Due to their low chemosensitivity and radiosensitivity, many malignant metastasized endocrine carcinomas are refractory to conventional therapy; developing new approaches toward these cancer types is of particular importance (1). Immunotherapy in the form of dendritic cell vaccines or adoptive T-cell therapy may extend the range of current treatment options. However, applying these therapies effectively and monitoring induced immune response accurately requires specific tumor antigens to be identified. Several tumor-associated antigens recognized by T lymphocytes, such as MAGE1-MAGE3, GAGE1-GAGE6, etc., have already been described in these tumor types (2–4). Various studies have shown that immunity toward these tumor-associated antigens may even spontaneously develop in both nonendocrine (5) and endocrine (6) cancer patients. The functionality of these usually low-avidity T cells and their influence on the clinical course of the disease has, however, been questioned (7). The tumor area is a hostile and suppressive milieu for cytotoxic T lymphocytes (CTL), and tumor-associated antigen–directed T cells often show an anergic phenotype (5). In addition, regulatory T cells and inhibitory cytokines present in the tumor area are important factors in down-regulating any possible tumor-associated antigen–directed immune response (8). Specific vaccinations toward highly expressed tumor cell-specific antigens with high-expression profiles on tumor cells may help overcome this devastating situation.

Because most neuroendocrine malignancies secrete large amounts of polypeptide hormones, they may represent specific target molecules (9). One example is insulin. This polypeptide secreted by pancreatic β-cells has already been shown to play a prime role in inducing antigen-specific CTL in nonmalignant neuroendocrine disease with cytotoxic autoimmune character, namely type 1 diabetes mellitus (10–13). In contrast, when coupled to antigen-presenting cells, insulin has been shown to inhibit pathogenic T cells, leading to disease remission (14).

Abstract

Purpose: No relevant breakthrough has yet been achieved in the identification of tumor antigens in many neuroendocrine cancer types that exist, such as malignant gastrinoma, insulinoma, or medullary thyroid carcinoma. The aim of this study was to proof the concept of dendritic cell immunization with a tumor cell-specific polypeptide hormone as a target molecule in a transgenic mouse model for medullary thyroid carcinoma (Ret/Cal mice).

Experimental Design: Ret/Cal mice were repeatedly immunized for up to 6 months with amino acid–modified (xenogenic) calcitonin-pulsed dendritic cells. Xenogenic calcitonin was chosen for immunization due to its higher immunogenicity as compared with murine calcitonin.

Results: Lymph nodes from control protein-immunized mice did not show any macroscopic abnormalities, whereas tumor peptide-treated mice revealed in general profoundly enlarged lymph nodes. In tetramer analysis of paratumorous lymph nodes, 1.9% to 3.1% of all infiltrating CD8+ T cells were specific for one of three tumor epitopes tested. Analysis of the activated IFN-γ-secreting component in splenic cells revealed an average of 2.8% tumor epitope-specific CD8+ cells. Immunohistochemistry revealed strong CD8+ tumor infiltration in calcitonin-vaccinated mice. In addition, these cells also showed strong in vitro lysis capacity at up to 63.3%. Most importantly, calcitonin-immunized mice revealed largely diminished tumor outgrowth (-74.3%) compared with control mice (P < 0.0001). Likewise, serum calcitonin levels in calcitonin-vaccinated Ret/Cal mice were lower than in the control group.

Conclusion: These results have a major effect, as they are the first to establish a role for xenogenic polypeptide hormones as target molecules for immunotherapy in endocrine malignancies.
Protooncogene at codon 634 as is present in most patients with MEN2A mutation (substitution of cysteine for arginine) within the mouse model for MTC (Ret/Cal mice) displaying the identical tumor reduction was monitored by different assays. Ret/Cal mice were immunized over 6 months with amino acid–pulsed dendritic cells in six i.p. injections (4-5 × 10^7 cells per vaccination) for 6 months at intervals of 4 weeks between each injection. Another 20 male and female Ret/Cal mice were treated using human albumin (control protein)–pulsed dendritic cells. I.p. injections were used for immunization rather than i.v. or intralymphatically vaccinations because of a successful use of this approach in another animal model reported previously (22). An additional 30 mice were followed for up to 6 months without any treatment for evaluating tumor size. Ten mice were treated with calcitonin-pulsed dendritic cells for 2 to 5 months to evaluate tumor-infiltrating T lymphocytes.

With this in mind, tumor-specific peptides have already been used for vaccination in patients with parathyroid cancer using parathyroid hormone (15, 16) or in patients with medullary thyroid carcinoma (MTC) using calcitonin-pulsed monocyte-derived dendritic cells (17). Inducing polypeptide hormone-specific cytotoxic immunity led to long-term cancer regression as well as tumor marker expression normalization in parathyroid cancer patients (16) and a calcitonin-specific Th1 immunity in some of our patients (18). As yet, however, no systematic in vivo study has been done as a proof-of-concept for polypeptide hormone-triggered cytotoxic immune response in endocrine malignancies.

To test this hypothesis, we have now employed a transgenic mouse model for MTC (Ret/Cal mice) displaying the identical mutation (substitution of cysteine for arginine) within the RET protooncogene at codon 634, as most patients with multiple endocrine neoplasia type 2A (MEN2A) also share this mutation (19). Like patients with hereditary MTC, Ret/Cal mice develop multifocal and bilateral MTC with tumor prevalence at 100%, also leading to increased serum calcitonin levels (20). Ret/Cal mice were immunized over 6 months with amino acid–modified (xenogenic) calcitonin-pulsed dendritic cells and tumor reduction was monitored by different assays.

Materials and Methods

Animals. Transgenic Ret/Cal mice were first described by Michiels et al. (20) as displaying the same mutation within the RET protooncogene at codon 634 as is present in most patients with MEN2A (substitution of Cys^{634} for arginine; TGC→CGC). All mice therefore display overt bilateral C-cell hyperplasia as early as 3 weeks after birth and subsequently develop multifocal and bilateral MTC and an increased serum calcitonin levels. As these mice were backcrossed to C57BL/6 mice, they display a H2-Kb phenotype (see Supplementary Fig. S1). The animal experiments were approved by the Animal Care Committee of North Rhine-Westphalia (reference no. 50.05-230-77/05).

Generation of dendritic cells and calcitonin peptide pulsing. Bone marrow–derived dendritic cells from wild-type C57BL/6 mice were generated as described previously (21). Briefly, bone marrow cells were prepared from femurs and cultured in complete medium (CellGrowth; CellGenix) with granulocyte macrophage colony-stimulating factor (R&D Systems; 1,000 units/mL). After 3 days, nonadherent cells were harvested, washed in medium, and pulsed with amino acid–modified full-length calcitonin (Calcitonin-Ratiopharm, Ratiopharm; 100 μg/mL; endotoxin content <0.050 IU/mL) representing the same amino acid sequence as salmon calcitonin. This xenogenic peptide revealed differences at 17 amino acid positions compared with murine calcitonin (see Supplementary Fig. 2A). Binding affinity was calculated using SYFPEITHI<sup>5</sup> epitope prediction software. Human albumin (Aventis) was used as a control protein (100 μg/mL). After 2 h, cells were harvested, washed four times in isotonic NaCl, and resuspended in 50 μL of 0.9% NaCl. After that, the cells were used for vaccination. In all preparations, cell viability was ≥95%.

Phenotypic analysis of dendritic cells by flow cytometry analysis. Surface marker expression characteristic for dendritic cells was determined by flow cytometry (see Supplementary Fig. S3). Cell staining was done using different fluorochrome-conjugated monoclonal antibodies: hamster anti-mouse CD80, CD11c, CD29, CD54, CD69, rat anti-mouse CD45, CD45-B220, MHC class II (I-A<sup>B</sup>), MHC class I (RT-1<sup>B</sup>), CD86, CD40, CD11b, CD41, NK1.1, CD49b, CD62L, and CD3, CD4, CD8, CD19, and Ly6c (all BD Bioscience). Samples were analyzed using a FACSCalibur device with CellQuest<sup>®</sup> software (BD Biosciences).

Immunization of Ret/Cal mice with dendritic cells. Thirty male and female 4-week-old Ret/Cal mice were treated with calcitonin-pulsed dendritic cells in six i.p. injections (4-5 × 10^7 cells per vaccination) for 6 months at intervals of 4 weeks between each injection. Another 20 male and female Ret/Cal mice were treated using human albumin (control protein)–pulsed dendritic cells. I.p. injections were used for immunization rather than i.v. or intralymphatically vaccinations because of a successful use of this approach in another animal model reported previously (22). An additional 30 mice were followed for up to 6 months without any treatment for evaluating tumor size. Ten mice were treated with calcitonin-pulsed dendritic cells for 2 to 5 months to evaluate tumor-infiltrating T lymphocytes.

Purification of activated IFN-γ–secreting CD8<sup>+</sup> T cells. CD8<sup>+</sup> T cells were purified from spleen cell preparations by negative selection using a PanT-cell isolation kit and anti-CD4 microbeads (Miltenyi Biotec) resulting in purity levels of >95% in CD8<sup>+</sup> T cells. After overnight incubation with interleukin-2 (25 U/mL), activated IFN-γ-secreting CD8<sup>+</sup> T cells were purified. These cells were used for cytotoxicity assays. IFN-γ expression was monitored in control experiments using FITC-labeled rat anti-mouse IFN-γ antibodies (BD Bioscience).

Tetramer analysis. Three calcitonin-specific H2-K<sup>B</sup> tetramers covering the amino acids 2 to 9 (tetramer T1), amino acids 5 to 12 (T2), and amino acids 12 to 19 (T3) sequences in amino acid–modified calcitonin were provided by Orpegen. These tetramers were chosen because they offered the best probability of binding antigens as calculated by SYFPEITHI epitope prediction software (see Supplementary Fig. S2B). Another two calcitonin-specific H2-K<sup>B</sup> tetramers covering the amino acids 2 to 9 (human tetramer 1) and amino acids 8 to 15 (human tetramer 2) sequences from human calcitonin were used as negative controls.
Tetramer staining in CD8+ cells was done in PBS supplemented with 10% bovine serum albumin (FCS) by incubating these cells with phycoerythrin-labeled tetramer complexes (10 pmol/mL) at 4°C for 1 h. Afterwards, samples were incubated with anti-CD8-FITC at 4°C for 20 min, washed, and resuspended in 0.5% FCS in PBS for subsequent fluorescence-activated cell sorting analysis. Control T cells from TCR transgenic OT-1 mice were used in tetramer analyses for control (see Supplementary Fig. S4).

**Cytotoxicity assay.** Murine MTC cells (ATCC: CRL 1806) were labeled with 100 μCi 51Cr for 1 h at 37°C, washed three times, and resuspended in complete medium. Thereafter, cells (10⁴ per well) were incubated with varying numbers of IFN-γ-secreting CD8+ T effector cells for 16 h. Then, the supernatant was collected and radioactivity was measured using a Wizard Gamma Counter (Wallac). Percentage cytotoxicity was calculated as follows: cytotoxicity (%) = [(experimental group counts/min - spontaneous counts/min) / (total counts/min - spontaneous counts/min)] × 100. Data were expressed as mean ± SD from triplicate wells. YAC-1 murine lymphoma cell line (ATCC: TIB-160) was used as a negative control.

**Immunohistochemical analysis and tumor size measurement.** Cryostat sections (7 μm) of thyroid glands were serially cut, blocked 30 min with normal porcine serum (3%; DakoCytomation), and stained with polyclonal rabbit anti-human calcitonin antibodies or rat anti-mouse CD8 monoclonal antibodies (Abcam) at 4°C overnight. Biotin-labeled antibodies were used for visualizing anti-rat IgG amplified by avidin-biotin enzyme complex (Vector Laboratories), and red staining was done by high-sensitivity 3-amino-9-ethylcarbazole and substrate chromogen (DakoCytomation).

**Tumor outgrowth and serum calcitonin measurement.** Tumor sizes were measured independently (Leica QWin V3) by two investigators analyzing tumors from both thyroid lobes. Tumor sizes are given in square micrometers. Serum calcitonin was measured as recommended by the manufacturer (Roche). The lower detection limit of the assay was 2 pg/mL.

**Statistical analysis.** The results were analyzed for statistical significance by paired t test using Prism computer software (GraphPad Software).

**Results**

**Dendritic cell vaccination in Ret/Cal mice.** Altogether, 30 Ret/Cal mice were immunized with bone marrow–generated dendritic cells pulsed with non-species-specific amino acid–modified calcitonin. As shown in Fig. 1, untreated transgenic Ret/Cal mice develop MTC with a tumor prevalence of 100% (20). After 6 months, all of the Ret/Cal mice were still alive and were sacrificed for further analysis. As shown in Fig. 2A, xenogenic calcitonin peptide-treated mice showed profoundly enlarged lymph nodes characteristic of strong immune response. In contrast, control mice did not show any macroscopic abnormalities, whether nonimmunized or control protein immunized (Fig. 2B).

**Detection of calcitonin epitope-specific T cells.** For counting of epitope-specific CD8+ T cells, three different calcitonin-specific tetramers (T1-T3) with highest binding affinities to the MHC class I molecule H-2Kb were designed and used for measurement (Supplementary Fig. S2B). Only a negligible number of calcitonin-specific T lymphocytes could be detected in control mice spleens (0.11 ± 0.13%) or inguinal or cervical lymph nodes (0.2 ± 0.17% and 0.3 ± 0.14%, respectively; Fig. 3, left). In contrast, tumor peptide-treated mice showed significantly more CD8+ T cells, recognizing different calcitonin epitopes. Splenic cells from tumor-peptide vaccinated mice showed an increase in calcitonin-specific CD8+ T cells at up to 0.41% depending on the tetramer tested (mean, 0.25 ± 0.37%). An increase of tetramer-positive cells was seen in any of these mice tested (Fig. 3A, right). These results were also highly significantly different compared with untreated and control peptide-vaccinated mice (P values between 0.0003 and 0.0159).

Strikingly, at least 1.9% of all CD8+ cells recognized one of the three calcitonin epitopes in paratumorous cervical lymph nodes (Fig. 3C; P values from <0.0001 to 0.0003). Interestingly, the highest counts were detected for an epitope specific for the amino acids 5 to 12 sequence, the average lying at 3.1% for all CTL recognized this region. In other words, >6% of all CD8+ T cells in paratumorous lymph nodes were specific for the tumor antigens tested (Fig. 3C, right). Importantly, we found average values among 2.1 ± 1.7%, 2.5 ± 0.9%, and 3.9 ± 1.6% tetramer-positive CD8+ T cells, respectively, by analyzing the activated component CD8+ splenic cells (Fig. 4A and B). By using human calcitonin-specific tetramers, we did not detect any tumor epitope-specific CD8+ T cells (Fig. 4C). Strikingly, the subset of CD8+ cells of xenogenic calcitonin-immunized mice also showed high IFN-γ expression (Fig. 4D). Control protein-immunized mice did not reveal any significant number of calcitonin-specific cells (0.0-0.2% average) within the activated fraction (data not shown).

In parallel to these data, we also evaluated the presence of CD4+ calcitonin-specific T cells. Splenic T lymphocytes from calcitonin-immunized mice were purified and cultured with interleukin-2 and calcitonin. Detailed fluorescence-activated cell sorting analyses revealed that up to 40.2% of all IFN-γ expression cells were also CD4+ (Supplementary Fig. S5). These results indicate that our vaccination approach induced a broad T-cell response including the induction of antigen-specific CD4+ helper T cells.

![Fig. 2. Cervical and axillary lymph nodes in control protein-immunized and calcitonin-immunized mice. Ret/Cal mice immunized with amino acid–modified calcitonin-pulsed dendritic cells frequently revealed greatly increased lymph nodes here shown for the paratumorous cervical and axillary region (A, arrows). In contrast, control protein (human albumin)–immunized mice did not show any macroscopic abnormalities (B).](http://www.aacrjournals.org/doi/fig)
Detection of tumor-infiltrating CD8+ T cells. Additional 10 Ret/Cal mice were immunized with xenogenic calcitonin-pulsed dendritic cells and sacrificed at ages between 2 and 5 months. We found C-cell hyperplasia or small MTC in all of the mice (Fig. 5A). Interestingly, infiltrations with CD8+ T cells could be detected in most of them (Fig. 5B), indicating that these cells are attracted by tumor cells and may recognize them. In contrast, control mice did not show any tumor infiltration with CD8+ T lymphocytes at all (Fig. 5C and D). In addition, CD4+ T cells were neither detected within the tumors.

Tumor-specific CD8+ T cells lyse MTC cells. To analyze whether CD8+ T cells in tumor peptide-treated mice were able to kill MTC cells in vitro, IFN-γ-secreting CD8+ T cells were separated from interleukin-2-cultured spleen cells, and cytotoxicity assays were done on a murine MTC cell line for targeting (see Supplementary Fig. S6). Chromium release assays revealed strong dose-dependent cytotoxic activity in CD8+ T cells from tumor peptide-treated Ret/Cal mice, whereas CD8+ T cells from nonimmunized and control protein-immunized mice did not reveal any significant lysis activity (Fig. 6A). Additional controls were done by using the murine lymphoma cell line YAC-1, where no lysis activity was seen (data not shown).

Diminished tumor outgrowth and tumor marker decrease. Calcitonin-immunized mice revealed a greatly diminished tumor outgrowth (-74.3%) as compared with nonimmunized and control protein-immunized Ret/Cal mice (Fig. 6B, left; \( P < 0.0001 \)). Calcitonin staining was completely negative in 6 of 30 tumor peptide-vaccinated Ret/Cal mice, indicating complete tumor rejection in these animals (Fig. 6C). In accordance with these findings, serum calcitonin levels in tumor peptide-vaccinated Ret/Cal mice were almost as low (2.39 ± 0.84 pg/mL) as those from nontransgenic C57BL/6...
Fig. 4. Tetramer analysis for IFN-γ-secreting calcitonin epitope-specific CD8+ T cells. A, left, mean tetramer-positive IFN-γ-secreting CD8+ spleen cells calculated from eight experiments; middle and right, representative fluorescence-activated cell sorting of a forward/side scatter and analysis of viable (propidium iodide – negative) CD8+ spleen cells (gated on R1). B, representative fluorescence-activated cell sorting analysis shows significant amounts of specific activated T cells, which were detected using three different tumor peptide-specific tetramers (for gate R2). C, propidium iodide – negative cells from tumor peptide-treated Ret/Cal mice were analyzed with isotype controls as well as with human calcitonin-specific tetramers revealing negative results (for gate R2). D, IFN-γ was detected in both regions (right, R1; middle, R2) using FITC-labeled rat anti-mouse IFN-γ antibodies for intracytoplasmatic detection (gray histogram) after 1-day culture with interleukin-2 (viable cells gated on activated cells (R1) and resting cells (R2); middle). Negative controls were done using FITC-labeled rat isotype controls antibodies (IgG1, unfilled histogram). Tetramer analyses of control protein-vaccinated mice only revealed a negligible number of activated CD8+ cells specific to one of three tetramers tested (data not shown).
Calcitonin, a 32–amino acid polypeptide hormone secreted in substantial amounts by neuroendocrine thyroidal C cells, represents a highly sensitive tumor marker for detecting MTC and monitoring metastatic spread (9, 19). Epitope mapping with monoclonal antibodies revealed the highest immunogenicity toward the central region of calcitonin (amino acids 13–21 sequence; ref. 28). In vitro testing showed that coculturing with specific antibodies covering this region (amino acids 10–19) inhibited proliferation of a MTC cell line (29). Another group showed antigen-specific immune response following cDNA vaccination in a murine model using the preprohormone (preprocalcitonin) of calcitonin (30). Preprocalcitonin cDNA administration was combined with either granulocyte macrophage colony-stimulating factor or IFN-γ to enhance immune response, showing preprocalcitonin-specific proliferative cellular and antibody responses in the granulocyte macrophage colony-stimulating factor group. In contrast, codelivery of IFN-γ expression plasmid resulted in decreased antibody response against preprocalcitonin, albeit in enhanced Th1-like immunity. Together, with our results from a transgenic mouse model and the human data mentioned previously (15–17), these results show that polypeptide hormones may serve as target molecules for cytotoxic immunity.

In the past, we used species-specific calcitonin immunizing some MTC patients, which led to calcitonin-specific Th1 immunity in some (18) and partial tumor regression in one patient (17). In contrast to these previous reports, we now have applied non-species-specific amino acid–modified (xenogenic) calcitonin for immunization to increase binding affinity to MHC class I molecules by using a non-self peptide (31) while improving vaccination strategy. Comparable approaches with xenogenic tumor epitopes have already been successfully applied in other nonendocrine malignancies (32–34). In these reports, single amino acid substitutions of the original epitope improved tumor antigen recognition by CTL (32–34). In these reports, single amino acid substitutions of the original epitope improved tumor antigen recognition by CTL (32–34). This might be explained by an increased binding affinity to the MHC molecule itself or by stabilizing the MHC/peptide complex by binding anchor positions in the MHC molecule.

Amino acid–modified hormone peptides were also used for immunization in endocrine malignancies in two parathyroid cancer patients (15, 16). In both cases, single substitutions by different amino acids at position 2 within the bioactive site of parathyroid hormone (amino acids 1–34 peptide sequence) were done and used for vaccination. These patients showed a clear long-term clinical response most likely due to the high MHC binding affinity of the polypeptide hormone peptides used. The data collected here from the murine system support this concept.

Importantly to note, calcitonin receptors are known to be present on osteoclasts but also on lymphocytes (35) as well as on monocytes (36). Recently, it has also been shown that activated lymphocytes suppress osteoclastogenesis by diverting early monocyte/macrophage progenitor lineage commitment toward dendritic cell differentiation (37). These cells show a largely diminished calcitonin receptor expression (37). We were able to reproduce these results based on fluorescence-activated cell sorting analyses (Supplementary Fig. S7). A substantial question is whether direct binding of calcitonin to its receptor may influence the immunostimulatory capacity of dendritic cells. Based on a mixed leukocyte reaction by coincubation of dendritic cells with allogenic T cells, we could show a slight cell...
concentration-dependent decrease of lymphocytic proliferation, indicating a marginal influence of the direct binding of calcitonin to its receptor on the functional immunostimulatory abilities of dendritic cells (Supplementary Fig. S7).

Another important question is whether antitumor immunity, as described here, may occur without breaking self-tolerance especially in endocrine organs where autoimmune phenomena are well known. In the past, it has already been shown that knockout of SOCS1, an inducible negative feedback inhibitor of the JAK/STAT signal pathway (38), leads to an uncontrolled IFN-γ signaling (39, 40) and to the induction of an enhanced antigen-specific CTL response and antitumor immunity (41, 42). Moreover, it has been shown that SOCS1 represents an important negative regulator of antigen presentation by dendritic cells and that silencing of SOCS1 enhances antigen presentation by dendritic cells and antigen-specific antitumor immunity (42). In line with these data, the necessity of inhibiting SOCS1 has been proven to break self-tolerance and to induce effective antitumor responses (43, 44). In our present study, we also investigated whether any autoimmune phenomena might be detectable in immunized Ret/Cal mice (data not shown). However, neither in the paratumorous thyroid gland (as a sign of autoimmune thyroiditis) nor in the Langerhans' islets of the pancreas (as a sign of type 1 diabetes mellitus) any T-cell infiltration was detected. In addition, we also examined the calcium homeostasis: we found elevated serum calcium levels as well as increased parathyroid hormone values consistent with a primary hyperparathyroidism, which belongs to the multiple endocrine neoplasia type 2 (MEN2) syndrome caused by a (transgenic) RET protooncogene mutation (19). In other words, an autoimmune hyperparathyroidism could also be excluded in our treated mice. Our data suggest that an antitumor immunity may also occur without induction/activation of autoreactive T cells. The discrepancy between the aforementioned and our observations might be due to a multitude of different reasons. Important to note, however, our vaccination was done over a period of 6 months while tumors were growing and not, as frequently described, against an existing tumor mass. Because of that, we might postulate that antitumor immune response as described here might not be as efficient as under other conditions.

In summary, our systematic analysis leads us to the conclusion that immunization with a polypeptide hormone induces activated IFN-γ-secreting CTL, which directly lyses target carcinoma cells, thereby serving as target molecules for immunotherapy in neuroendocrine cancer. Our findings carry

![Fig. 6.](image-url)
major effect, as our approach may also be applicable to other devastating endocrine cancers such as metastatic insulinoma, malignant gastrinoma or glucagonoma, or others known to secrete various polypeptide hormones. No relevant tumor antigens have been identified yet in any of these tumors. Based on these results, we have conducted new immunotherapy trials in patients with metastasized MTC using xenogenic HLA-restricted calcitonin peptides.

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Dendritic Cell Vaccination with Xenogenic Polypeptide Hormone Induces Tumor Rejection in Neuroendocrine Cancer

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