
Original article

Long-Term Observation of Natural Killer and Lymphokine-Activated Killer Activities in a Patient Who Survived from Serious Bladder Cancer

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Abstract

Here, we describe natural killer (NK) and lymphokine-activated killer (LAK) activities in a patient who recovered from bladder cancer. The patient is a 62-year-old male who was diagnosed having the cancer (transitional cell carcinoma) in 1980 (age, 40 years). He underwent several chemotherapies and operations. The last operation was in 1985. Since 1986, neither relapse nor metastasis has occurred. The patient is now (2003) in a tumor-free state. We first determined his NK and LAK activities in 1992 and found very high killer activities. His lymphocytes contained high percentages of CD16⁺ cells and CD8⁺CD16⁺ cells. Therefore, the high killer activities were thought to be due to the high percentages of them. During the past 10 years, we repeatedly determined the NK and LAK activities and obtained almost the same results every time. The CD16⁺ cells and CD8⁺CD16⁺ cells also maintained nearly the same percentages until now. These findings suggest that the high killer activity of his lymphocytes may be in relation with the reasons he survived. We have investigated his medical conditions for these ten years, founding no hematological abnormalities such as leukemia or monoclonal proliferation of NK cells.

Introduction

Natural killer (NK) cells can kill NK-sensitive tumor cells, and it is thought that NK cells may play an important role in tumor suppression *in vivo*¹⁻³⁾. Lymphokine-activated killer (LAK) cells, which are generated from peripheral blood lymphocytes (PBLs) by culturing with interleukin-2 (IL-2), can kill not only NK-sensitive tumor cells but also NK-non-sensitive tumor cells^{4, 5)}. NK and LAK activities indicate the protective condition of cancer patients. LAK cells are used for adoptive immuno-therapy by injecting them

into cancer patients^{6, 7)}.

Well, we have been observing a patient who recovered from bladder cancer. The patient was diagnosed having the cancer in 1980 (age, 40 years), and underwent several chemotherapies and operations. Since 1986, no relapse or metastasis occurred. This recovery was curious, because the cancer was very serious (transitional cell carcinoma). In 1992, we first determined the NK and LAK activities in this patient, and found very high killer activities⁸⁾. In addition, we noticed that his PBLs contained high percentages of CD16⁺ cells and CD8⁺CD16⁺ cells. CD16 is a marker of NK cells, and CD8 is a marker of suppressor/killer cells. For the past 10 years, we have repeatedly determined his NK and LAK activities. In the present paper, we describe the results of the long-term observations, and discuss the relationship between these findings and his survival from the cancer.

Materials and Methods

Case:

The patient (S.T.) was diagnosed with bladder cancer in 1980 (age, 40 years), and transurethral resection of the bladder tumor (TUR-BT) was performed. Transitional cell carcinoma (G2-3) was confirmed. The TUR-BT was repeated several times for 1 year. However, bleeding from the bladder mucosa continued and the tumor soon reappeared. Because he refused the total removal of his bladder, the adjuvant chemotherapies, including the injection of Mitomycin C into the bladder and the intra-arterial administration of Doxorubicin and Cisplatin, were repeated until 1985. By that time, the capacity of his bladder had been reduced and an infiltration of cancer into the surrounding areas was found. Consequently, after radiation therapy (total dose, 4000 cGy), the patient underwent total bladder removal and construction of an ileal conduit. Administration of Doxorubicin and Cisplatin was performed through

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the following year. Since 1986, neither relapse nor metastasis of the cancer has occurred. The patient is now (2003) in a tumor-free state.

Isolation of Peripheral Blood Lymphocytes and Generation of LAK Cells:

PBLs were isolated from heparinized peripheral blood of the patient by Ficoll-Hyperque gradient centrifugation. Then, adherent cells were removed by adherence to a plastic culture flask for 1 hr. The PBLs were used for determination of NK activity. For generation of LAK cells, the PBLs were suspended in a culture medium RPMI 1640 + 10% fetal calf serum (FCS) containing 400 IU/ml recombinant IL-2 (Shionogi Pharmaceutical Co. Ltd., Osaka, Japan), and were then incubated for 1 week at 37°C in a 5% CO₂ atmosphere. As a control, PBLs obtained from 8 healthy volunteers were used.

Determination of Cell Surface Markers:

CD4, CD8 and CD16 cell surface markers of the PBLs were determined by a flowcytometry method. Phycoerythrin-conjugated and fluorescein isothiocyanate-conjugated anti-CD4, anti-CD8 and anti-CD16 antibodies (DAKO A/S Produkt, Glostrup, Denmark) were used. The PBLs were incubated with these antibodies for 30 min at 4°C and washed once with phosphate-buffered saline (PBS). After that, the percentages of immunofluorescence-stained cells were assayed using the Ortho Cyturon flowcytometer (Ortho Diagnostic Systems, Raritan, NJ, USA).

Determination of NK and LAK Activities:

NK and LAK activities were determined by a 4h-⁵¹Cr-release assay. As target cells, the commercially available myeloblastoid cell line K562, the lymphoblastoid cell line Raji and the glioblastoma cell line T98G (American Type Culture Collection, Rockville, MD, USA) were used. These target cells were labeled with Na₂⁵¹CrO₄ for 1 hour and washed twice with PBS. Then, the target cells (T), alone or with the effector cells (E), were added into a microtiter plate (E: T ratio=5:1, 10:1 or 20:1) and incubated for 4 hours at 37°C. Next, radioactivity of supernatants was determined using a gamma counter. Maximum CPM was produced by target cells incubated with 1% Nonidet P40. Spontaneous CPM was produced after incubation of target cells in medium alone. Experimental CPM was produced by the mixtures of target cells and effector cells. The ⁵¹Cr-release rate (killer activity) was determined using the following calculation: [(Experimental CPM - Spontaneous CPM) / (Maximum CPM - Spontaneous CPM)] × 100 (%). Each determination was performed in triplicate.

Determination of Specific Killing against Auto-Tumor Cell Line H41:

Because an auto-tumor cell line H41 of this patient was established at the first operation and stored in liquid Nitrogen, we determined the specific killing of the patient's PBLs against the H41 by the 4h-⁵¹Cr-release assay as described above. The establishment was performed as follows; briefly, a tumor tissue specimen was minced and treated with collagenase and DNase. The tumor cell-enriched fraction was then separated with steel mesh and cultured in RPMI 1640 + 20% FCS at 37°C in a 5% CO₂ atmosphere. After 2 months, continuously growing cell line was obtained. The shape of the cells was elliptic, being distinguishable from a fibroblast. After the establishment as a cell line, reculturing was repeated every 3 days in RPMI 1640 + 10% FCS for another month and they were then frozen in liquid nitrogen.

Separation of CD4⁺, CD8⁺, CD16⁺ and CD8⁺CD16⁺ Cells:

Because the patient's PBLs contained high percentages of CD16⁺ cells and CD8⁺CD16⁺ cells, his PBLs were separated into CD4⁺, CD8⁺, CD16⁺ and CD8⁺CD16⁺ subsets, and LAK cells from each subset were generated. Then, the killer activities against H41 and T98G were determined. CD4⁺ and CD8⁺ subsets were separated using the Micro CELLector capture flasks (Applied Immune Sciences, Inc., Menlo Park, CA, USA). CD16⁺ subset was isolated using commercially available antibody-coated beads (Dynal AS, Oslo, Norway). CD8⁺CD16⁺ subset was obtained by removing CD4⁺ cells and CD16⁺ cells. The purity of separated subsets was determined by the flowcytometry method. Both CD4⁺ cells and CD8⁺ cells were separated at a 93% of purity. The purity of CD16⁺ cells was 96%, CD8⁺CD16⁺ cells 94%.

The results were shown as mean (M) ± SD, and statistical difference was calculated using Student's t-test; $p < 0.05$ indicating significance. The significant difference between patient's PBLs and control's PBLs was also evaluated when the values in the patient were 3 SD greater than the mean average in the control.

Results

1. Percentages of CD16⁺ and CD8⁺CD16⁺ Cells in the Patient's PBLs

Cytograms of PBLs from the patient (in 1992) and one of the volunteers (control) on the flowcytometer are shown in Fig. 1. The patient showed high percentage of CD16⁺ cells, accounting for 52.3% (control, 13.5 ± 1.2%; $n=8$). Half of the CD16⁺ cells overlapped with CD8; CD8⁺CD16⁺ cells accounted for 26.7% (control, 4.3 ± 0.3%). The CD16⁺ cells did not overlap with CD4 (data not shown). The percentages of CD16⁺ cells

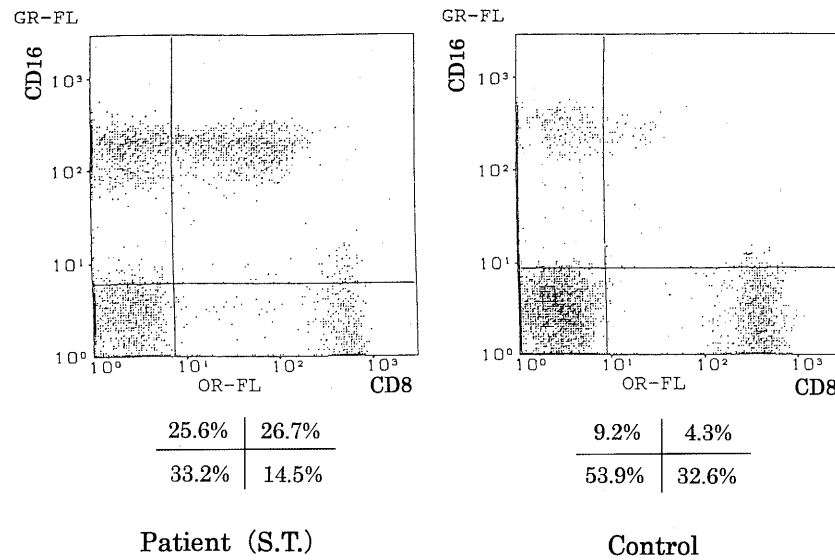


Fig. 1. Cytograms of CD8/CD16 for the PBLs of the patient (S.T.) and one of the volunteers (control) on the flowcytometer. The surface markers were determined using the Ortho Cytoron. The patient showed high percentages of CD16⁺ cells and CD8⁺CD16⁺ cells.

and CD8⁺CD16⁺ cells in the patient were significantly higher than the control, because the results in the patient were 3 SD greater than the mean averages of the control.

2. NK and LAK Activities

NK activity of the patient's PBLs was determined by the 4h-⁵¹Cr-release assay (⁵¹Cr-release rate). As shown in Table 1, the control's PBLs killed the NK-

Table 1. NK Activity of the Patient's PBLs

E:T	Patient (S.T.)			Contol (n=8)		
	K562	Raji	T98G	K562	Raji	T98G
5:1	22.5	8.3	6.6	7.5±0.8	1.1±0.1	0.7±0.0%
10:1	76.4	23.6	21.2	45.3±3.4	1.7±0.1	1.5±0.1%
20:1	87.2	30.5	28.7	67.5±5.6	2.8±0.2	2.3±0.2%

n=8, M±SD

Killer activity was determined by a 4h-⁵¹Cr-release assay. The patient's PBLs generally demonstrated higher killer activities than the control's PBLs.

Table 2. LAK Activity of the Patient's PBLs

E:T	Patient (S.T.)			Contol (n=8)		
	K562	Raji	T98G	K562	Raji	T98G
5:1	23.8	18.3	15.5	13.6±1.1	11.4±1.0	8.5±0.7%
10:1	84.6	73.7	70.8	56.7±4.7	53.5±4.5	51.6±4.3%
20:1	91.5	88.4	86.7	71.3±5.6	70.5±5.8	68.4±5.2%

n=8, M±SD

LAK cells were generated from the PBLs by incubating them in a culture medium containing 400 IU/ml recombinant IL-2. The patient's LAK cells showed higher killer activities than the control's LAK cells.

sensitive cell line K562 but scarcely killed the NK-non-sensitive cell line Raji or T98G. Meanwhile, the patient's PBLs killed not only K562 but also Raji and T98G. The killer activity was determined using E:T ratios of 5:1, 10:1 and 20:1, and all results in the patient demonstrated significantly higher levels than those in the control.

Killer activity of LAK cells was also determined. As shown in Table 2, the LAK cells in the patient and in the control killed both NK-sensitive and NK-non-sensitive cell lines. The patient's LAK cells exhibited higher killer activities than did the control's LAK cells.

3. Long-Term Determinations

Because the patient's PBLs contained high percentages of CD16⁺ cells and CD8⁺CD16⁺ cells in 1992, the determination was repeatedly performed during the past ten years. Nearly the same results were obtained every time, as shown in Table 3.

We also determined the NK and LAK activities of the patient, and obtained almost the same results as shown in Table 4. In this Table, the killer activities

Table 3. Long-Term Determinations of the Patient's PBLs during the Past Ten Years

	1992	1993	1994	1995	1997	1999	2001	2003
WBC	6,880	7,870	6,500	6,100	7,400	6,700	6,200	7,800/ μ l
Lymph	30	28	35	36	32	38	34	33%
CD16 ⁺ cells	52.3	53.3	52.8	52	51.1	51.5	48.3	47.5%
CD8 ⁺ CD16 ⁺ cells	26.7	28.5	27.3	26.2	25.8	25.5	25.1	24.6%

The patient exhibited high percentages of CD16⁺ cells and CD8⁺CD16⁺ cells.

The high percentages have maintained for these ten years.

Table 4. Long-Term Determinations of the Patient's NK and LAK Activities during the Past Ten Years

	1992	1993	1994	1995	1997	1999	2001	2003
NK activity								
against K562	76.4	78.3	77.5	76.1	75.3	73.8	72.4	69.6%
against Raji	23.6	27.4	25.5	24.4	22.7	20.4	21.6	20.3%
against T98G	21.2	25.1	24.8	23.8	20.6	19.3	20.4	18.2%
against H41	38.3	41.5	39.6	38.9	38.4	37.2	37.9	37.6%
LAK activity								
against K562	84.6	86.5	85.4	83.7	83.4	82.5	81.7	80.7%
against Raji	73.7	75.2	76.1	74.2	72.8	73.5	70.6	69.1%
against T98G	70.8	75.3	74.9	74.6	70.1	72.4	68.8	67.9%
against H41	87.3	91.5	88.4	87.7	86.6	87.3	87.5	86.4%

E : T = 10 : 1

The patient has shown high levels in the NK and LAK activities at every determination for these ten years.

Table 5. Laboratory Tests of the Patient in 2003

RBC	456×10 ⁴ /μl	BUN	16.6 mg/dl
Hb	14.5 g/dl	Cr	0.8 mg/dl
WBC	7800/μl	UA	4.2 mg/dl
Hemogram		AST	29 IU/l
Gran	60.5 %	ALT	16 IU/l
Mo	5.3 %	T. Bil	0.6 mg/dl
Lymph	33.2 %	Glu	98 mg/dl
Eo	1.0 %	CRP	0.1 mg/dl
Baso	0 %	IgG	1250 mg/dl
Plt	25.5×10 ⁴ /μl	IgA	240 mg/dl
TP	7.8 g/dl	IgM	110 mg/dl
Alb	4.3 g/dl	Bone marrow	normal

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No hematological abnormalities such as leukemia or monoclonal proliferation of NK cells were recognized.

against H41 were also shown. The H41 is an auto-tumor cell line of this patient. The patient's NK and LAK cells could kill the auto-tumor cell line markedly.

Because the percentages of CD16⁺ and CD8⁺CD16⁺ cells in this patient were conspicuously high compared to the control, we investigated his medical conditions for a long-term. Recent laboratory tests were shown in Table 5. We detected no hematological abnormalities such as leukemia or monoclonal proliferation of NK cells. No leucocytosis was also observed. As described above, he is now in tumor-free state. Therefore, it was thought that the high percentages of them were not due to hematological disorders.

4. Specific Killer Activity against Auto-Tumor Cell Line H41

Specific killer activity of the patient's PBLs against auto-tumor cell line H41 was investigated. The results were shown in Fig. 2. For the patient's killer activities in this Figure, mean averages of them during these ten years were exhibited. The patient's PBLs showed higher killer activities than the control's PBLs, and they

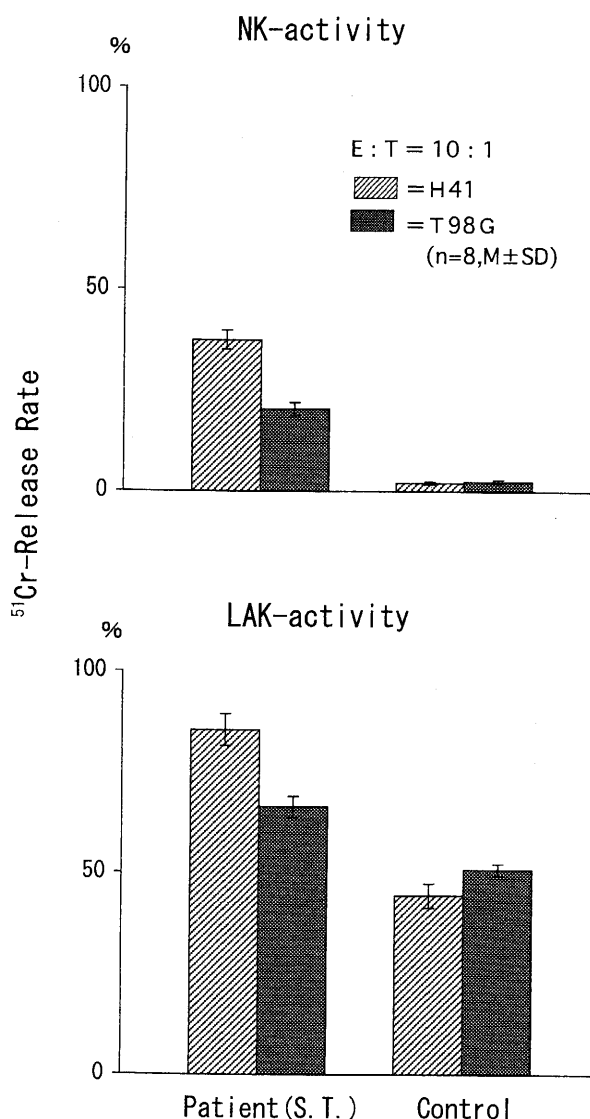


Fig. 2. NK and LAK activities against the auto-tumor cell line H41 and allogeneic tumor cell line T98G. H41 was established from his cancer tissue at the first operation. The patient's PBLs generally showed higher killer activities than the control's PBLs, in particular against H41.

Table 6. Killer Activities of Separated and Non-Separated LAK Cells

	Patient (S.T.)		Control	
	H41	T98G	H41	T98G
Non-Separated LAK	86.5	67.3	45.3±3.7	52.2±4.7%
CD4 ⁺ LAK	4.7	3.5	0	0 %
CD8 ⁺ LAK	66.4	35.7	30.2±2.5	34.8±3.3%
CD16 ⁺ LAK	89.4	74.7	nt	nt
CD8 ⁺ CD16 ⁺ LAK	97.3	75.2	nt	nt

E : T = 10 : 1, n = 8, M ± SD, nt = not tested (because the subsets could not be separated sufficiently)

The separation was performed at higher percentages of purity than 93%. CD16⁺ and CD8⁺CD16⁺ subsets in the patient exhibited higher killer activities against H41 than against T98G. In particular, CD8⁺CD16⁺ subset demonstrated the highest killer activity against H41.

demonstrated higher killer activity against H41 than against T98G.

Because the patient's PBLs contained high percentages of CD16⁺ cells and CD8⁺CD16⁺ cells, his PBLs were separated into CD4⁺, CD8⁺, CD16⁺ and CD8⁺CD16⁺ subsets, and LAK cells from each subset were generated. Then, the killer activities against H41 and T98G were compared. As shown in Table 6, the patient's LAK cells generally showed higher killer activities than the control's LAK cells. CD16⁺ and CD8⁺CD16⁺ subsets of the patient exhibited higher killer activities against H41 than against T98G. In particular, CD8⁺CD16⁺ subset demonstrated the highest killer activity against H41.

Discussion

In the present report, we described a patient who recovered from bladder cancer. The recovery was very curious because the cancer (transitional cell carcinoma) is very serious, of which 5-year survival rate is estimated to be approximately 17 to 20%^{9, 10)}. The patient underwent several therapies including radiation and operations. At the last operation (1985), total bladder removal and construction of an ileal conduit were performed. Since 1986, neither relapse nor metastasis has been observed. He is now in a tumor-free state.

In 1992, we first determined his NK and LAK activities, and found high killer activities. An unique finding in this patient was that his PBLs contained high percentages of CD16⁺ cells and CD8⁺CD16⁺ cells. We revealed that the high killer activities were carried by these CD16⁺ cells and CD8⁺CD16⁺ cells. During the past ten years, we have repeatedly determined the percentages of these subsets, and have obtained almost the same percentages. We have also investigated the killer activities of this patient, and recognized nearly the same activities every time. Therefore, the recovery of this patient from the cancer was thought to be in relation with the high killer activity. The relationship between the NK activity and tumor-free state has already been known^{11, 12)}. Unfortunately, we are unable to describe the time when the percentages of CD16⁺ cells and CD8⁺CD16⁺ cells increased, because we did not determine them in the early period of his disease course.

This case brought us several informations as follows:

1. The patient underwent chemotherapies using Doxorubicin and Cisplatin after radiation and operation. These radiation, operation and chemotherapies might have a role for reduction of bladder tumor in a part. However, it is usually uncommon that the tumor which infiltrated into surrounding area is completely suppressed by these therapies. In this patient, Doxorubicin and Cisplatin were already used before the last operation, but the infiltration occurred. Thus, it is impossible to explain the disappearing of the tumor by these therapies alone.
2. The mechanism by which the CD16⁺ and CD8⁺CD16⁺ cells increased is unclear. We have investigated the clinical conditions in this patient for these ten years. But we have found no specific abnormalities. He is now in a healthy state, although he carries a urine bag. Thus, it is not likely that the high percentages of CD16⁺ cells and CD8⁺CD16⁺ cells may be due to hematological disorder such as leukemia or monoclonal proliferation of NK cells. On the other hand, this patient suffered from intermittent high fever after the last operation. The etiology of the high fever was not confirmed, but several factors such as urinary tract infection, side effect of the chemotherapies, tissue injury by the therapies and common cold were speculated. For these ten years, we investigated whether NK cells after high fever increases or not. In general patients and healthy volunteers, we noticed that the NK cells after high fever (such as pyelonephritis) increased. Usually, the increasing continued for 2 to 4 months. In rare cases, it continued for 1 year. After then, it returned to the regular range. We will further investigate about the mechanism of high percentage of NK cells in this patient.
3. Generally, NK cells can kill NK-sensitive tumor cells, but not kill NK-non-sensitive cells¹³⁻¹⁵⁾. K562 is a representative NK-sensitive cell line. K562 does not possess major histocompatibility complex (MHC)-class I antigens on the cell surface. The NK-sensitivity of this cell line is thought to be due to the depression of MHC-class I antigens. However, the present patient's PBLs could kill not only NK-sensitive cell line K562 but also NK-non-sensitive cell lines Raji and T98G. Raji and T98G possess complete MHC-class I antigens^{16, 17)}. Thus, the killing of these cell lines cannot be explained by the depression of MHC-class I antigens. This finding suggests that a different pathway between the effector cells and target cells may participate to the killing.
4. Fortunately, we could use the auto-tumor cell line H41 for experiments, and could reveal the specific killing of this patient's PBLs against the H41. Some authors described that specific killing against auto-tumor cells may be carried by CD8⁺ subset^{18, 19)}. While, in this patient, we revealed that its killing was carried by the CD8⁺CD16⁺ subset preferably. It provided us with useful information that the patient possessed high percentage of this subset in his PBLs.
5. Adoptive immuno-therapy using LAK cells has been performed to treat cancer patients by injecting

the LAK cells into them^{6,7}. This therapy is thought to be theoretically useful. A lot of cases with complete remission or partial remission have already been reported. The present case may support the theoretical usefulness of this therapy. In this therapy, a large number of LAK cells are necessary. In particular, specific killer cells against auto-tumor cells are required^{4, 20}. The present case also indicates that CD8⁺CD16⁺ subset may be advantageous for this therapy.

In conclusion; it is thought that the recovery of this patient from bladder cancer may have a relationship with the high killer activity of his PBLs. The high killer activity was carried by high percentages of CD16⁺ cells and CD8⁺CD16⁺ cells, and furthermore, the patient's PBLs, in particular CD8⁺CD16⁺ cells, killed auto-tumor cell line H41 specifically.

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