Generation of Leukemia-reactive Cytotoxic T Lymphocyte Clones from the HLA-identical Bone Marrow Donor of a Patient with Leukemia

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Summary

Allogeneic bone marrow transplantation (BMT) has been associated with a graft-vs.-leukemia (GVL) reactivity. Since T cell depletion of the bone marrow graft has decreased the risk of graft-vs.-host disease (GVHD), but has been associated with higher rates of leukemia relapse, GVL reactivity is probably caused by donor-derived T lymphocytes. Previously, we demonstrated that minor histocompatibility (mH) antigen-specific cytotoxic T lymphocyte (CTL) clones, generated from patients after BMT, are capable of major histocompatibility complex (MHC) restricted lysis of (clonogenic) myeloid leukemic cells. Here, we investigated whether donor-derived leukemia-specific CTL clones can be generated in vitro, before BMT, using irradiated leukemic cells from a patient with acute myeloid leukemia as stimulator cells, and peripheral blood or bone marrow from the HLA genotypically identical sibling donor as responder cells. Several CTL lines were generated that showed specific lysis (>50%) of the recipient leukemic cells in a 51Cr-release assay. Two of these CTL lines were cloned by limiting dilution in the presence of the irradiated recipient cells. Multiple leukemia-reactive, HLA class I and II-restricted clones with various specificities could be established. These alloreactive, antileukemic CTL clones may cause GVL reactivity after BMT, and may be used as adjuvant immunotherapy in the treatment of leukemia.

Allogeneic bone marrow transplantation (BMT) has been associated with a graft-vs.-leukemia (GVL) reactivity (1–4). This GVL reactivity is probably caused by donor-derived T lymphocytes from the graft since T lymphocyte depletion of the bone marrow graft is correlated with an increased risk of leukemic relapse after BMT, and an inverse correlation has been found between the occurrence of GVHD and relapse of leukemia in HLA-identical BMT (5–7). Alloantigen-specific CTLs may be responsible for the GVL reactivity, since this GVL effect has not been observed in transplants between homozygous twins.

Minor histocompatibility (mH) antigens appear to play a major role in the etiology of GVHD, graft rejection, and GVL reactivity after BMT between HLA genotypically identical siblings, and MHC-restricted mH antigen-specific CTL lines can be isolated after HLA-identical BMT from patients with GVHD or graft rejection (8–13). We recently demonstrated that mH antigen-specific CTL lines can be generated in vitro, without in vivo priming, by stimulation with HLA-identical bone marrow cells, and that mH antigen-specific CTL clones are capable of antigen-specific MHC–restricted lysis of (clonogenic) normal and leukemic precursor cells (14–16). Here, we have investigated whether it is possible to generate leukemia-specific CTL clones from the HLA genotypically identical sibling donor of a patient with acute myeloid leukemia (AML).

Materials and Methods

Generation of Leukemia-specific CTL Clones. After informed consent, bone marrow and peripheral blood from a patient with AML and from her HLA-identical sibling donor were obtained. The patient was a 29-yr-old female with AML, subtype M5 according to the FAB classification, and cytogenetic analysis revealed a translocation t(8;16)(p11;p13). Complete remission was achieved after treatment with Daunorubicin and arabinosyl-cytosine (ARA-C). After a consolidation course with high dose ARA-C and amsacrin (Amsa), allogeneic BMT with the bone marrow from her HLA genotypically identical sister was performed. HLA typing was performed by standard serological methods. The HLA typing of the family was as follows: father A2 B7 Cw7 DR15(2) DQ6/A2 B27 CW1 DR1 DQ5; mother A1 B8 CW7 DR3 DQ2/A24 B8 CW7 DR3 DQ2; sib 1 (patient) A2 B7 CW7 DR15(2) DQ6/A24 B8 CW7 DR3 DQ2; and sib 2 (donor) A2 B7 CW7 DR15(2) DQ6/1283

1 Abbreviations used in this paper: AML, acute myeloid leukemia; BMT, bone marrow transplantation; CML, chronic myeloid leukemia; GVL, graft-vs.-leukemia; LCL, lymphoblastoid cell line; mH, minor histocompatibility; MNC, mononuclear cells; TCGF, T cell growth factor.
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A24 B8 Cw7 DR3 DQ2. This shows that there is no homozygosity of a complete haplotype of the parents of the patient and her HLA identical sibling, which illustrates that the patient and her donor are not only phenotypically, but also genotypically HLA identical. Pretransplant primary MLR and cell-mediated lysis assays between donor and patient were negative.

2 x 10⁶ irradiated (30 Gy) leukemic bone marrow or peripheral blood mononuclear cells (MNC) >95% morphologically recognizable leukemia cells) from the patient were used as stimulator cells, and 10⁶ bone marrow or peripheral blood (MNC) from her HLA genotypically identical sibling donor, were used as responder cells. The cells were cultured in 50-ml flasks in medium consisting of RPMI plus 15% prescreened human AB serum. On day 6, 20% T cell growth factor (TCGF; Biotest, Offenbach, Germany) was added to the medium, and on day 9 the cells were restimulated with 10-fold of the irradiated leukemia cells from the recipient. Between days 16 and 20 the generated CTL lines were tested in a ⁵¹Cr-release assay. Two of the CTL lines that showed specific reactivity (specific lysis >50%) with the recipient leukemia cells were further cloned by limiting dilution (effector cell concentration 1:0.3 cell/well) in the presence of the irradiated recipient leukemia cells (30 Gy) and in the presence of 20% TCGF. The generated CTL clones were further expanded in the presence of the irradiated (30 Gy) leukemia cells from the recipient in the presence of irradiated (30 Gy) allogeneic peripheral blood MNC.

In the same way, CTL lines were generated after BMT using 10⁶ peripheral blood MNC from the recipient, collected 6 mo after BMT, as responder cells, and 2 x 10⁶ irradiated (30 Gy) recipient leukemia bone marrow cells as stimulator cells.

The phenotypes of the CTL clones were analyzed on a FACScan® (all from Becton Dickinson & Co., Mountain View, CA) using CD3, CD4, and CD8 mAbs.

**Target Cells.** Lymphocytes were expanded by stimulating 10⁶ peripheral blood MNC from the patient or donor or from various HLA genotypically identical sibling pairs with 0.2% PHA (Difco Laboratories, Detroit, MI) in RPMI plus 15% human AB serum for 3 d. The cells were then washed and further cultured in the absence of PHA in the presence of human recombinant IL-2 300 IU/ml, for at least three more days.

Stable EBV-transformed B cells (EBV-lymphoblastoid cell lines [LCLs]) were established by in vitro transformation of 10⁶ peripheral blood MNC with EBV supernatant for 1.5 h. The cells were washed and further cultured in RPMI plus 10% FCS (Gibco Laboratories, Grand Island, NY).

Samples of leukemic cells from patients with AML or chronic myeloid leukemia (CML) were obtained, after informed consent, from the peripheral blood or bone marrow of the patients before treatment. Patients with >95% morphologically recognizable leukemia cells in their peripheral blood or bone marrow were selected. The cells were centrifuged over Ficoll Isopaque (density 1.077 g/cm³, 1,000 g, 20 min) and the interphase cells were harvested. For cryopreservation, the cells were resuspended in medium consisting of 65% RPMI, 25% FCS, and 10% DMSO, frozen, and preserved in liquid nitrogen. Before use, the cells were thawed, washed twice, and resuspended in RPMI containing 15% prescreened AB serum.

**⁵¹Cr-release Assay.** Standard ⁵¹Cr-release assays were performed as described (17). Briefly, target cells consisting of lymphocyte populations, leukemic cells, or EBV-LCLs were labeled with 0.1 ml Na⁵¹CrO₄ (100 μCi) for 1 h at 37°C, washed three times, and resuspended in RPMI plus 15% AB serum at a concentration of 5 x 10⁴ viable cells/ml. 0.1 ml of the effector population and 0.1 ml of the target suspension were added to each well of a round-bottomed microtiter plate at an E/T ratio ranging from 5:1 to 1:1. Target and effector cells were incubated for 4 h at 37°C. To measure spontaneous release of ⁵¹Cr, 0.1 ml of the target suspension was added to 0.1 ml RPMI plus 15% AB serum without effector cells. Maximum release was determined by adding 0.1 ml of the target suspension to 0.1 ml zaponine solution.

The percent specific lysis obtained in a ⁵¹Cr-release assay was determined as follows: 100 x [(experimental release cpm - spontaneous release cpm)/(maximum release cpm - spontaneous release cpm)].

To determine CD4 and class II or CD8 and class I restriction of the recognition of the target cells, blocking studies were performed. Effector cells were incubated with saturated concentrations of anti-CD4 or anti-CD8 mAbs (RIV6 and FK18, respectively, [18, 19]) or target cells with anti-class I or anti-class II mAbs (W6/32 and PdV5.2, respectively [20, 21]) at a final dilution of 1:100 for 30 min before the effector and target cells were cocultured.

To determine whether the antigen, recognized by one CTL clone and presented by different targets, was similar, a cold target inhibition assay was performed. Non-²⁵¹Cr-labeled (cold) target cells were added to a specific combination of effector cells and ²⁵¹Cr-labeled (hot) target cells (E/T ratio 5:1). The cold/hot target ratios used were 100:1, 30:1, 10:1, and 3:1.

The percent inhibition of lysis of hot targets by cold targets was measured as follows: 100 x [(% lysis of hot targets only - % lysis of hot targets with presence of cold targets)/(% lysis of hot targets only)].

**Results**

Several donor-derived CTL lines were generated, that showed specific lysis (specific lysis >50%) of the recipient leukemia cells in a ⁵¹Cr-release assay. Two of these CTL lines were cloned by limiting dilution in the presence of the irradiated leukemia cells from the recipient and 70 clones could be isolated, of which 13 showed specific reactivity (specific lysis >50%) with the leukemic cells from the recipient. Of these 13 clones, three types of clones could be recognized:

| Table 1. Specific Recognition by the Three Types of Donor-derived CTL Clones, Generated against the Leukemic Cells from the HLA Genotypically Identical Recipient |
|---------------------------------|-----------------|-----------------|
| **Specific lysis**               | **Target cells** | **I** (clone H) | **II** (clone Y) | **III** (clone S) |
| Lymphocyte recipients            | + +             | + +             | + +             |
| Lymphocyte donor                 | -               | -               | -               |
| AML recipient                    | + +             | + +             | + +             |
| EBV-LCL recipient                | + +             | -               | + +             |
| EBV-LCL donor                    | + +             | + +             | + +             |

Lysis of the target cells was measured using a standard ⁵¹Cr-release assay in duplicate at E/T ratio 5:1.

+ + specific lysis >50%.
- specific lysis <20%.
Spontaneous release cpm/maximum release cpm ratio <25%.
Figure 1. Blocking studies of clone H, an example of a type I clone, CD8 positive, that recognized the recipient lymphocytes, leukemic cells, and EBV-LCL. To determine class I restriction of the recognition of the target cells by clone H, blocking studies were performed. The specific lysis of the targets was measured using a standard 3HCr-release assay in duplicate at an E/T ratio of 5:1. The specific lysis of the recipient EBV-LCL was blocked with anti-CD8 and anti-class I mAbs. Spontaneous release cpm/maximum cpm ratio <25%.

Table 2. Specificity of Clone H, An Example of a Type I Clone, CD8 Positive, that Recognized the Recipient Lymphocytes, Leukemic Cells, and EBV-LCL

| HLA antigens shared with the donor | Specific lysis | No. of pairs | Sibling 1 | Sibling 2 |
|-----------------------------------|---------------|--------------|-----------|-----------|
| A2C7                              | + +           | 1            | -         | -         |
| A2B7B8C7                          | + +           | 1            | +         | +         |
| A2B8C7                            | + +           | 1            | -         | -         |
| A2B7C7                            | + +           | 1            | +         | +         |
| A2B7C7                            | + +           | 1            | +         | -         |
| A2B7C7                            | + +           | 2            | -         | -         |
| A2B7C7                            | + +           | 2            | -         | -         |
| A2                             | + +           | 3            | -         | -         |
| A2B7C7                            | + +           | 1            | +         | +         |
| A2B7C7                            | + +           | 1            | +         | -         |
| A2B7C7                            | + +           | 1            | +         | -         |
| A2B7C7                            | + +           | 2            | -         | -         |
| A2B7C7                            | + +           | 2            | -         | -         |
| None                              | -             | 2            | -         | -         |

Lysis of lymphocytes from unrelated HLA-identical sibling pairs sharing one or more HLA antigens with the recipient and donor, was measured using a standard 3HCr-release assay in duplicate at E/T ratio 5:1. HLA recipient/donor: A2 A2B7 B8 Cw7 DR3 DR15(2) DQ2 DQ6. + + specific lysis >50%. + specific lysis >20%. - specific lysis <20%. Spontaneous release cpm/maximum release cpm ratio <25%.

Table 3. Specificity of Clone Y, An Example of a Type II Clone, CD8 Positive, that Only Recognized the Recipient Leukemic Cells

| HLA antigens shared with the donor | No. of pairs | AML classification | Specific lysis |
|-----------------------------------|--------------|--------------------|---------------|
| A2                                | 1            | M5                 | + +           |
| A2                                | 5            | M2/M5              | -             |
| A24                               | 1            | M5                 | -             |
| M2A24                             | 1            | M5                 | -             |
| A2B7C7                            | 1            | M5                 | -             |
| A2B8C7                            | 1            | M5                 | -             |
| A24B8C7                           | 2            | M5                 | -             |
| B7C7                              | 1            | M3                 | + +           |
| B7C7                              | 2            | M5                 | -             |
| B8C7                              | 2            | M5                 | -             |
| B7B8                              | 2            | M5                 | -             |
| None                              | 2            | M1/M5              | -             |

Lysis of leukemic cells from 21 unrelated patients with AML, sharing one or more HLA antigens with the recipient and donor, was measured using a standard 3HCr-release assay in duplicate at E/T ratio 5:1. HLA recipient/donor: A2 A24 B7 B8 Cw7 DR3 DR15(2) DQ2 DQ6. + + specific lysis >50%. + specific lysis >20%. - specific lysis <20%. Spontaneous release cpm/maximum release cpm ratio <25%.

Figure 2. Specificity of clone Y, an example of a type II clone, CD8 positive, that only recognized the recipient leukemic cells. To determine the possibility of non-MHC-restricted lysis by clone Y the lysis of several NK or lymphokine-activated killer cell reactive targets was measured using a standard 3HCr-release assay in duplicate at an E/T ratio of 5:1. Clone Y exhibited no MHC nonspecific activity. Spontaneous release cpm/maximum release cpm ratio <25%.
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Recipient by clone H could be blocked with CD8 and anti-class I mAbs illustrating class I-restricted recognition (Fig. 1). Clone H was tested in a 51Cr-release assay against a panel of lymphocytes from unrelated HLA identical sibling pairs sharing one or more HLA antigens with the recipient and donor (Table 2). Only target cells expressing HLA-B7 antigens were lysed by clone H. Possible reactivity against a subtype of HLA-B7 could be excluded, since donor and recipient were genotypically identical, and the HLA-B7 locus was present only once in the parental haplotypes. Furthermore, not all target cells expressing the HLA-B7 antigens were lysed by clone H. Differential recognition of the lymphocytes of one of the unrelated genotypically identical sibling pairs expressing the HLA-B7 antigen illustrated that clone H was directed against a mH antigen, and that the recognition was HLA-B7 restricted.

Clone Y, an example of the type II clone, CD8 positive, which only recognized the recipient leukemic cells, was tested in a 51Cr-release assay against a panel of leukemic cells from 21 patients with AML; Fab classification M2, M3, or M5 (Table 3). Two AML targets sharing no HLA antigens with the recipient and donor were not lysed by clone Y. Of six targets sharing HLA-A2 with the recipient and donor, one was recognized by clone Y. Of 13 other targets sharing certain HLA antigens with the recipient and the donor in different combinations, one AML target sharing HLA-B7C7 with the recipient and donor was lysed by this CTL clone. Clone Y exhibited no MHC nonspecific activity as demonstrated by the lack of reactivity with K562, Mol t 4, Daudi, and HL60 cells in a 51Cr-release assay (Fig. 2). In contrast, the specific lysis of these targets could be inhibited with anti-CD8 and anti-class I mAbs (Fig. 3A). No blocking was observed with anti-CD4 and anti-class II mAbs.

Figure 3. Blocking studies of clone Y, an example of a type II clone, CD8 positive, that only recognized the recipient leukemic cells. To determine class I restriction of the recognition of the target cells by clone Y, blocking studies were performed. The specific lysis of all three leukemic targets, recognized by clone Y, was blocked with anti-CD8 and anti-class I mAbs in a standard 51Cr-release assay (4). To determine whether the antigen, recognized by clone Y and presented by three different targets, was similar, a cold target inhibition assay was performed. Non-51Cr-labeled (cold) target cells were added to a specific combination of effector cells and 51Cr-labeled (hot) target cells (E/T ratio 5:1). The cold targets were the leukemic cells of the recipient (O), the two AML samples recognized by clone Y (sharing HLA-B7 (+) or HLA-A2 (+) with the recipient) and an AML sample not recognized by clone Y (sharing the HLA-A24 (□) with the recipient). As hot targets, the three leukemic samples, recognized by clone Y, were also used: the leukemic cells (B), the AML sample sharing the HLA-B7 with the recipient (C), and the AML sample sharing the HLA-A2 with the recipient (D). The cold/hot target ratios used were 100:1, 30:1, 10:1, and 3:1. When the three targets, recognized by clone Y, were used as cold targets, the specific lysis of the hot target, the recipient leukemic cells, could be inhibited by two of the cold targets, the cold target AML-A2 (□) was not clearly inhibitory (B). When the AML-B7 or the AML-A2 was used as hot target, all three specific cold targets could inhibit the specific lysis of these two hot targets (C, D). In all three assays, the cold target AML-A24 (□) could not significantly inhibit the specific lysis of the hot targets (B–D). Spontaneous release cpm/maximum release cpm <25%.
When the targets, recognized by clone Y, were used as competitors in a cold target inhibition assay, the specific lysis of the hot target, the recipient leukemic cells, could be inhibited by both cold targets, the recipient leukemic cells (O) and the AML-B7 (+) (Fig. 3 B). The cold target AML-A2 (□) was not clearly inhibitory (Fig. 3 B). The specific recognition of the AML-A2 leukemic sample was lower than that of the AML-B7 leukemic sample (Fig. 3 A). When the AML-B7 target was used as hot target, the cold targets, the recipient leukemic cells, the AML-B7, and the AML-A2 could strongly inhibit the specific lysis (Fig. 3 C). The specific lysis of hot target AML-A2 could also be inhibited by all three specific cold targets, the recipient leukemic cells, the AML-B7, and the AML-A2 (Fig. 3 D). In all three assays, the cold target AML-A24 (♣), the target that was not recognized by clone Y, could not significantly inhibit the specific lysis of the hot targets (Fig. 3, B, C, and D).

Clone S, an example of the type III clone, CD4 positive, showed specific lysis of the recipient leukemic cells and EBV-LCL from both recipient and donor (Table 1). The lysis by clone S of the recipient leukemic cells and EBV-LCL could be blocked with anti-CD4 and anti-class II mAbs, illustrates the class II-restricted lysis (Fig. 4). No blocking was observed after incubating of effector or target cells with anti-CD8 and anti-class I mAbs. To analyze the possible restriction element, clone S was tested against a panel of EBV-LCLs, homozygous for the various HLA-class II antigens. The specific lysis by clone S was shown to be HLA-DR2-restricted, and not restricted by HLA-DP or HLA-DQ (Fig. 5). Clone S also recognized other EBV-LCLs, heterozygous for the HLA-DR2 antigen (data not shown). The antigen recognized by clone S was not specific for an EBV-associated antigen because the recipient leukemic cells were shown not to be infected by EBV as measured by PCR or in situ hybridization (22). Furthermore, DOHH2, an immunoblastic lymphoma cell line, EBV and HLA-DR2 positive, was not lysed by clone S. The antigen was further recognized on one other AML sample, expressing the HLA-DR2 restricting element. Two CML samples, HLA-DR2 positive, were not recognized by clone S (Fig. 5).

The patient described was transplanted with the bone marrow from her HLA genotypically identical sister. She developed clinical stage I acute GVHD of the skin 30 d after BMT, which disappeared after treatment with prednisone. She is now 24 mo after BMT and in good health. After BMT of this patient with her HLA identical donor sibling, leukemia-reactive CTL lines could also be generated from the recipient, using the peripheral blood MNC from the recipient, collected 6 mo after BMT, as responder cells, and the irradiated original leukemic bone marrow from the recipient as stimulator cells (Fig. 6). It was not possible to expand these generated CTL lines for further analysis of their specificity.

Discussion

Alloreactive, antileukemic CTL clones could be generated in vitro from the HLA genotypically identical sibling donor of a patient with AML, using the irradiated leukemic cells from the recipient as stimulator cells and the bone marrow or peripheral blood MNC from the donor, collected before BMT, as responder cells. Of the 13 cytotoxic clones, which could be generated in vitro, three types of clones could be recognized. The specific recognition of the target cells by clone H, an example of a CD8 positive recipient-specific clone, appeared to be restricted by HLA-B7 expression. Differential recognition of lymphocytes from HLA genotypically identical pairs revealed that this CTL clone exhibited a HLA-B7-restricted mH antigen-specific recognition. Previously, it has been described that antihost mH antigen-specific reactivity can be found after HLA-identical BMT not only in patients with GVHD, but also in patients without clinical signs of GVHD, or after resolution of acute GVHD (23).

Clone Y, an example of a CD8 leukemia-specific clone,
only showed reactivity with leukemic samples from two other patients with AML. Because these two samples shared different HLA antigens with the recipient and donor, the possibility of non-MHC-restricted lysis by clone Y was analyzed using several NK or lymphokine-activated killer cell reactive targets. Since clone Y showed no reactivity with K562, Molt 4, Daudi, and HL-60 cells in a 51Cr-release assay, nonspecific lysis due to NK activity was excluded. Furthermore, blocking studies showed that the specific lysis by clone Y of the recipient leukemic cells, but also of the other two AML samples, could be inhibited with anti-CD8 and anti-class I mAbs, illustrating HLA-class I-restricted recognition. Cold target inhibition assays indicated that the antigen recognized by clone Y was similar on all the three leukemic examples. These results suggest that different MHC determinants may similarly present the same antigen. Previously, it has been described that one T lymphocyte clone can recognize an antigen presented by different restricting HLA-class I antigens (24). Our results indicate that clone Y may recognize an antigen that is not the specific product of the described t(8;16) translocation, since the two other AML samples did not exhibit this specific translocation.

Clone S, an example of a CD4-positive clone, recognized EBV-LCLs expressing the HLA-DR2 antigen, which suggests that the antigen recognized by clone S was EBV associated. However, using the PCR with EBV-specific primers and in situ hybridization, EBV infection of the leukemic cells of the recipient could not be demonstrated. Furthermore, a human immunoblastic B cell line (DOHH2), EBV and HLA-DR2 positive, was not recognized by clone S. The antigen recognized by clone S and restricted by HLA-DR2 expression, was also presented by one other AML (M5) sample, but not by two HLA-DR2 positive chronic myeloid leukemic samples. To exclude the possibility of nonspecific recognition of a protein from the human serum or culture medium used, the assays were repeated varying the culture medium (IMDM instead of RPMI), using culture medium with and without antibiotics, or with BSA instead of human serum (data not shown). The results were similar, showing that clone S revealed specific lysis of recipient leukemic cells and of the EBV-LCL of both recipient and donor. Thus, clone S showed HLA-DR2 restricted reactivity with leukemic cells from at least two patients with AML (M5) and EBV-LCLs. Therefore, the antigen recognized appeared not to be an EBV antigen.

Not only before, but also after BMT, CTL lines could be generated, using the recipient peripheral blood MNC, collected after BMT, as responder cells. These generated CTL lines also showed the specific reactivity with the recipient leukemic cells and not with the IL-2-stimulated T lymphocytes from the recipient. Therefore, these T cells may have exhibited antileukemic reactivity after BMT, and have attributed to the persistent remission.

In conclusion, we demonstrated that various MHC class I and II-restricted leukemia-specific CTL clones can be generated in vitro from an HLA genotypically identical sibling of a patient with AML. The donor-derived CTL clones with reactivity with the recipient leukemic cells may explain GVL reactivity after BMT. In the treatment of leukemia, donor-derived CTL clones that recognize the leukemic target cells from the recipient and not the normal host cells of the tissues that are involved in GVHD, may be used as adjuvant immunotherapeutic agents.

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