**NOTE** *Internal Medicine*

# Malignant Transformation of T-cell Large Granular Lymphocyte Leukemia in a Dog

Tomoko TAKAHASHI[1)], Isao OTANI[2)], Masaru OKUDA[3)], Masashi INOUE[4)], Kanako ITO[4)], Manabu SAKAI[1)], Hiroshi KOIE[1)], Yoshiki YAMAYA[1)], Toshihiro WATARI[1)], Tsuneo SATO[5)], Kiichi KANAYAMA[2)] and Mikihiko TOKURIKI[1)]

*[1)]Laboratories of Comprehensive Veterinary Clinical Studies and [2)]Veterinary Physiology, [4)]Animal Medical Center and [5)]Laboratory of Veterinary Pathology, Department of Veterinary Medicine, College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa, Kanagawa 252–8510 and [3)]Laboratory of Veterinary Internal Medicine, Faculty of Agriculture, Yamaguchi University, 1677–1 Yoshida, Yamaguchi, Yamaguchi 753–0077, Japan*

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ABSTRACT. An 8-year-old spayed female Golden Retriever was referred to us for evaluation of mild lymphocytosis. The peripheral lymphocytes were comprised of mostly large granular lymphocytes (LGLs), and flow cytometry showed that they were mostly CD3+8+ T lymphocytes. Clonal rearrangement of the T-cell receptor gene was identified in the peripheral blood, and the dog was therefore diagnosed with LGL chronic leukemia. The dog was subclinical without treatment until hospitalization on day 154, at which point the lymphocytes looked like lymphoblasts and the surface markers changed to CD3–8–. This was regarded as malignant transformation from LGL chronic leukemia to the acute type. Sequential chemotherapy was started, but the dog died on day 190. Necropsy revealed tumor cell infiltration into the heart, skin, and brain.

KEY WORDS: flow cytometry, large granular lymphocyte leukemia, malignant transformation.

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Large granular lymphocytes (LGLs) are defined as large lymphocytes characterized by the presence of azurophilic granules in the cytoplasm. Two subsets of LGLs exist in humans, based on expression of leukocyte differentiation (cluster of differentiation, CD) antigens, the T-cell receptor (TCR) complex, and *in vitro* cytotoxicity. T-cell LGLs express the CD3/TCR complex and rearranged TCR genes and most are positive for cytotoxic T-cell phenotype (CD3+8+). A small proportion of them are natural killer cell LGLs that do not express the CD3/TCR complex, but they do display the germline configuration of TCR genes. In human medicine, T-cell LGL leukemia is defined as a disease caused by clonal proliferation of CD3+8+ T cells, representing an indolent disorder in most patients [10]. The few reports describing T-cell LGL proliferative disorders in dogs have identified LGL acute lymphoblastic leukemia (ALL), LGL chronic lymphocytic leukemia (CLL), and reactive LGL proliferation by chronic ehrlichiosis [15]. These pathologies are not as well defined as in human medicine.

This report describes a unique case of malignant transformation from indolent LGL CLL to life-threatening LGL ALL and illustrates the usefulness of flow cytometric methods in monitoring pathological changes.

An 8-year-old, 29.6-kg, spayed female Golden Retriever was referred to the Animal Medical Center of Nihon University, Japan, for evaluation of mild lymphadenopathy and mild lymphocytosis. The dog's general physical condition was good, and physical examination revealed generalized lymphadenopathy (the right superficial cervical lymph node was the most enlarged; 29 × 27 × 17 mm) and slight splenomegaly. Her white blood cell count (WBC) was normal at 10,900/$\mu l$, but the number of lymphocytes was slightly increased at 5,014/$\mu l$, with the majority of lymphocytes being LGLs (3,434/$\mu l$; Figs. 1A, 2). LGLs displayed abundant cytoplasm, fine azurophilic granules, and nuclei that were round, indented, or irregularly shaped with moderately clumped chromatin. The dog's complete blood count (CBC) was within the normal range (hematocrit [Ht], 44.0%; red blood cell count [RBC], $6.07 \times 10^6/\mu l$; platelet count [PLT], $334 \times 10^3/\mu l$), and her blood chemistry panel was within the reference range. Thoracic and abdominal radiography did not reveal any abnormalities. An excisional biopsy was performed to remove the right popliteal lymph node, and hyperplasia was diagnosed based on histopathological analysis. LGLs were only scarcely observed in bone marrow aspirate from the right femur, and the myeloid-to-erythroid ratio was normal. The LGLs manifesting in the peripheral blood were mainly considered to have proliferated in the spleen based on evaluation of splenic cytology, which indicated infiltration of numerous LGLs similar in cytological appearance to those seen in the peripheral blood. *Ehrlichia* infection was ruled out based on serology. Flow cytometric analysis was performed using monoclonal antibodies as described previously [9]. The results indicated that most peripheral blood lymphocytes (PBLs) were of the CD3+8+ phenotype (Figs. 3A, B, 4), although this phenotype was in the minority in the frozen sections of resected lymph node using monoclonal antibodies (anti-human CD3 [clone CD3-12], anti-canine CD21 [clone CA2.1D6], anti-canine CD4 [clone YKIX302.9], and anti-canine CD8 [clone YCATE55.9]; Serotec, Oxford, UK; data not shown). T-cell LGL CLL was diagnosed based on the above results.

Monthly examinations revealed slight increases in the number of LGLs in the peripheral blood and appearance of CD3–21– lymphocytes (Figs. 2, 4A). At the same time, the

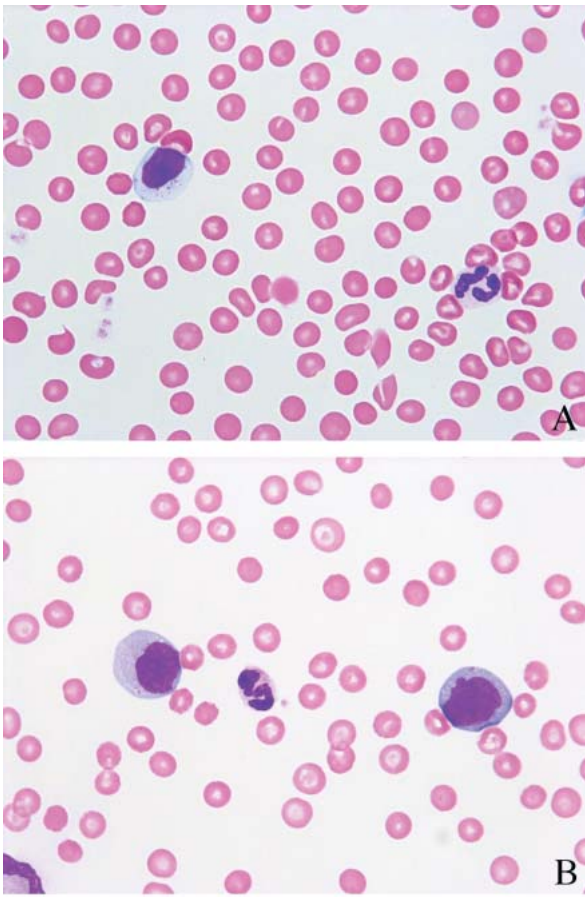


Fig. 1. Blood smears on days 13 (A) and 154 (B). (A) A large lymphocyte with abundant cytoplasm displaying fine azurophilic granules. The nucleus is irregularly shaped with moderately clumped chromatin. (B) Lymphocytes are markedly large with deeply basophilic abundant cytoplasm showing few azurophilic granules. Nuclei are pleomorphic with large prominent nucleoli. Anemia is evident. Wright-Giemsa staining (× 1,000).

morphology of the LGLs changed gradually, with some becoming large and lymphoblast-like. On day 128, the dog's CBC decreased slightly (Ht, 38.0%; RBC, $5.50 \times 10^6/\mu l$; PLT, $123 \times 10^3/\mu l$), but she remained asymptomatic.

The dog was subclinical without treatment until hospitalization on day 154. On presentation, she could not walk and physical examination revealed pale mucous membranes, tachycardia, and a weak femoral pulse. Superficial lymph nodes were slightly enlarged as before. Her CBC revealed an increased WBC, non-regenerative anemia, and a decreased PLT (WBC, 33,700/$\mu l$; Ht, 23.0%; RBC, $3.26 \times 10^6/\mu l$; PLT, $33 \times 10^3/\mu l$). Analysis of blood smears revealed numerous markedly large lymphoblasts (4,515/$\mu l$) with basophilic cytoplasm including few azurophilic granules (Figs. 1B, 2). Flow cytometry indicated that the surface markers of nearly 50% of the PBLs had shifted to CD3–8– (Figs. 3C, D, 4). The dog's blood chemistry profile revealed hypoalbuminemia (serum albumin, 1.46 g/d*l*), azotemia (blood urea nitrogen, 80.2 mg/d*l*), and elevated enzyme lev-

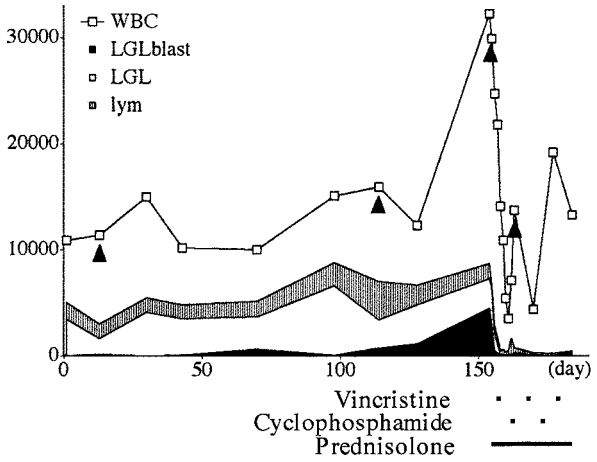


Fig. 2. Changes in white blood cell count (WBC). Arrowheads represent points at which flow cytometric analyses were performed (see Figs. 3, 4). LGL blast indicates large lymphocytes with prominent nucleoli, LGL indicates normal lymphocytes with azurophilic granules, lym indicates normal lymphocytes without azurophilic granules. The chemotherapeutic agents used are shown below the graph. The population of lymphocytes changed dramatically on day 154 when the dog was hospitalized. Sequential chemotherapy resulted in a continuous decrease in WBC and almost complete disappearance of all categories of lymphocytes. Based on the blood films, the dog's leukemic condition was well controlled.

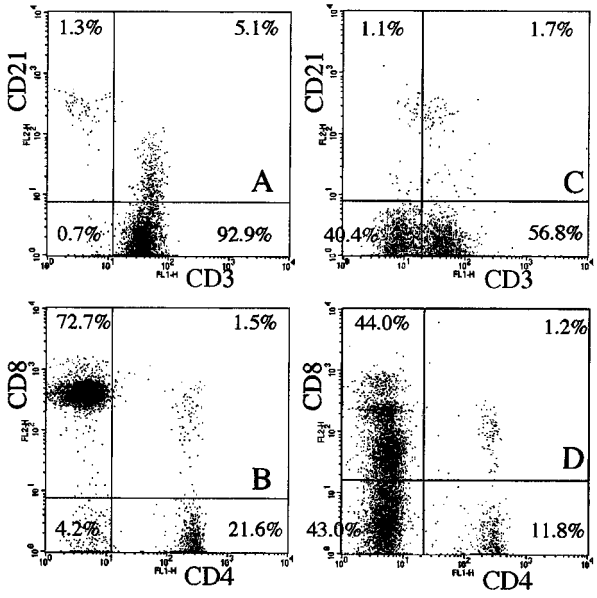


Fig. 3. Two-color flow cytometric dot plot analyses of peripheral blood lymphocytes (PBLs) on days 13 (A, B) and 154 (C, D) and the proportion of CD3/CD21-positive (A, C) and CD4/CD8-positive cells (B, D). Most of the PBLs expressed CD3 and CD8 on day 13. The surface markers of the PBLs shifted to CD3-negative and CD8-negative on day 154.

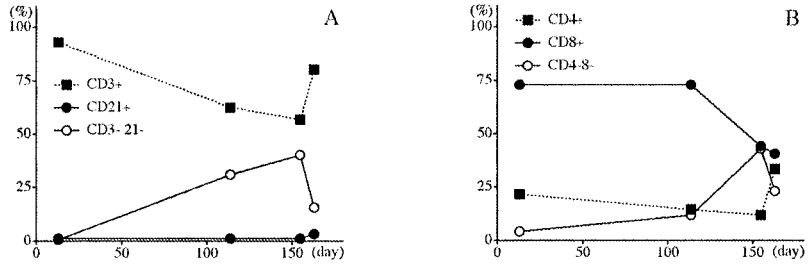


Fig. 4. Changes in the proportion of surface markers in peripheral blood lymphocytes (PBLs) analyzed by flow cytometry. (A) Percentage of CD3/CD21-positive PBLs and (B) percentage of CD4/CD8-positive PBLs. Analyses were performed on the days indicated in Fig. 2 (arrowheads). Almost no CD3–21– PBLs were present in the early stage but then the proportion gradually increased. At the same time, the proportion of CD4–8– PBLs increased. CD21-positive PBLs were scarce at all time points. The proportions seemed to return to those seen before malignant transformation when chemotherapy was started after day 154.

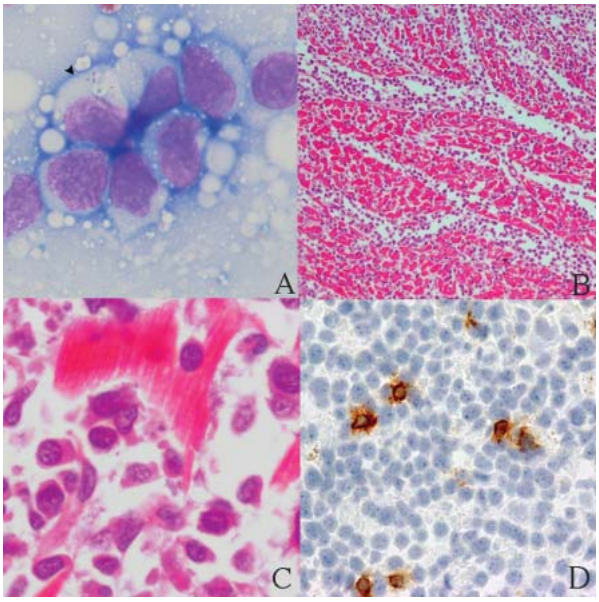


Fig. 5. Photomicrographs of tissues. (A) Fine needle aspiration cytology of the skin mass on day 179. Many large lymphoid cells with pale cytoplasm were obtained. A small number of cells exhibited fine azurophilic granules in the cytoplasm (arrowhead). Wright-Giemsa staining (× 1,000). (B), (C), and (D) Tissue sections of the heart at necropsy. (B) and (C) show diffuse infiltration of round lymphoid cells into the myocardium. HE staining (B × 100, C × 1,000). (D) Some cells display strong positive staining for CD3 (immunostaining, × 400).

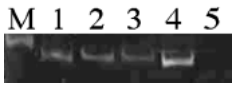


Fig. 6. Polymerase chain reaction results using primers for T-cell receptor gamma. DNA was derived from the following sources: M, molecular marker (100 bp); lane 1, peripheral blood on day 128; lane 2, heart at necropsy; lane 3, skin at necropsy; lane 4, positive control; and lane 5, negative control. Lanes 1, 2, and 3 are rearrangement-positive with bands of identical size.

els (alkaline phosphatase, 297 U/*l*; alanine aminotransferase, 904 U/*l*; aspartate aminotransferase, 783 U/*l*; lactate dehydrogenase, 1,965 U/*l*; creatine kinase, 836 U/*l*). Her calcium levels were within the normal range (8.23 mg/d*l*). No hemorrhages were apparent, but her blood coagulation profile revealed disseminated intravascular coagulation (DIC) (fibrin/fibrinogen degradation products, 10 $\mu$g/m*l*; prothrombin time, 7.5 sec; activated partial thromboplastin time, 19.9 sec; fibrinogen concentration, 163 mg/d*l*; antithrombin III activity, 57%). Radiography of the thorax disclosed an enlarged heart. The dog's heart rate was > 300 beats/min, and electrocardiography revealed severe ventricular tachycardia. A tentative diagnosis of DIC secondary to malignant transformation of LGL leukemia was made.

Symptomatic treatment was started with whole blood transfusion and injection of heparin sodium, gabexate mesilate, lidocaine hydrochloride, aminophylline, famotidine, and antibiotics. The dog appeared to be stabilized temporarily, and her ventricular tachycardia improved but never completely disappeared. At the same time, the dog was treated with sequential chemotherapy comprising vincristine (0.7 mg/m$^2$ body surface area [BSA] intravenously on days 156, 170, and 184), cyclophosphamide (250 mg/m$^2$ BSA intravenously on days 163 and 177), and prednisolone (1 mg/kg bodyweight subcutaneously or orally from day 154 until death). This resulted in a prompt decrease in the number of atypical lymphocytes in peripheral blood (Fig. 2). The number of PBLs decreased, and the proportions of surface markers for PBLs returned to those seen before malignant transformation (Fig. 4). On day 162, the superficial lymph nodes shrank and could not be palpated. On day 170, the dog began to display ataxia and dizziness, which gradually worsened and resulted in dysstasia. Numerous firm skin nodules appeared along her spine on day 179. Fine needle aspiration of the mass revealed numerous large mononuclear cells (Fig. 5A). The dog's clinical condition declined approximately 1 month after diagnosis of malignant transformation, and she died on day 190.

Necropsy revealed tumor cell infiltration into the cardiac muscle, skin, and brain. Extensive tan areas were identified

in the myocardium, and histopathology revealed diffuse infiltration of neoplastic lymphoid cells into myocardium (Figs. 5B, C). Immunohistochemistry of frozen sections from the heart and skin identified most lymphoid cells as negative for CD3, CD21, CD4, and CD8. A small number stained positively for CD3 (Fig. 5D). Diffuse proliferation of large neoplastic lymphoid cells was observed in the subarachnoid cavity and perivascular space of the cerebrum and subarachnoid cavity of the cerebellum. The bone marrow of the right femur was largely replaced by fat, and no significant tumor cell growth was detected. The spleen and liver were unaffected. The number of macrophages containing hemosiderin was increased in the hepatic lymph node, although no other lymph nodes were swollen.

Molecular analysis using polymerase chain reaction (PCR) demonstrated clonal rearrangement of TCR genes with bands of identical size for PBLs on day 128 and for the heart and skin at necropsy (Fig. 6) [2].

This was a rare case of T-cell LGL leukemia in which transformation from indolent CLL to life-threatening ALL was well documented by morphology, immunophenotyping, and molecular analysis of TCR chain genes. Flow cytometry was particularly useful in monitoring the stage of leukemia. We were able to directly attribute the dog's ventricular tachycardia to infiltration of tumor cells into cardiac muscle rather than to thromboembolism secondary to DIC. While the leukemic condition in the peripheral blood was well controlled by chemotherapy, tumor cell proliferation in the heart, skin, and brain could not be prevented.

Lymphoid neoplasms that predominantly involve bone marrow and peripheral blood are considered to be leukemias, while lymphoma represents a group of lymphoid neoplasms that originate in lymph nodes, other primary lymphoid organs including the spleen, or extranodal sites. In the present case, neoplastic lymphoid cells did not proliferate in the bone marrow, but instead proliferated in the spleen during the early stage, expanded into the peripheral blood, and ultimately expanded to multiple organs. It remains uncertain whether invasion from the spleen to the bone marrow occurred, since bone marrow aspiration could not be performed at the time of malignant transformation. Few neoplastic cells were present in the bone marrow at necropsy, which was consistent with the description of Kleiter [5]. Although the number of neoplastic cells in the PBL was relatively low in terms of leukemia, the clinical course was defined as leukemia. McDonough *et al.* reported that the degree of lymphocytic infiltration into bone marrow did not appear to be sufficient to cause myelophthisis in LGL ALL cases with anemia and thrombocytopenia [8]. Vernau *et al*. also noted marked splenic infiltration with LGL, whereas bone marrow involvement was either minimal or inapparent in both LGL ALL and CLL [14]. The classical definition and concept of leukemia may need to be broadened in cases of LGL neoplasia.

The immunophenotypes of canine leukemia have been described previously [11, 14]. T-lymphocyte proliferation, particularly LGL proliferation, seems to be common in both ALL and CLL. The phenotype changed dramatically in the present case, with most PBLs microscopically identified as LGLs during the indolent stage and comprising mostly CD3+8+ T lymphocytes; morphology and phenotype gradually changed to blast-like LGLs displaying CD3–8– over the course of 5 months. The immunophenotype of the neoplastic cells that infiltrated into the heart and skin was also CD3–8–. The change in scatter plot patterns from flow cytometry looked continuous (Fig. 3). It is possible that 2 groups of PBLs were present with phenotypes of CD3+8+ and CD3–8–; however, we believed that the expressions of CD3 and CD8 were downregulated, probably as a result of cell activation. We selected PBLs for flow cytometry by gating cells according to forward and side scatter [9]. Later stage neoplastic lymphocytes were enlarged, and we believed we might not be able to analyze whole PBLs. Unusually large lymphocytes, in which CD3 was downregulated, were mixed with other leukocytes and could not be analyzed due to unavailability of the specific lymphocyte marker. If all of the PBLs could have been examined, the percentage of expression for each surface marker would have been different from those in the data shown here. However, we would like to stress that the expressions of CD3 and CD8 were downregulated even in lymphocytes of relatively normal size and that flow cytometry was useful in monitoring these unusual changes. In dogs, the immunophenotype of CLL cases reportedly does not change significantly over time or with disease progression, and many dogs remain alive for more than 3 years [14]. As in the present case, immunophenotypic alteration could occur during the course of canine leukemia. Shifts (loss or acquisition) in expression of immunological markers have also been well demonstrated in human acute leukemia [12].

In human medicine, the criteria for diagnosis of LGL leukemia include the presence of cytopenia of one or more cell lines with evidence of a clonal CD3+8+57+ T-cell population by either PCR or Southern blotting [10]. Assessment of clonality recently became available for canine lymphoid neoplasms and could provide an objective and accurate predictor of neoplasia [2]. In the present case, clonal proliferation of T lymphocytes was determined using PBLs and PCR methods before the dog became clinically ill, although the number of neoplastic lymphocytes was relatively low. Clonal growth of the same population was detected in the heart and skin. This might indicate that leukemic condition should be screened not only by the number of PBLs, a proportion of malignant cells in bone marrow, or immunohistochemistry, but also by assessment of clonality.

Unfortunately, proving whether homology existed in the TCR gene rearrangement was impossible among the PBLs, heart, and skin due to the conditions of the samples. Each PCR sample had a band of identical size, but this was insufficient to determine whether the CD3+8+ LGLs and CD3–8– blast-like LGLs were genetically identical. More advanced analyses might be necessary. Burnett *et al.* described a case of B-cell lymphoma that developed into multiple myeloma without any changes in immunoglobulin

gene rearrangement [1]. Several other reports have illustrated likely cases of blast transformation [5, 7]. In human patients, development of large-cell lymphoma from CLL/small lymphocytic lymphoma is known as Richter's transformation and occurs in approximately 5% of patients with CLL [13]. The present case illustrates that LGL leukemia in dogs may transform, although rarely, in a phenomenon similar to Richter's transformation in human CLL.

Chemotherapy was not initiated on day 128 even though a limited number of lymphoblasts and TCR gene rearrangement were revealed on this day. This was because it is difficult to decide to initiate treatment in the absence of clinical signs. Furthermore, no treatment has been established for LGL proliferating disease in dogs, and spontaneous remission may occur even when the clinical course is indolent [3]. Some symptomatic cases with anemia have been treated with good results using prednisone and chlorambucil or other combination chemotherapies [4, 8]. In most human patients, LGL leukemia is an indolent disorder, and significant improvement of cytopenias can be achieved with immunosuppressive agents such as steroids [10]. Conversely, LGL ALL is a clinically aggressive disease and most dogs either die or are euthanized within 3 months of diagnosis [8, 14]. In the present case, sequential chemotherapy seemed effective based on blood films, but it could not prevent tumor cell proliferation in the heart, skin, and brain. Considering that ordinary chemotherapeutic agents do not penetrate the blood-brain barrier, highly lipid-soluble nitrosoureas such as lomustine would have been suitable in this case. Moreover, long-term complete remission has been achieved in one case of LGL ALL through chemotherapy followed by splenectomy [6]. In our case, tumor cell proliferation in the spleen was well controlled by chemotherapy alone, as the number of neoplastic lymphocytes in the PBLs decreased and no tumor proliferation was apparent in the spleen or bone marrow at necropsy. Further study is needed to identify an optimum treatment against LGL ALL.

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