotropic and SJL-151 viruses. With a panel of monoclonal antibodies the recovered dualtropic viruses showed an antigenic profile similar to that of the originally injected SJL-151 virus. Whereas virus-injected mice did not show lymphoma induction or acceleration, a remarkable decrease in spontaneous lymphoma incidence was observed in the SJL/J(v⁺) mice receiving SJL-151 virus alone. A virus-specific antibody response was detectable in these mice, but a similar serum reactivity was also demonstrated in SJL/J(v⁺) mice coinfecting with SJL-ecotropic virus and SJL-151 virus, which subsequently developed lymphomas with the usual high incidence, thus rendering an antibody-mediated protective mechanism untenable. The possibility of viral interference, as an alternative mechanism for lymphoma prevention, is discussed in view of the findings that persistence of SJL-151 virus or *de novo* generation of dualtropic virus did not occur in aged SJL/J(v⁺) mice injected neonatally with SJL-151 virus alone.

The discovery of a virus class possessing the host range properties of both ecotropic and xenotropic viruses, as well as the capacity to induce a cytopathic effect in mink lung cells (1) was an important acquisition in studies on leukemogenesis by murine type C retroviruses. Molecular studies indicate that these mink cell focus-inducing (MCF) dualtropic viruses originate from a recombination of ecotropic and endogenous xenotropic viral sequences in the *env* gene (2, 3). As a rule, dualtropic viruses are detected only in preleukemic and leukemic tissues of high leukemia incidence mice, and some, but not all, isolates are endowed with leukemogenic activity (4). Furthermore, in conventional murine leukemia virus (MuLV) stocks, such as Moloney, Friend, Rauscher, and Gross MuLV, the presence of a recombinant virus component has been well documented (5-10). These findings, together with the observation that endogenous ecotropic viruses are usually not tumorigenic, support the hypothesis that dualtropic viruses are the proximal cause of the leukemic disease.

In most studies of spontaneously occurring leukemias and lymphomas, oncogenic dualtropic viruses have been isolated from the thymus and have been etiologically associated with T-cell neoplastic disease. Hence, our interest was stimulated when

we isolated dualtropic viruses from the neoplastic spleens and lymph nodes of SJL/J(v⁺) mice in which lymphoma had occurred spontaneously (11). As known, SJL lymphomas show immunologic and histopathologic features of B-cell neoplasms (12-14), much like the human polymorphic lymphoplasmacytoid lymphomas (15).

The biochemical, morphological, and immunological characterization of the SJL dualtropic isolates has been described (16). In the present study we report data indicating that a cloned SJL-dualtropic virus is devoid of oncogenicity when injected into newborn mice; more interesting, it is able to prevent spontaneous lymphoma development.

MATERIALS AND METHODS

Mice. The origin of SJL/J(v⁺) mice has been reported (17), and CBA/J mice were purchased from The Jackson Laboratory. Control and treated mice were observed weekly for signs of disease and were killed when enlarged lymph nodes or splenomegaly was evident. After autopsy, spleen, lymph nodes, and thymus specimens were examined histologically.

Cells and Viruses. Mouse SC-1, rat XC, CCL64 mink lung (ML), and mouse 3T3FL (3T3) cell lines were grown and maintained as described (11). SJL ecotropic (SJL-eco) virus was obtained from tail extracts of normal adult SJL/J(v⁺) mice and subsequently cloned on SC-1 cells by limiting dilution and propagated on SC-1 cells. SJL-151 dualtropic virus was obtained and cloned as described (11) and propagated on ML cells.

Virus Injection. Within 48 hr after birth, SJL/J(v⁺) and CBA/J mice were injected subcutaneously in the dorsal region with 0.10 ml of dualtropic SJL-151 virus, 0.05 ml of SJL-eco virus, or both. Titers were 8.32×10^5 focus-forming units/ml for SJL-151 virus and 6.40×10^6 plaque-forming units/ml for SJL-eco virus (see below). Mice of groups coinfecting with SJL-151 and SJL-eco viruses received closely spaced consecutive injections of each virus preparation in the same dorsal subcutaneous area. Control animals were similarly injected with the SC-1 or ML uninfected culture supernatants. Other groups of mice were also given four subcutaneous doses of dualtropic SJL-151, either alone or in combination with ecotropic virus, every other day starting 24 hr after birth.

Virus Assays. Cloned SJL-eco and endogenous ecotropic virus infectivity was tested on SC-1 cells by the UV-XC procedure (18). Cloned dualtropic virus and endogenous XC-negative (XC⁻) virus infectivity was tested on ML cells by induction of cytopathic effect, by immunofluorescence (IF), or by both techniques. Infectious center assay by lymphoid cell suspensions was carried out as reported (11, 19). The dualtropic viruses re-

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Abbreviations: MuLV, murine leukemia virus; MCF virus, mink cell focus-inducing virus; SJL-eco virus, SJL ecotropic virus; SJL-151 virus, SJL dualtropic virus; ML cells, CCL64 mink lung cells; IF, immunofluorescence; FITC, fluorescein isothiocyanate.

covered from lymphoid organs were defined as XC⁻ viruses able to infect both ML and 3T3 cells, as determined by IF.

IF Assay. Direct IF assay was performed by using fluorescein isothiocyanate (FITC)-conjugated goat antiserum directed against Tween/ether-disrupted Moloney MuLV, at 1:40 dilution on acetone-fixed cells, as described (11, 20). Indirect IF assay was performed according to Hilgers *et al.* (21). Virus-specific monoclonal antibodies (22), kindly supplied by R. C. Nowinski, were used at 1:300 dilution. FITC-conjugated goat anti-mouse IgG was used at 1:40 dilution. The FITC-conjugated antibodies were furnished by Becton Dickinson Research Center under a contract from the Division of Cancer Cause and Prevention, National Cancer Institute (Bethesda, MD).

¹²⁵I-Labeled Protein A Binding Assay. Specific antibodies bound to sucrose density gradient-purified eco- or dualtropic virus were evaluated by the staphylococcal protein A binding assay as described (23). Protein A (SpA, Pharmacia) labeled with ¹²⁵I by the Chloramine-T method (24), was used at 3 ng per well (3 × 10⁴ cpm/ng).

RESULTS

Recovery of Dualtropic Virus from Young Virus-Injected Mice. AKR MCF viruses, which efficiently accelerate lymphoma development in mice of the original strain, replicate in thymic cells when they are inoculated into newborn recipients (25). Furthermore, the MCF virus replication rate is greatly increased by simultaneous infection and replication of ecotropic virus (26–27). Hence we investigated whether dualtropic SJL-151 virus injected in newborns, either alone or in combination with SJL-eco virus, could be recovered 4 to 8 weeks after the injection. In these studies, besides SJL/J(v⁺), CBA/J mice were also employed because of their susceptibility to lymphomagenesis by recombinant viruses (28).

Because the spleen is one of the target tissues for neoplastic transformation as well as for dualtropic virus growth in SJL/J(v⁺) mice, animals of the different experimental groups were hemisplenectomized to allow evaluation of lymphoma appearance, and the spleen cells were cocultivated with SC-1 and ML indicator cells to detect growth of ecotropic and dualtropic viruses, respectively.

The results of this experiment (Table 1) show that 4- to 8-week-old uninjected SJL/J(v⁺) mice express a high frequency of endogenous ecotropic virus (70%), whereas CBA/J mice are negative; neither strain exhibits any detectable expression of xenotropic and dualtropic viruses, confirming previous findings (11).

Table 1. Virus recovery from SJL/J(v⁺) and CBA/J mice neonatally injected with ecotropic and dualtropic SJL-151 viruses

Viruses	No. virus-positive mice/ no. mice tested*			
	SJL/J(v ⁺)		CBA/J	
	SC-1 [†]	ML [‡]	SC-1 [†]	ML [‡]
None	7/10	0/10	0/5	0/5
SJL-eco	3/4	0/4	5/5	0/5
SJL-151	5/9	1/9	0/9	0/9
SJL-151 (four doses)	3/6	0/6	0/6	0/6
SJL-eco + SJL-151	10/11	8/11	7/7	6/7
SJL-eco + SJL-151 (four doses)	5/6	3/6	6/6	5/6

* The mice were hemisplenectomized at 4 to 8 weeks of age and the spleen cells were cocultivated with SC-1 and ML indicator cells.

[†] The presence of XC⁺ ecotropic virus was determined on SC-1 cells by the UV-XC method.

[‡] The presence of dualtropic virus was determined on ML cells by IF assay. For further details see *Results*.

Ecotropic virus was easily recovered from virus-injected SJL/J(v⁺) mice of the various groups; no significant differences were observed in the percentage of positive mice or in the titers of isolated SJL-eco. In the treated animals, obviously, it was not possible to distinguish between the endogenous and the “exogenously” injected ecotropic virus; however, CBA/J spleen cells were constantly negative when obtained from control donors, but released virus in all cases when obtained from SJL-eco-injected mice. This last result was expected because SJL-eco virus shows Fv-1 tropism (17).

Dualtropic SJL-151 virus injected alone, either in a single dose or in four consecutive doses, did not replicate in SJL/J(v⁺) and CBA/J mice as shown by negative spleen-ML cocultures in all cases, except for one SJL/J(v⁺). But, when SJL-151 and SJL-eco viruses were injected together in single or repeated doses, high percentages of positive cocultures were observed in both mouse strains. In agreement with Haas and Patch (26), this finding indicates that ecotropic viruses may facilitate dualtropic virus replication, possibly through genomic masking.

Groups of six SJL/J(v⁺) mice each injected with dualtropic virus either alone or in combination with SJL-eco virus were also studied at 8 weeks of age for virus production by thymic cells on ML and SC-1 cells. Whereas recovery of dualtropic virus was constantly negative, ecotropic virus expression could be detected in 33% of the mice, and this percentage was approximately the same as that in untreated SJL/J(v⁺) controls of the same age (data not shown). This suggests that, unlike the AKR system, thymic cells do not represent the appropriate targets for SJL dualtropic virus infection and replication.

Characterization of Dualtropic Virus Isolated from Virus-Injected SJL/J(v⁺) Mice. In order to obtain further evidence that the viruses isolated from treated SJL/J(v⁺) mice and successfully growing in ML cells were indeed dualtropic in nature, randomly chosen supernatants of two cultures that had given positive results after cocultivation with spleen cells from SJL/J(v⁺) mice inoculated with SJL-eco and SJL-151 viruses were used as source of virus for cloning by limiting dilution (29). The cloned viruses grew equally well on heterologous (ML) and homologous (SC-1 and 3T3) cells, as evaluated by IF assay; they also induced cytopathic foci on ML cells and were constantly negative in the UV-XC assay.

With a panel of monoclonal antibodies that recognize different epitopes on the gp70 and p15(E) viral envelope proteins (22), two cloned viruses (SJL-68/82 and SJL 77/82) were analyzed by IF on chronically infected acetone-fixed ML cells. Table 2 shows that both viruses reacted in a manner similar to the reference SJL-151 virus, indicating that the new dualtropic isolates had the same antigenic profile as the SJL-151 virus originally injected. However, the possibility that these recovered

Table 2. Monoclonal antibody specificity for dualtropic viruses from SJL/J(v⁺) mice dually injected with SJL-eco and SJL-151 viruses

Monoclonal antibody	Epitope identified by monoclonal antibody*	IF assay on SJL isolates [†]			
		eco	151	68/82	77/82
19-A2	gp70 ^c	+	—	—	—
16-C1	gp70 ^b	+	—	—	—
16-B7	gp70 ^a	+	—	—	—
9-E8	p15(E) ^a	+	—	—	—
19-VIII-E8	p15(E) ^b	+	+	+	+
19-F8	p15(E) ^c	+	+	+	+

* Data obtained from Lostrom *et al.* (22).

[†] The IF assay was carried out on SC-1 cells chronically infected by SJL-eco virus and on ML cells chronically infected by dualtropic cloned isolates 151, 68/82, and 77/82.

viruses represent *de novo* generated dualtropic agents different from the SJL-151 viruses could not be ruled out because previous results had shown antigenic similarities between SJL-151 and SJL-149 viruses, another cloned dualtropic isolate (16).

Decrease of Spontaneous Lymphoma Incidence in SJL/J(v⁺) Mice Injected with SJL-151. Different groups of virus-injected mice were studied for lymphoma development, and the cumulative incidence of lymphomas during a 20-month observation period is reported in Fig. 1. Mice injected with SJL-eco alone, and those with SJL-eco together with SJL-151 virus showed the same incidence as untreated controls; mice injected with supernatants of uninfected SC-1 or ML cultures also exhibited the same lymphoma incidence as control mice (data not shown). In contrast, SJL/J(v⁺) mice injected with a single dose of SJL-151 showed a substantial reduction in lymphoma development, and the three mice that developed lymphoma exhibited a longer latency than controls. Moreover, mice injected with four doses of SJL-151 showed no lymphomas up to 14 months of age, while mice receiving repeated doses of SJL-eco and SJL-151 showed the same lymphoma incidence as untreated controls for the same time period. Mice of these two groups are still under observation, whereas survivors of the other experimental groups were sacrificed at 20 months of age.

On histological examination, virus-injected lymphomatous mice in the majority of cases showed B-cell neoplasms typical for SJL/J(v⁺) mice (13, 14). No neoplastic disease was recorded in CBA/J mice (matched groups of about 20 animals each) similarly injected with SJL-151 alone or in association with SJL-eco virus (data not shown).

These findings clearly indicate that dualtropic SJL-151 virus, even when injected together with SJL-eco and in repeated doses, is devoid of oncogenicity. On the other hand, the marked decrease in lymphoma incidence in SJL/J(v⁺) mice injected with dualtropic virus alone speaks in favor of a protection conferred by this virus.

Failure to Detect Dualtropic Viruses from Aged SJL/J(v⁺) Mice Injected Only with SJL-151. Dualtropic viruses are consistently isolated from neoplastic and preneoplastic tissues of aged SJL/J(v⁺) mice (11), but whether their *de novo* generation rep-

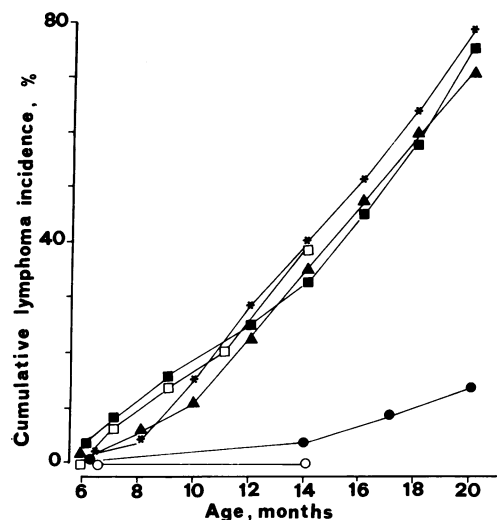


FIG. 1. Protective effect of SJL-151 virus on spontaneous lymphoma development in SJL/J(v⁺) mice. Mice were injected subcutaneously within 48 hr of birth with a single dose (●; 23 mice) or four consecutive doses (○; 16 mice) of SJL-151 virus. Mice of other groups were similarly injected with SJL-eco alone (▲; 17 mice) or together with SJL-151 in a single dose (■; 24 mice) or in repeated doses (□; 15 mice). There were 67 mice as untreated controls (*).

Table 3. Detection of dualtropic viruses in untreated and neonatally virus-injected, aged SJL/J(v⁺) mice

Viruses injected	No. virus-positive mice/ no. mice tested*
None	19/22
SJL-151	0/6
SJL-eco + SJL-151	5/6

*Ten- to 14-month-old mice were hemisplenectomized and spleen cells were cocultivated with SC-1 or ML indicator cells. Virus was detected as indicated in Table 1.

resents a necessary event for SJL/J lymphoma development is still an open question. We therefore investigated if dualtropic virus expression could be detected in spleen cells of aged SJL-151 virus-injected mice, which had been spared lymphoma appearance. As reported in Table 3, spleen cells from 10- to 14-month-old mice injected with SJL-151 together with SJL-eco expressed viruses that infect and replicate in ML cells as did spleen cells from untreated controls. On the other hand, no dualtropic virus was detected in spleen cells from mice injected with SJL-151 virus alone. These data imply that prevention of dualtropic virus replication, and possibly *de novo* generation, is related to the striking decrease in SJL/J lymphoma appearance.

Virus-Specific Antibody Response in Mice Injected with SJL-eco Virus, SJL-151 Virus, or Both. In an attempt to clarify the mechanisms underlying this apparent protection, the sera of neonatally virus injected SJL/J(v⁺) mice were collected 6 to 8 weeks after injection and individually assayed for the presence of specific antibodies against SJL-eco and SJL-151 viruses. Because similar results were obtained in mice given single or repeated virus doses, these data have been pooled.

Sera from mice belonging to the virus-injected groups as well as sera from untreated controls showed no differences in anti-SJL-eco activity (Fig. 2A). This last finding may be related to endogenous ecotropic virus activation, which commonly takes place in SJL/J(v⁺) mice after the third week of life (17). A remarkable antibody response against SJL-151 was detected in sera of mice injected with this virus alone, as well as in mice injected with SJL-151 together with SJL-eco (Fig. 2B). It is worth recalling that the latter mice, in contrast with the former, subsequently developed lymphomas with the same incidence as untreated controls. On the other hand, no significant response was detected in untreated or SJL-eco-injected SJL/J(v⁺) mice.

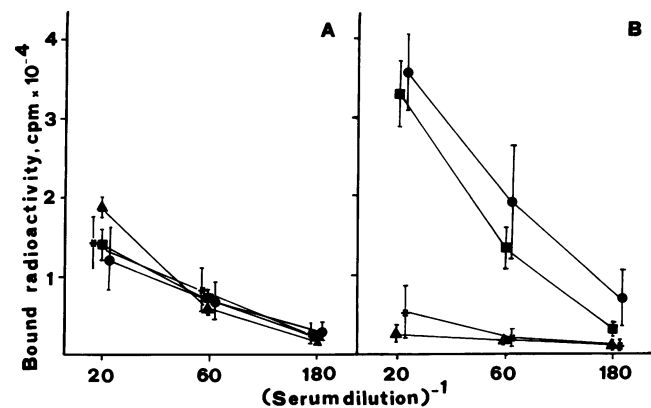


FIG. 2. Binding activity of sera obtained from 6- to 8-week-old SJL/J(v⁺) mice against SJL-eco virus (A) and SJL-151 dualtropic virus (B), both adsorbed on a solid surface. Mice were neonatally injected with SJL-eco (▲), SJL-eco together with SJL-151 (■), or SJL-151 (●). *, Untreated mice. Standard error is represented by vertical bars (5-10 mice per group).

Finally, sera from 6- to 8-week-old virus-injected CBA/J mice of different groups showed a pattern of reactivity essentially similar to that of SJL/J(v⁺) mice, whereas control untreated animals were negative for antibody production against both viruses (data not shown).

On the whole, these results indicate that, although SJL-151 is quite immunogenic, the antibodies produced play no role in lymphoma prevention.

DISCUSSION

The dualtropic MCF viruses thus far isolated fall into two major classes: those capable of producing or accelerating leukemias and lymphomas and those devoid of such capacity. Our results indicate that dualtropic SJL-151 virus belongs to the second, nononcogenic, class because lymphoma induction or acceleration was not observed in CBA/J and SJL/J(v⁺) mice that had been neonatally injected with this agent. Even though different routes of inoculation (e.g., intravenous or intraperitoneal) and various eco/dualtropic virus dose ratios might favorably influence the tumorigenic response, the lack of oncogenicity observed does not seem to be due to a poor virus replication rate in the host cells or to virus inactivation by the oncornavirus-inactivating factor, which is present in the serum of normal adult mice of most strains (30), including SJL/J(v⁺) (unpublished results). In fact, when SJL-151 was injected together with SJL-eco virus, either in single or in repeated doses, it was easily recovered 4–8 weeks later from spleen cells. The coinfection procedure was adopted because it increases dualtropic virus infectivity, most probably through ecotropic pseudotype formation, which may also protect the masked dualtropic virus from oncornavirus-inactivating factor (26, 31). It has also been shown that, whereas lymphomagenic dualtropic viruses are rendered far more oncogenic by genomic masking, the nonlymphomagenic dualtropic viruses do not acquire oncogenic potential after their conversion into ecotropic pseudotypes (26). Thus, the fact that mice dually infected with SJL-eco and SJL-151 viruses exhibited neither induction nor acceleration of lymphomas, despite easy dualtropic virus recovery, strongly argues against the lymphomagenic properties of SJL-151. Because specific differences between pathogenic and nonpathogenic dualtropic viruses have been detected in the *env* gene recombination sites (32, 33), it should be of interest to investigate whether SJL-151 also possesses a genomic structure specific for the nonpathogenic virus class.

A dramatic reduction in B-cell lymphoma incidence was observed in SJL/J(v⁺) mice injected with SJL-151 virus alone. However, this unexpected finding is in line with the report of Stockert *et al.* on inhibition of AKR leukemogenesis by a dualtropic virus (SMX-1) originally derived from Moloney MuLV stocks (34). Intrathymic injection of SMX-1 in young adult AKR mice not only prevented acceleration of leukemia produced by dualtropic leukemogenic AKR virus but also reduced spontaneous leukemia incidence when injected alone, as compared to AKR control mice. Leukemogenesis inhibition appeared specific for SMX-1 because other isolates, including a dualtropic one, failed to behave similarly. In this regard, it is interesting that in our study another cloned dualtropic virus, SJL-149 (11, 16), exerted the same protection against lymphoma development as SJL-151 when injected into newborn SJL/J(v⁺) mice (unpublished results). Moreover, because the present study was originally designed to ascertain the oncogenicity of SJL-151 virus, mice were treated with the virus only at a neonatal age—i.e., when endogenous ecotropic virus is not yet activated in SJL/J(v⁺) mice (17). It would perhaps be worthwhile to investigate whether SJL-151 injection of older SJL/J(v⁺) mice al-

ready positive for ecotropic virus production is also followed by a decrease in lymphoma incidence. Furthermore, it would be of interest to ascertain whether treatment with SJL-151 is equally efficient in conferring protection from spontaneous lymphoma development in conventional SJL/J mice (from which our colony was derived), which apparently show lower frequencies of endogenous ecotropic virus expression (17).

To study the mechanisms underlying lymphoma prevention, we tested mice for virus-specific antibody production. We found that, even though SJL-151 is quite immunogenic, there was no substantial difference in serum activity from SJL-151-injected SJL/J(v⁺) mice, which later showed a marked decrease in lymphoma incidence, compared to sera from dually virus-injected mice, which developed lymphomas at the usual high rate. Thus, while this finding does not preclude the possibility that cell-mediated immune reactions are at work in determining SJL/J(v⁺) lymphoma prevention, it does indicate that antibody production is not relevant for the protective effect. The same conclusion was reached by Stockert *et al.* (34), who failed to detect specific antibodies in AKR mice injected with SMX-1. These workers hold that the protective action of SMX-1 involves some aspect of viral interference that could harness the oncogenic potential of a leukemogenic dualtropic virus at any one of several steps from formation to infectivity.

At variance with Stockert *et al.* (34), however, we were unable to detect dualtropic virus in the spleen cells of mice injected with SJL-151 alone. More interesting, in contrast with the easy detection of dualtropic viruses from target tissues of 10- to 14-month-old untreated SJL/J(v⁺), no such agents were isolated from age-matched mice injected with SJL-151 alone, thus suggesting that *de novo* generation or release of dualtropic virus is inhibited. How these findings may be interpreted in terms of interference is a matter of speculation. It has been suggested that the expression of MCF-virus-related envelope glycoproteins in the absence of complete virus production may interfere with dualtropic virus replication or spread (35, 36). Determining whether a similar glycoprotein is also expressed on the target cell surface after injection of SJL-151 in SJL/J(v⁺) mice is an attractive possibility for further experimental exploration.

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