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Original Article

Gaucher disease is associated with lymph node reactive follicular hyperplasia with tingible body macrophages of M2 phenotype

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Abstract: In Gaucher disease (GD), deficiency of the lysosomal enzyme, acid β-glucocerebrosidase (GCase), and subsequent accumulation of its substrate in macrophages leads to inappropriate immune activation. GCase deficiency affects the differentiation of the mononuclear phagocyte lineage with the resultant dysfunction of reticuloendothelial system organs including liver, spleen and lymph nodes. While lymphadenopathy is often observed in GD, the underlying mechanisms are unknown. We studied the pathophysiology of lymph node enlargement in a case with Gaucher disease (N370S/RecNciI) and monoclonal gammopathy of undetermined significance (MGUS). Reactive follicular hyperplasia with intrafollicular monotypic plasma cells (IgG kappa) was observed in lymph node biopsy with presence of variably sized follicles in polarized germinal centers and macrophage infiltration. Dual fluorescence labeling with macrophage (M2) marker and Ki67, a marker of proliferative activity, indicated that the lesion was composed of active and proangiogenic macrophages. The immunophenotyping of peripheral blood showed clonal B-cell proliferation, and a reduced number of circulating marginal zone memory B-cells. M2 macrophages promote tumor formation through cell-to-cell interaction by differentiating into tumor-associated macrophages. M2 cells also promote vascularization and formation of lymphatic vessels. This data not only highlights the mechanisms of lymphadenopathy in GD, but also may bridge the gap between the cell types interplaying in the development of B cell related disorders.

Keywords: Gaucher disease, lymphadenopathy, M2 macrophages

Introduction

Gaucher disease (GD), the most common lysosomal storage disorder, is caused by the deficiency of the enzyme β-glucocerebrosidase. Accumulation of the substrate, glycosylceramide, most notably in macrophages, leads to the pathognomonic Gaucher cells with resultant diverse immune phenotypic effects, including dysregulation in differentiation of the mononuclear phagocytic cell lineage [1]. Gaucher cells occur predominantly in liver, spleen and bone marrow, resulting in the most common clinical features: organomegaly, cytopenias, and skeletal involvement with bone crisis, chronic pain and fractures.

In addition to the classical symptoms, comorbidities, especially cancer, occur in patients with GD [2]. Patients with GD have an increased risk for the development of specific malignant disorders, commonly B-cell or plasma cell malignancies, multiple myeloma (MM) [3], and less commonly, chronic lymphocytic leukemia, acute myeloid leukemia, acute lymphoblastic leukemia, large B-cell lymphoma and Hodgkin lymphoma. Current data has shown that in GD, splenectomy is specifically associated with the development of a malignancy [2]. It has been hypothesized that loss of splenic function shifts the predominant site of hemolysis from extravascular to intravascular tissues, causing plasma hemoglobin levels to rise and promote smooth muscle proliferation, and activation of platelets and endothelial cells [2]. Due to the success of enzyme replacement therapy (ERT), splenectomy is now rarely performed in GD.

While lymphadenopathy is commonly reported in patients with GD, the underlying mechanisms are not yet explored. This is the first report investigating the role of activated macrophages in the development of comorbidities including lymphadenopathy and tumor development in GD. This data not only highlights the mechanisms of lymphadenopathy in GD, but also may bridge the gap between the cell types interplaying in the development of B cell related disorders.

Material and methods

Tissue samples and immunostaining

The lymph node core biopsy and surgically-extracted right submandibular lymph node were fixed in 10% neutral formalin, embedded in paraffin, and stained with hematoxylin-eosin or antibodies CD20, CD3, immunoglobulin (Ig) light chains kappa & lambda, IgG, IgA, IgM, and IgD. Immunofluorescent staining (IFC) was performed with antibodies against markers for cell proliferation (Ki67) and M2 macrophages (CD163, CD68). The marker indices for Ki-67, CD163, and CD68 were defined as the percentage of positively stained cells in representative areas of the lymph nodes and were evaluated by counting using digital image analysis (ImageJ ITCN plugin).

Immunoglobulin PCR

DNA extraction was performed from paraffin embedded lymph node surgical tissue sections. To measure the immunoglobulin heavy (IGH) locus, two separate reactions were performed using two primer sets. The first set of primers covered framework region III and the joining region of the immunoglobulin heavy chain gene (FRIII-IGH-PCR), while the second set of primers covered framework region II and the joining region of the immunoglobulin heavy chain gene (FRII-IGH-PCR) [4]. For both reactions, the joining region primers were covalently linked to fluorescent dye FAM for fluorescence detection following the protocol [5]. Testing for the IGκ locus was performed at laboratory of pathology, National Cancer Institute (NCI), NIH as described previously [5].

Western blot

Whole cell extracts of PBMCs (30 μg) were separated on NuPAGE 4-12% gel and blotted to PVDF membranes. Membranes were probed with antibody Ki67 (Abcam, Cambridge, MA). Immunoblots were scanned using Bio-Rad GelDoc (Bio-Rad, Hercules, CA).

Flow cytometry immunophenotyping

Direct immunofluorescence with specific antibodies was performed on peripheral blood as previously described [6, 7]. Briefly, 100 μl of washed blood was used per tube, and samples were acquired on Accuri C6 flow cytometer (BD Bioscience, San Jose, CA) and analyzed using FCS express software (De Novo software, Glendale, CA).

Ethical approval

All research procedures have been performed under the IRB approved protocol NCT02000310, clinicaltrials.gov.

Statistical analysis

Data are expresses as average ± standard error of the mean. Statistical significance was determined using Student's t-test and statistical significance p value is <0.05.

Results

Clinical presentation

During annual evaluation, a 46-year old female with Type 1 GD presented with an enlarged right submandibular lymph node, measuring 3 by 6 cm, not freely movable and medium in consistency. The diagnosis of GD was at age 19, followed by splenectomy. She was lost to follow up until she presented with thrombocytopenia and transfusion dependent anemia due to bone marrow failure, but was successfully treated with ERT for the past 2 years with normalization of hematological parameters. The current work-up showed increased IgG, 1792 (700-1,600 mg/dl), decreased IgM, 25 (26-217 mg/dl), and an observed serum M spike. The immunofixation showed monoclonal protein with light chain specificity. Urine free kappa light chains and lambda light chains were elevated, 41.37 (3.30-19.40 mg/L) and 27.65 (5.71 and 26.3 mg/L) respectively (**Figure 1A**). She was diagnosed with monoclonal gammopathy of undetermined significance (MGUS) and lymphadenopathy. For a

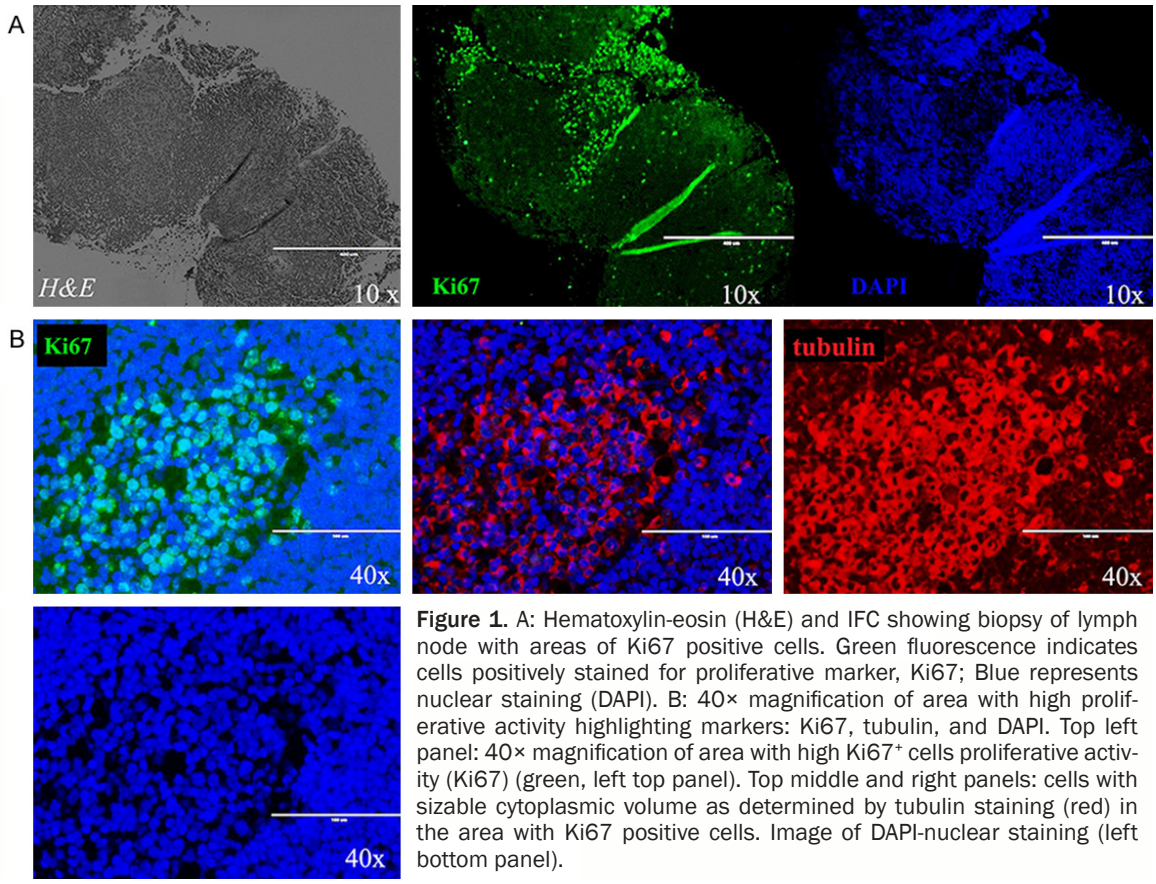

Figure 1. A: Hematoxylin-eosin (H&E) and IFC showing biopsy of lymph node with areas of Ki67 positive cells. Green fluorescence indicates cells positively stained for proliferative marker, Ki67; Blue represents nuclear staining (DAPI). B: 40× magnification of area with high proliferative activity highlighting markers: Ki67, tubulin, and DAPI. Top left panel: 40× magnification of area with high Ki67$^+$ cells proliferative activity (Ki67) (green, left top panel). Top middle and right panels: cells with sizable cytoplasmic volume as determined by tubulin staining (red) in the area with Ki67 positive cells. Image of DAPI-nuclear staining (left bottom panel).

suspected hematologic malignancy, a core biopsy of the lymph node was performed followed by the surgical removal of the lymph node.

Histopathological analysis

The histopathological analysis of the core biopsy specimen showed areas with high proliferative activity (87% of Ki67 positive cells) (**Figure 1A**). Unlike the surrounding cells, Ki67$^+$ cells had sizeable cytoplasmic volume (tubulin staining), suggesting a predominant population of large cells (**Figure 1B**). The analysis of the remaining lymph node that was surgically removed showed preserved nodal architecture with many variably-sized follicles, polarized germinal centers with tingible body macrophages [6], and intact mantle zones. There was a focal paracortical expansion by a polymorphic population, as well as increased population of large cells in germinal centers and inter-follicular areas (**Figure 2A**). The lymph node mass was primarily comprised of infiltrated macrophages.

Molecular studies

Lymph node enlargement with large, foamy macrophages is frequently observed in GD patients. To determine the contribution of the macrophages to tumor formation and lymphadenopathy in GD, we performed IFC staining with CD68 and CD163 antibodies [8], both markers for M2 type macrophages [9]. The results showed CD68 and CD163 positive macrophages were present in follicular centers and interfollicular zones, confirming the H&E histopathology analysis (**Figure 2B**). Scoring, on the basis of the percentage of positively stained cells, demonstrated a significantly increased number of CD68 and CD163 positive cells in the lymph node (**Figure 2C**). Ki67 stains demonstrate a pattern of polarity and could provide a distinction between reactive follicular hyperplasia and follicular lymphoma in tumor samples [10]. Ki67 staining verified oval structure of follicle centers with polarized centers with clear definition of “light” and “dark” zones (**Figure 2D**). In this current case, 26% of the cells of follicular center were positive for Ki67 (**Figure 2D**), as opposed to 3% in the control

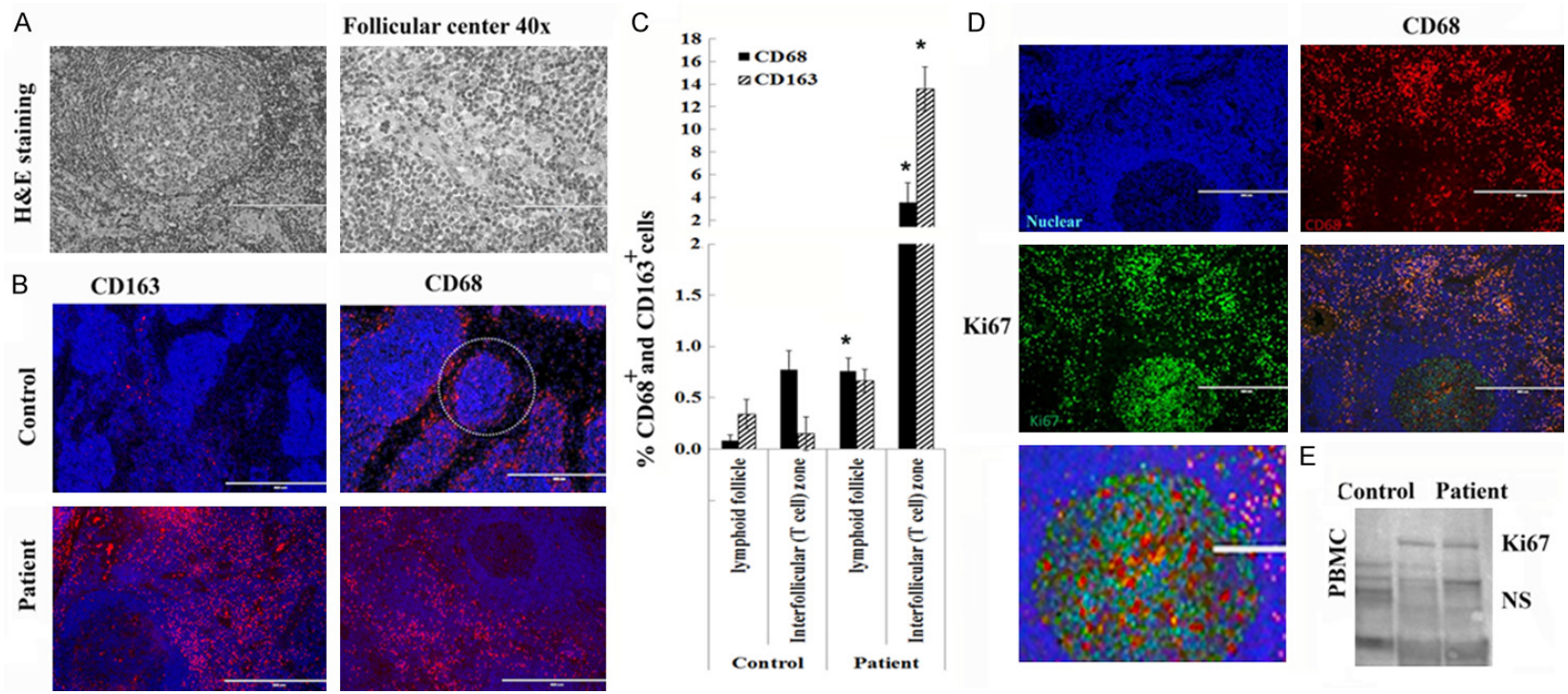

Figure 2. Histopathology of lymph node surgical tissue samples. A: Hematoxylin-eosin (H&E) staining showing nodal architecture with polarized germinal centers (left panel) and infiltration of macrophages (right panel: 40x magnification). B: IFC staining with macrophage markers CD68 and CD163 (red fluorescence) shows macrophages in follicular centers and interfollicular zones in patient sample (bottom panels), as compared to controls (top panels); blue represents nuclear staining (DAPI). C: Histogram showing CD68 and CD163 staining in the lymphoid follicles and interfollicular zones of both control and patient samples. D: ICF on lymph node biopsy. Top left panel: DAPI nuclear staining (blue). Top right panel: Staining with macrophage marker CD-68 (red). Bottom left panel: Staining with proliferative marker Ki67 (green). Bottom right panel: Dual Ki67 and CD68 staining (orange) confirms reactive follicular hyperplasia. E: Western blot analysis shows expression of Ki67 in patient PBMCs, but not in PBMCs from healthy control (NS: nonspecific signal).

samples. Higher number of Ki67 positive cells in follicular center suggested the possibility of reactive follicular hyperplasia than follicular lymphoma [11]. The dual-labeling of CD68 with proliferative marker Ki67 demonstrated that 43% and 12% of CD68 positive macrophages expressed Ki67 proliferative marker in follicular centers and interfollicular zones, respectively (**Figure 2D**). Ki67 expression profile in whole blood is a diagnostic tool for evaluation of lymphoproliferative disorders [10]. To further explore Ki67 expression magnitude, western blot analysis showed expression of Ki67 in peripheral blood cells (PBMC) (**Figure 2E**). The B (CD20) and T (CD3) cells were in appropriate distribution in lymph node mass (data not shown). However, further IHC examination showed monotypic IgG kappa plasma cells in the germinal centers and fewer IgM and IgA positive plasma cells. IgD staining highlighted the presence of intact mantles in the lymph node structure.

Due to the identification of IgM and IgA, immunoglobulin gene rearrangement studies were performed using PCR for framework region III and region II on DNA isolated from the right submandibular lymph node tumor. A polyclonal rearrangement pattern was detected in both FRII-IGH and FRIII-IGH reactions. IG PCR detected a clonal population comprising 2%-10% (FRIII-IGH=3%, FRII-IGH=10%) of the total B-cell population. The combined analysis of IGH and IGκ identified 85-90% of clonal B-cell proliferation. This finding was consistent with reactive follicular hyperplasia with intrafollicular IgG kappa monotypic plasma cells.

Flow cytometry on peripheral blood confirmed the normal range of T-/B-lymphocytes and NK/ and NKT cell fractions with reduced dendritic cell fraction. Further analysis of subgroups showed that there are no dysregulations in T-lymphocytes. In contrast, $CD27^+$/IgM producing B-cells were lower, 2.45 compared to a reference range of 5.6-27.2%, indicating reduced number of circulating marginal zone memory B-cells. This could be an effect of previous splenectomy [6]. In addition, kappa and lambda light chain analyses were performed on peripheral blood. The ratio of kappa to lambda was found to be 2:1 which is in the normal range.

Discussion

Lympadenopathy is commonly observed in GD, but rarely reported. It is the authors' experience that lymph node enlargement can occur at any anatomical site in GD, but limited case reports mention abdominal and mesenteric lympadenopathy causing morbidity as a space occupying lesion, and occasionally diagnosed as lymphoma. GD associated lymphadenopathy is known to be resistant to therapy [12, 13]. One report mentions collections of macrophages and scattered storage-type cells after needle core biopsies of abdominal lymphadenopathy in patient with Gaucher disease [14]. However, the origin and properties of these macrophages in relation to GD have never been studied. Our study demonstrates that $CD68^+$ and $CD163^+$ macrophages expressed Ki67, a marker of proliferative activity. M2 macrophages are known to promote tumor formation through cell-to-cell interaction by differentiating into tumor-associated macrophages. M2 cells also promote vascularization and formation of lymphatic vessels, and the above findings could reflect active pro-angiogenic properties of M2-tumor associate macrophages [15, 16].

While the majority of patients with GD and N370S/N370S genotype display late onset and predominantly skeletal disease, there is a higher incidence of gammopathies, multiple myeloma, and an increased risk of other cancers in this population [17]. It is known that the compound heterozygosity for the common mutation N370S with other severe mutations may be associated with an earlier disease onset, as well as osteonecrosis and hematologic complications [18]. The data for cancer risk in GD is not fully developed and primarily pertains to N370S/N370S patients due to it being the most common type of *GBA* mutation. Therefore, finding valid information about cancer incidence rates for GD patients is challenging for clinicians and researchers.

The mechanisms linking GD and cancer are not known, but several pathways may merit further investigation. One factor suggested to promote malignancy in GD is the chronic activation of endoplasmic reticulum (ER) stress especially for N370S/N370S mutations [17]. A second factor may be the microenvironment conducive to malignancy progression with the interaction of pathologically activated M2 macrophages [19, 20]. In this case, while the histopathology was consistent with reactive follicular hyperpla-

sia with intrafollicular monotypic plasma cells (IgG kappa), IFC analyses demonstrated the presence of activated macrophages of M2 phenotype. In a solid tumor microenvironment, macrophages were shown to differentiate into subgroups with different functional characteristics: classically-activated M1 and alternatively-activated M2 macrophages [15]. M2 cells can promote vascularization and formation of lymphatic vessels [16]. Therefore, because M2 macrophages have the tendency to promote tumor formation and hyperplasia through cell-to-cell interaction, they differentiate into tumor associated macrophages (TAM) [21, 22]. In the present case, we found that the population of M2 macrophages was relative to the proliferative state of the tumor. This is not the first clinical case where this phenomenon has been mentioned [8, 23]; however, there are no fundamental studies directly investigating how or why M2 macrophages express proliferative marker Ki67 [15]. Until recently, macrophages were regarded as end-differentiated cells without mitotic activity. Since self-renewal of M1 and M2 macrophages has been described in the literature, positive staining of macrophages with Ki67 may indicate proliferative activity or, at least, an activation state [24]. Also, Ki67 stains demonstrated a pattern of polarity and distinction between reactive follicular hyperplasia and follicular lymphoma [10]. Higher number of Ki67 positive cells in follicular center was consistent with reactive follicular hyperplasia than follicular lymphoma.

Lymph node enlargement has been reported in patients with MGUS and MM, sometimes due to localized amyloidosis [25]. The development of normal plasma cells involves lymph nodes in addition to the bone marrow [26]. In the bone marrow, the common lymphocyte progenitor cells differentiate into pro-B cells that undergo immunoglobulin heavy chain (IgH) gene rearrangement and differentiate into pre-B cells. Pre-B cells migrate into the lymph node from the bone marrow, where the IgM^+ B cells encounter antigen and become activated. In this current case, a polyclonal rearrangement pattern was detected in both FRII-IGH and FRIII-IGH reactions, and IG PCR detected a clonal population comprising 2%-10% of the total B-cell population. The combined analysis of IGH and IGκ identified 85-90% of clonal B-cell proliferation, which was consistent with reactive follicular hyperplasia. Abnormalities in the adaptive immune system in patients with GD, which is exacerbated by splenectomy could be an alternative hypothesis for co-occurrence of MGUS and lymphadenopathy and peripheral B cell abnormalities as demonstrated by immunophenotyping. In general population, hematologic malignancies: non-Hodgkin lymphoma, Hodgkin lymphoma, multiple myeloma, acute myeloid leukemia, chronic lymphocytic leukemia, chronic myeloid leukemia, and any leukemia are shown to be increased after 10 years or more after splenectomy [27]. Whereas in GD, splenectomy, once employed as a therapeutic tool, is also suggested as a contributing factor to malignancy [28]. Splenectomized patients with GD are more likely to have a malignancy or multiple malignancies [2]. However, the mechanisms how splenectomy may have contributed to the formation or progression of malignancy have not been studied. Most cancers observed in patients with GD originate from mature B cells, and it is well known that the disruption of B-cell differentiation and activation leads to lymphomas and leukemia's [29]. We have recently shown that the splenectomized patients with GD have significantly lower percentages of $CD27^+$ memory B-cells. The difference in $CD27^+$ B-cells was specifically due to a very low number of circulating non-isotype switched splenic memory B-cells ($CD27^+/IgM^+$) [6]. Lyso-sphingolipids have been shown as the primary antigenic stimulus in Multiple Myeloma associated with Gaucher disease [30]. The contribution of the immune dysfunction exacerbated by splenectomy requires further exploration in the development of malignancy in patients with GD.

While GD has been regarded as a simple Mendelian disorder with one gene-one protein deficiency, the comorbidities associated with GD continue to amaze both the clinician and researcher paving the way to understand multiple mechanisms interplaying in common disorders such as cancer.

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staining with CD20, CD3, kappa, lambda, IgG, IgA, IgM, and IgD.

Disclosure of conflict of interest

None.

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