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Molecular Single-Cell Analysis of Hodgkin- and Reed-Sternberg Cells Harboring Unmutated Immunoglobulin Variable Region Genes

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SUMMARY: Hodgkin- and Reed-Sternberg (H/RS) cells in classical Hodgkin's disease of the B lineage are the clonal progeny of antigen-experienced B cells harboring highly mutated immunoglobulin variable (V) region genes. Based on the detection of obviously destructive somatic mutations in a fraction of cases, we speculated that H/RS cells may be derived from a pre-apoptotic germinal center B cell. Seemingly contradicting this speculation, we present here the first case of classical Hodgkin's disease with H/RS cells harboring unmutated, potentially functional V region genes, which may indicate the derivation of the H/RS clone from a naive B cell. However, germinal center founder cells, which have not yet acquired somatic mutations, already have the intrinsic propensity to die by apoptosis. Thus, the rare occurrence of H/RS cells with unmutated V genes is expected if the H/RS cells are derived from the pool of pre-apoptotic germinal center B cells. (*Lab Invest 2001, 81:289–295*).

In classical Hodgkin's disease (cHD), Hodgkin- and Reed-Sternberg (H/RS) cells typically account for less than 1% of cells within a complex admixture of lymphocytes, plasma cells, histiocytes, and eosinophils (Burke, 1992). In most cases of cHD, H/RS cells do not express B lineage markers, although in more than 90% of cHD cases, H/RS cells represent the outgrowth of a tumor clone derived from mature antigen-experienced B cells (Küppers and Rajewsky, 1998), and derivation of H/RS cells from the T lineage is rare (Müschen et al, 2000). This conclusion is based on the amplification of clonally related immunoglobulin (Ig) gene rearrangements from single micromanipulated H/RS cells in 45 of 46 analyzed cases (Bräuninger et al, 1999a; Irsch et al, 1998; Kanzler et al, 1996a, 1996b; Küppers et al, 1994; Marafioti et al, 1999, 2000; Müschen et al, 2000; Ohno et al, 1998; Vockerodt et al, 1998). In all informative B-lineage cases (41 in total), the H/RS cells harbored somatically mutated variable (V) region genes, indicating their (post-) germinal center (GC) B-cell origin. In 12 of 41 cases, the originally productive Ig V diversity (D) joining (J)-gene rearrangements were rendered nonfunctional by the introduction of stop codons, deletions, or destructive promoter mutations generated within the GC (Jox et al, 1999; Kanzler et al, 1996a; Küppers et al, 1994; Marafioti et al, 2000; Müschen et al, 2000). H/RS cells for which B-cell receptor (BCR) function was compromised by destructive somatic mutations are derived from GC B cells as BCR-deficient B cells are eliminated within the GC, and thus prevented from entering the memory compartment (Küppers and Rajewsky, 1998).

GC B cells are programmed to die unless positively selected by an antigen, thus the expression of a high-affinity BCR is critical for GC B-cell survival (Liu et al, 1989). A GC B cell within the highly competitive microenvironment of the GC can be eliminated for many reasons (Liu et al, 1997; Rajewsky, 1996). For instance, a lack of positive selection of a GC B cell can be caused by destructive somatic mutations (eg, mutations generating stop codons or frame shifts), an impairment of BCR expression (eg, by transcriptional deregulation [Marafioti et al, 2000] or destructive promoter mutations), a lack of co-stimulatory signals (eg, in case of autoreactivity), or insufficient antigen affinity (eg, by introduction of replacement mutations that reduce affinity).

Possible reasons for the lack of positive selection by antigen other than obviously destructive mutations (ie, promoter or splice site mutations and mutations generating stop codons, large deletions, duplications, and/or frameshifts) evade the technical means of analysis at the single-cell level. In approximately one third of cHD cases, H/RS cells harbor obviously destructive Ig gene mutations [such mutations are rare

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(< 5%) in normal GC B cells (Goossens et al, 1998; Küppers et al, 1993, 1997)]. Thus, the hypothesis that H/RS cells may commonly represent the outgrowth of a pre-apoptotic germinal center B cell was developed (Kanzler et al, 1996b; Küppers and Rajewsky, 1998).

Here we describe the first case of B lineage cHD in which the H/RS cells harbor virtually unmutated Ig V region genes.

Results

The H/RS cells in the cHD case presented here exhibit a typical immunophenotype, with co-expression of CD30 and CD15 (Fig. 1). The H/RS cells have phenotypic features of both the B and the T lineage in that they partly express the B-cell antigen CD20 and the cytotoxic T-cell markers, granzyme B and TIA-1. B and T lineage markers are found only in a minority of cHD and have not been reported to be expressed by H/RS cells in the same case (Burke, 1992; Küppers and Rajewsky, 1998). The H/RS cells are Epstein-Barr virus (EBV)-positive as assessed by amplification of a specific fragment of the EBV-nuclear antigen-1 gene by single-cell PCR (Table 1) and by in situ hybridization for EBV-encoded small RNA. Because the majority of anaplastic large-cell lymphomas express one or more of the cytoxic T-cell markers studied here, diagnosis of cHD was further corroborated by demonstrating that the H/RS cells lack expression of the nucleophosmin-anaplastic lymphoma kinase molecule (Weiss et al, 1995). The nucleophosmin-anaplastic lymphoma kinase fusion protein arises from the translocation t(2; 5) (p23; q35) which is typically seen in anaplastic large-cell lymphomas but not in cHD (Weiss et al, 1995).

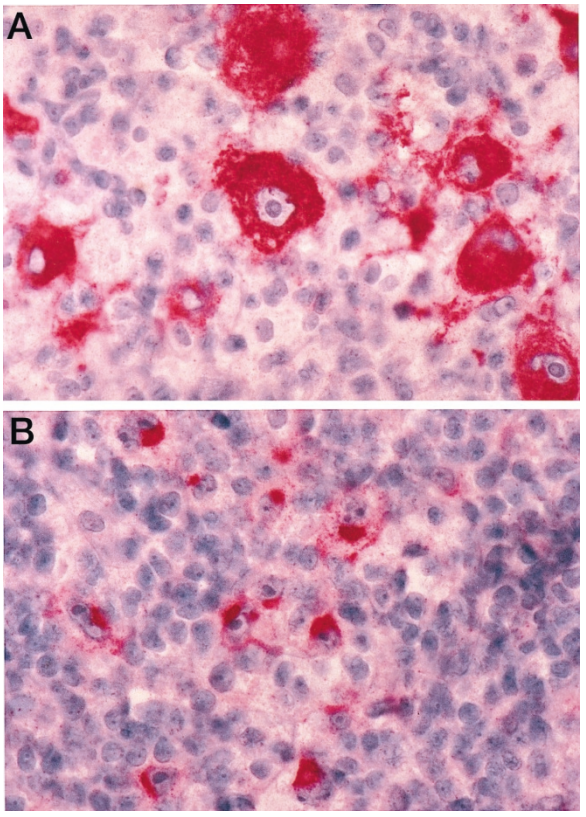

Figure 1.
Immunophenotype of Hodgkin- and Reed-Sternberg (H/RS) cells. CD30 immunoreactivity of paraffin-embedded sections from the case of cHD studied here (subtype mixed cellularity diagnosed on cervical lymph node biopsy at first presentation) (A, original magnification, ×80). Hodgkin cells show membrane-bound and intracytoplasmic immunoreactivity for CD15 (B, original magnification, ×60).

A total of 120 H/RS cells were micromanipulated from sections stained for CD30 and granzyme B in three experiments (39, 51, and 30 H/RS cells, respectively) and analyzed by single-cell PCR. The first experiment (39 H/RS cells) was omitted from analysis because there was an indication of PCR contamination (see "Materials and Methods"). Derivation from either the B or the T lineage is possible for H/RS cells expressing cytotoxic T-cell markers (Müschen et al, 2000). Micromanipulated H/RS cells were analyzed for Ig and TCRβ gene rearrangements (Table 1).

No TCRβ VDJ- or DJ-gene rearrangements were obtained from the analysis of 51 H/RS cells (Table 1), although fragments specific for germline configuration of the TCR Cβ1 and Cβ2 loci were repeatedly amplified (Table 1). However, three clonal Ig-gene rearrangements, namely a V_H1-, a Vκ2-, and a Vκ3-gene rearrangement, were amplified from 81 H/RS cells. Hence, this case represents another example of cHD in which the H/RS cells expressed T lineage markers although derived from a mature B cell (Müschen et al, 2000).

The V_H1-46 gene rearrangement was in-frame and potentially functional. The Vκ2 family pseudogene A18 was rearranged out-of-frame resulting in a nonfunctional rearrangement on one allele. However, the other allele carried a potentially functional rearrangement of the Vκ3 family gene, L2. Thus, the configuration of the IgH- and Igκ-gene loci was compatible with expression of a functional BCR. Igλ-gene rearrangements were not obtained.

Within 320 bp of the $V_HD_HJ_H$-gene sequence and 580 bp of the VκJκ-gene sequence there was only one mismatch to the most homologous germline genes. This mismatch, at codon 11 of the V_H1-46 gene (resulting in a conservative val to leu replacement) (Table 2) might represent either a rare germline polymorphism or a single somatic mutation. To determine whether further differences from germline sequences were present within the rearranged IgH- and Igκ-genes, additional regions of the leader, leader intron, and framework region I of the V_H1-46, Vκ2 (A18), and Vκ3 (L2) genes were amplified and sequenced. Additionally, downstream J_H- regions and downstream Jκ-regions were analyzed because these regions are also possible targets for somatic hypermutation. However, all of these sequences were unmutated. In a total of 0.7 kilobases of IgH sequence there was only the single mismatch mentioned above. Within 1.5 kilobases of the rearranged Vκ2 and Vκ3 genes, including adjacent regions, there was no mismatch to the respective germline genes.

Table 1. Summary of Single Cell PCR Analysis of Micromanipulated H/RS Cells

			PCR products		Rearrangements/*germline* configuration[c]		
Experiment	Immunohistochemistry	Gene locus[a]	H/RS cells positive	Total	Sequenced	Repeated	Unique
I	CD30	IgH	9/41	9 V_H1	9	9 V_H1	
	Granzyme B	IgH	3/10	2 V_H1	2	2 V_H1	
				1 V_H3	1		1 V_H3
	CD30	Igκ	15/41	13 Vκ2	13	13 Vκ2	
				10 Vκ3	10	10 Vκ3	
			10/10	10 KDE *germline*[b]	10	10 KDE *germline*	
			9/10	9 JκCκ intron-KDE[b]	4	4 JκCκ intron-KDE[b]	
	Granzyme B	Igκ	3/10	3 Vκ2	3	3 Vκ2	
				2 Vκ3	2	2 Vκ3	
		Igλ	0/12				
	CD30	TCR Cβ1	15/41	15 *germline*		15 *germline*	
	Granzyme B	TCR Cβ1	7/10	7 *germline*		7 *germline*	
	CD30	TCR Cβ2	15/41	15 *germline*		15 *germline*	
	Granzyme B	TCR Cβ2	6/10	6 *germline*		6 *germline*	
	CD30	EBNA-1	21/41	21 EBNA-1			
	Granzyme B	EBNA-1	6/10	6 EBNA-1			
II	CD30	IgH	7/30	7 V_H1			
	CD30	Igκ	14/30	8 Vκ2			
				8 Vκ3			
				13 KDE *germline*	13	13 KDE *germline*	
			13/30	13 JκCκ intron-KDE	6	6 JκCκ intron-KDE	

Controls	Micromanipulated		Sorted by flow cytometry	
Gene locus	B cells	Buffer controls	B cells	T cells
IgH	10/30	0/12	3/4	
Igκ	2/10	0/12	2/4	
Igλ		0/4	1/3	
TCR Cβ1, 2		1/10 *germline*	4/6 *germline*	8/18VDJ
EBNA-1		0/6	2/2 (EBV$^+$ B cells)	

H/RS, Hodgkin- and Reed-Sternberg; EBNA-1, Epstein-Barr virus nuclear antigen-1; KDE, Kappa deleting element.

[a] For IgH and IgL loci only V(D)J-rearrangements, for TCRβ loci VDJ- and DJ rearrangements as well as *germline* configuration were analyzed. IgH- and Igκ- gene rearrangements were amplified using framework region I- and leader-specific primers.

[b] Only Vκ^+ cells were analyzed for KDE rearrangements.

[c] *Germline* configuration was identified by specific primer sets.

VκJκ joints can be transcriptionally silenced and exempted from somatic hypermutation by rearrangement of the kappa deleting element (KDE) to a recombination signal sequence in the Jκ-Cκ intron (Klein et al, 1998; Siminovitch et al, 1985). To exclude the possibility that the lack of somatic mutations in the VκJκ joints was caused by KDE recombinations, the rearrangement status of the KDE in the H/RS cells was studied. A fragment indicative of the germline configuration of the KDE and a clonal JκCκ intron-KDE rearrangement were repeatedly amplified (Table 1). This indicated that one of the two VκJκ joints was inactivated by KDE rearrangement, whereas the VκJκ rearrangement on the other allele was not. Thus, one of the two Vκ-gene rearrangements could be transcribed and should have been accessible to somatic hypermutation.

Given that the V_H and Vκ region genes were virtually unmutated, the possibility arose that the H/RS cells might stem from a naive rather than an antigen-experienced B cell. Therefore, the H/RS cells were investigated by immunohistochemistry using antibodies to Bcl-6 and CD138, which are thought to be markers of GC or post-GC origin of H/RS cells, respectively (Carbone et al, 1999). Approximately half of the H/RS cells were positive for Bcl-6 but not for CD138.

Table 2. Sequence Analysis of Clonal Rearrangements Obtained from H/RS Cells[a]

Gene locus	Rearrangement/ *germline*	In-frame	Potentially functional	% mutation	Intraclonal diversity	Remarks
IgH	V_H1-46-D3-22-J_H4b	Yes	Yes	0.1	No	1 nucleotide difference
Igκ	Vκ2 (A18)-Jκ5	No	No	0	No	Pseudogene; translational stop
	Vκ3 (L2)-Jκ2	Yes	Yes	0	No	
	JκCκ intron-KDE	n/a	n/a	0	No	Allele assignment unclear
TCRβ	Cβ1 cluster *germline*	n/a	n/a	0	No	
	Cβ2 cluster *germline*	n/a	n/a	0	No	

H/RS, Hodgkin- and Reed-Sternberg; KDE, kappa deleting element; Ig, immunoglobulin; TCRβ, T-cell receptor β.
[a] Sequence data from the four rearrangements are available at EMBL/GenBank under accession numbers 291990–291994.

Discussion

In all informative cases of B-cell lineage cHD (41 in total) analyzed so far (Bräuninger et al, 1999a; Irsch et al, 1998; Kanzler et al, 1996a, 1996b; Küppers et al, 1994; Marafioti et al, 1999, 2000; Müschen et al, 2000; Vockerodt et al, 1998), the H/RS cells harbor somatically mutated Ig genes with mutation frequencies of the V_H genes between 2% and 23% (average 10%; Klein et al, 1998). In the H/RS cells analyzed here, only one difference from the most homologous germline gene was detected in the Ig heavy chain gene rearrangement and no mutation was detected within the two Igκ light chain gene rearrangements. This might be indicative of an origin of the H/RS-cell clone from a naive B cell. Besides naive B cells, many "founder" cells of GC also carry unmutated Ig V region genes (Lebecque et al, 1997; McHeyzer-Williams et al, 1993). Therefore, derivation of the H/RS cells harboring unmutated V region genes from an early GC B cell is an alternative possibility. Moreover, the single nucleotide difference in the V_H region gene may represent a first point mutation introduced by the somatic hypermutation machinery. A potential GC B cell derivation of the H/RS cells in the present case is also supported by the expression of Bcl-6 in a fraction of the H/RS cells. Thus, the unprecedented lack of somatic mutation in the Ig V region genes in the present case remains an ambiguous finding and does not definitely indicate that a naive B cell was the precursor of the tumor clone. The finding that H/RS cells do not always harbor somatically mutated V region genes is reminiscent of chronic lymphocytic B-cell leukemia, which comprises two subsets that are thought to be derived from antigen-experienced and naive B cells, respectively (Damle et al, 1999; Hamblin et al, 1999). However, the cases of cHD in which the H/RS cells harbor unmutated Ig genes are apparently very rare, as opposed to chronic lymphocytic B-cell leukemia.

BCR function was impaired because of obviously destructive Ig gene mutations (see "Introduction") in 12 of 41 cases of cHD, defining a pre-apoptotic GC B cell as the precursor of the tumor clone (Jox et al, 1999; Kanzler et al, 1996b; Küppers et al, 1994; Marafioti et al, 2000; Müschen et al, 2000). In the case of cHD presented here, the Ig V region genes analyzed were virtually unmutated. This suggests that, in this case, cHD developed in the absence of obviously destructive somatic mutations or other mutations reducing affinity to antigen.

Does this argue against an origin of the disease in this patient from a pre-apoptotic GC B cell? We do not believe that this is the case. B cells seem to be programmed to undergo apoptosis in the absence of positive selection by antigen from the time of entry into the GC environment (Fig. 1), before the onset of somatic hypermutation (Lebecque et al, 1997). GC B cells are stringently selected for the acquisition of somatic mutations improving affinity for the immunizing antigen. Thus, it is expected that not only cells that have acquired mutations interfering with antigen binding, but also cells that carry neutral mutations or have not yet initiated hypermutation (ie, GC "founder" cells) and therefore fail to successfully compete for antigen with their companions, will be among those cells that undergo apoptosis in GC (Fig. 2). Thus, in accordance with the scenario of a derivation of H/RS cells from pre-apoptotic GC B cells, one would predict that in rare cases of cHD, GC "founder" B cells harboring as yet unmutated V region genes should be the precursors of the tumor clone.

Materials and Methods

Immunohistochemistry

Tissue sections were taken from a cervical lymph node of a 68-year-old patient at first presentation. Immunohistochemistry was performed on 7-μm-thick frozen tissue sections, with antibodies against CD30 (Fig. 1A), CD20, anaplastic lymphoma kinase (ALK)-1 (Dako, Glostrup, Denmark), CD15 (Becton Dickinson, Mountain View, California; Fig. 1B), CD3 (Ortho Diagnostic Systems, Raritan, New Jersey), Bcl-6 (Novocastra, Newcastle-upon-Trent, United Kingdom), CD138 (syndecan-1; Serotec, Oxford, United Kingdom), T-cell receptor (TCR) α/β (T Cell Diagnostics, Woburn, Massachusetts), perforin (Neo Markers, Union City, California), granzyme B (Monosan, Am Uden, The Netherlands), or TIA-1 (Immunotech, Marseilles, France).

Micromanipulation and Single-Cell PCR for Ig and TCRβ Genes

Immunoreactive cells were mobilized and aspirated with a micropipette fixed to a hydraulic micromanipu-

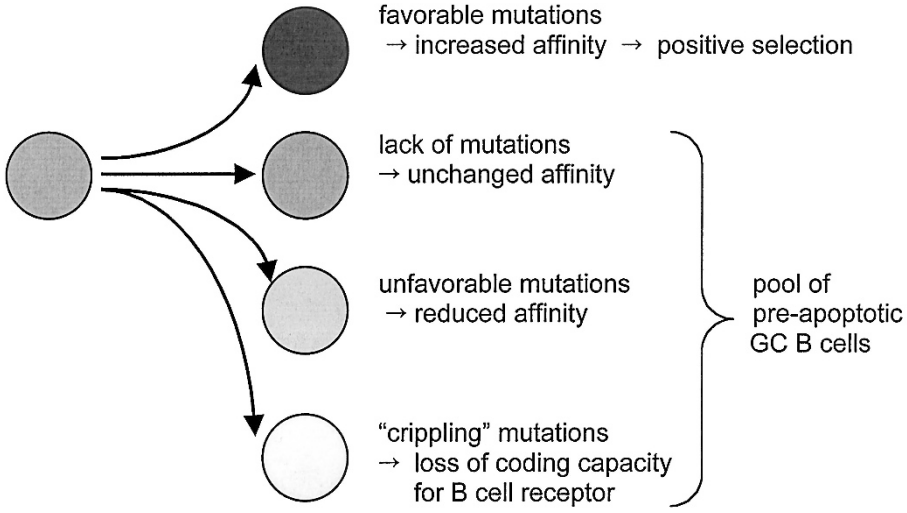

Figure 2.

Derivation of the H/RS cell clone from a pre-apoptotic germinal center (GC) B cell. GC B cells are committed to die by apoptosis unless they are positively selected for a B-cell receptor of high affinity to antigen. The failure to increase affinity to antigen during the GC reaction may have several reasons, one of which is the lack of somatic mutations within the Ig V region genes as discussed here.

lator (Küppers et al, 1993). Buffer covering the sections was aspirated for negative controls for the PCR analysis. For positive controls for PCR, single B and T cells were either micromanipulated or sorted by flow cytometry.

To analyze individual micromanipulated cells for IgH, Igκ, Igλ, and TCRβ VDJ- and DJ-gene rearrangements or TCRβ germline configuration, whole genome pre-amplification (Zhang et al, 1992) was performed with slight modifications. Aliquots of 4 μl from these reactions were then subjected to two rounds of semi-nested PCR amplification as previously described. Briefly, rearranged V_H-, Vκ-, and Vλ- genes were amplified using family-specific framework region I (FRI) V-gene primers and two sets of J_H-, Jκ-, and Jλ-primers in a semi-nested approach (Bräuninger et al, 1999b; Kanzler et al, 1996a). In some experiments, IgH- and Igκ-gene rearrangements were amplified using V_H leader- (Bräuninger et al, 1997) and Vκ leader-specific primers instead of V_H- and Vκ-FRI-primers. The following Vκ leader family-specific primers were used: 5′-GCTCAGCTCCTGGGGCTCCTGC-3′ for Vκ1L, 5′-CTCAGCTYCTGGGGCTGCTAATGCT-3′ for Vκ2L, 5′-CTCAGTTAGGACCCAGABGGAACCAT-3′ for Vκ3L, and 5′-GGGGCAGCAAGATGGTGTTGCAGA-3′ for Vκ4L. Within the IgH locus, the intronic sequence flanked by the J_H4- and the J_H5-gene was amplified using 5′-TCCTCAGGTGAGTCCTCAACCTCT-3′ and 5′-CTCACAACCTCTCTCCTGCTTTA-3′ as external and internal forward primers, respectively, and 5′-TAGGC-CTCTGGGGTCCAATGC-3′ as reverse primers. The intron between the VκJκ-joint and the next downstream Jκ-genes were amplified using primers specific for the rearranged Vκ-genes and primers for the next downstream Jκ-genes. Rearrangements of the KDE to a Vκ-germline gene segment or a recombination signal sequence site in the JκCκ intron, or to a specific fragment for germline configuration of this element, were amplified as previously described (Küppers et al, 1996).

The same cells were subjected to semi-nested amplification of TCRβ VDJ and DJ rearrangements and fragments specific for germline configuration of the Cβ1 and Cβ2 loci, as previously described (Müschen et al, 2000; Roers et al, 1998).

PCR products were gel-purified and directly sequenced using the BigDye Terminator cycle sequencing kit and an automated sequencer (ABI 377; Applied Biosystems, Weiterstadt, Germany). Sequences were compared with the EMBL IMGT database (http://www.genetik.uni-koeln.de/dnaplot/).

In a first experiment, one clonal V_H and two clonal Vκ gene rearrangements were repeatedly amplified from 39 micromanipulated H/RS cells. These rearrangements were also obtained when DNA isolated from whole tissue sections of the biopsy specimen was analyzed for V_H and Vκ gene rearrangements in one round of PCR with 35 cycles (data not shown). Because an additional V_H3 gene rearrangement (out-of-frame and unmutated) was amplified in this PCR, the whole genome preamplification aliquots of the 39 H/RS cells were analyzed again for V_H3 gene rearrangements by single-cell PCR. Whereas the first three attempts did not give rise to a single V_H3 amplificate, eight polyclonal V_H3 gene rearrangements (all unrelated to the V_H3 gene amplified from the whole-tissue DNA) were obtained in the fourth amplification. These amplificates probably represent a PCR contamination of the whole-genome preamplification reactions in the fourth amplification. For this reason, the first micromanipulation and PCR experiment was completely omitted from analysis.

Determination of Epstein-Barr Virus Infection

Epstein-Barr virus (EBV)-infection of H/RS cells was examined by in-situ hybridization for noncoding EBV-encoded small RNA and by amplification of a fragment of the EBV-nuclear antigen-1 gene from single micro-

manipulated H/RS cells using the previously published primers in a semi-nested PCR (Müschen et al, 2000).

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