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Cancer Res 2001;61:2080-2084.

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Oct-2 and Bob-1 Deficiency in Hodgkin and Reed Sternberg Cells¹

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ABSTRACT

Hodgkin and Reed Sternberg (H-RS) cells represent the malignant cells in classical Hodgkin's disease. Although derived from germinal center B cells, they do not express surface immunoglobulin. This has been explained by the presence of crippling mutations within the immunoglobulin genes in numerous cases of Hodgkin's disease. As immunoglobulin gene expression in B cells requires an interaction between octamer sites and the transactivating factors Oct-2 and Bob-1, this study addresses the expression of the transcription factors Oct-2 and Bob-1 in H-RS cells. In Hodgkin's disease-derived cell lines, low levels of Oct-2 transcripts but no Oct-2 protein were detected. Transcripts of Bob-1, a B-cell-specific cofactor of Oct-2, could not be observed in these cell lines. Absence of Oct-2 and Bob-1 protein expression in primary H-RS cells was demonstrated by performing immunohistochemistry in 20 cases of classical Hodgkin's disease. H-RS cells stained negative for both proteins in all of the cases analyzed. In conclusion, absence of functional Oct-2 and Bob-1 cells represents a novel mechanism for immunoglobulin gene deregulation in H-RS cells. Lack of Oct-2 and Bob-1 points to a defect in transcription machinery in H-RS cells and is associated with lack of immunoglobulin gene expression in these cells.

INTRODUCTION

H-RS³ cells represent the malignant cells in classical HD. In most cases, the H-RS cells are derived from GC B cells because they harbor somatically mutated immunoglobulin-V region genes (1–3), whereas derivation from T cells is rare (4). Notably, in a substantial proportion of classical HD cases, the H-RS cells had lost their capacity to express a functional B-cell receptor because of obviously destructive somatic mutations, rendering potentially functional immunoglobulin gene rearrangements nonfunctional (2). However, in other cases potentially functional immunoglobulin gene rearrangements were detected in H-RS cells (3, 5), indicating that in these cases, immunoglobulin gene expression might be possible in the lymphoma cells. However, *in situ* hybridization experiments failed to show detectable amounts of IgL and IgH mRNA in these cases. From these data, it was concluded that in these cases the immunoglobulin gene transcription is deregulated in H-RS cells (3).

Expression of rearranged *IgH* and *IgL* genes is critical for B-cell differentiation and is regulated by a complex interaction between regulatory DNA elements and transcription factors (6). Among the regulatory DNA elements necessary for B-cell-specific transcription, the octamer motif is an important transcriptional regulatory site that is part of promoters and enhancers of ubiquitously expressed genes.

Furthermore, this octamer motif is found in all of the IgH and IgL promoters and in the heavy chain and κ light chain enhancer elements (7). It has been shown to be essential for B-cell specificity and activity of the immunoglobulin promoter and enhancer (8, 9). The octamer site interacts with transcription factors belonging to the POU family of homeodomain-proteins binding specifically to this octamer motif via their POU domain (10).

Oct-1 was identified as a ubiquitous protein, whereas Oct-2 expression is restricted to B cells and neuronal cells (11). In B cells and neuronal cells, alternative splicing of Oct-2 generates several proteins (12, 13). On the basis of transfection experiments, for Oct-2 a critical role for immunoglobulin promoter transactivation was shown (12). Recent studies (14) demonstrated that in addition to Oct-2, a B-cell-specific cofactor, namely Bob-1, is required. Thus, B-cell specificity of immunoglobulin promoter activity is mediated by the expression of Bob-1 (OCA-B or OBF-1). Bob-1 associates with the POU domain of octamer proteins Oct-1 and Oct-2 and alters their recognition specificity (10). In a Bob-1-deficient mouse model, GC formation was drastically impaired, and class switch recombination was reduced substantially (15).

Because H-RS cells derive from GC B cells (16) but lack immunoglobulin gene expression, we now address the role of Oct-2 and Bob-1 for immunoglobulin gene transcription in classical HD. In this study, we discuss whether a disturbed expression of transcription factors is involved in the deregulation of immunoglobulin gene transcription in classical HD.

MATERIALS AND METHODS

Cell Lines. The characteristics of the seven HD-derived cell lines are summarized by Drexler (17) and Wolf *et al.* (18). IARC277 is an EBV-immortalized lymphoblastoid cell line (19), and BJA-B is an EBV-negative B-cell lymphoma cell line established from the biopsy of a five-year-old patient suffering from an EBV-negative African Burkitt lymphoma (20). The adherent fibroblastic cell line NIH/3T3 and the human T-lymphoblastic leukemia cell line Jurkat were obtained from the American Type Culture Collection (Manassas, VA). All of the cell lines were grown according to standard procedures.

Pathological Specimen. Twenty primary cases of classical HD and one nonneoplastic lymph node sample were analyzed by immunohistochemistry. Characteristics are listed in Table 1. Pathological specimen were classified according to the WHO classification (21). All of the diagnoses have been reviewed by the pathologist reference panel of the German Hodgkin's Lymphoma Study Group.

Cell Separation and Flow Cytometry. B-cell subsets were purified from a reactive tonsil of a child and from peripheral blood of an unrelated donor. CD38⁺CD77⁺ GC B cells were isolated according to Goossens *et al.* (22). After two cycles of magnetic cell sorting enrichment of CD77⁺ cells, purity of these GC B cells was more than 90%. After the separation of CD38⁺CD77⁺ cells, tonsillar naïve (IgD⁺CD27⁻) B cells were isolated from the flow-through fraction and separated from memory cells on the basis of their CD27 expression according to Klein *et al.* (23). The naïve phenotype (IgD⁺IgM⁺CD20⁺) was verified by flow cytometry. Purity of these two B-cell subsets was confirmed by amplifying and sequencing of the immunoglobulin V_H gene rearrangements from single sorted cells (8 naïve cells were all unmutated, and 19 GC B cells were all mutated).

Received 6/5/00; accepted 1/18/01.

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¹ Supported by the Deutsche Forschungsgemeinschaft through SFB502. D. R. is supported by the Friedrich and Maria Sophie Moritz'sche Stiftung (Cologne, Germany). M. M. holds a postdoctoral fellowship from the Cancer Research Institute (Tumor Immunology Program, New York, NY).

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³ The abbreviations used are: H-RS, Hodgkin and Reed Sternberg; HD, Hodgkin's disease; GC, germinal center; RT-PCR, reverse transcriptase PCR.

Table 1 Description of primary cases of classical HD and results of immunohistochemistry for Oct-2 and Bob-1

Case	HD-subtype	Age	Presentation	Localization	EBV ^a	Bob-1	Oct-2
4	NS ^b	13	First	Cervical	-	-	-
5	NS	13	First	Cervical	+	-	-
7	NS	35	First	Cervical	-	-	-
8	NS	28	First	Supraclavicular	-	ni ^c	-
9	NS	16	First	Infraclavicular	+	-	-
15	NS	32	First	Supraclavicular	-	-	-
19	NS	19	First	Cervical	+	ni	-
20	NS	66	First	Cervical	+	-	-
2	NS	22	First	Cervical	-	-	-
6	MC/LD	46	First	Paraaortal	+	-	-
1	MC	38	First	Supraclavicular	-	-	-
10	MC	79	First	Cervical	-	-	-
13	MC	58	First	Axillar	+	-	-
14	MC	36	First	Cervical	-	-	-
16	MC	62	First	Cervical	+	-	-
18	MC	61	First	Cervical	-	-	-
3	LR	32	First	Parasternal	+	-	-
11	LR	69	First	Cervical	-	-	-
12	LD	40	First	Cervical	-	-	-
17	LD	na	First	Axillar	+	-	-
21	Reactive	na	Axillar	na	nd	+	+

^a EBV infection was assessed by staining for LMP1; +, strong reactivity; (+), weak reactivity; -, no reactivity; nd, not done.

^b NS, nodular sclerosis; MC, mixed cellularity; LD, lymphocyte depleted; LR, lymphocyte rich classical HD; na, not assessed/not applicable.

^c ni = not informative.

Western Blot. Cellular protein extracts were prepared in Laemmli buffer, boiled for 10 min, and chilled on ice. Protein extract (20 μ g) were separated on a discontinuous denaturing SDS-PAGE containing 7.5 or 10% acrylamide. The gel was blotted onto nitrocellulose filters (Hybond C extra; Amersham-Pharmacia, Freiburg, Germany). Equal loading of the gel was verified by Ponceau S staining. Unspecific binding of the antibody was inhibited by incubating the blot for 1 h with blocking buffer. Subsequently, blots were incubated overnight at 4°C either with a rabbit polyclonal antibody (dilution 1:10,000) raised against a peptide mapping at the COOH terminus of Oct-2 (clone C-20; Santa Cruz, Heidelberg, Germany) or with a mouse monoclonal antibody (dilution 1:5,000) recognizing an NH₂-terminal segment of Oct-2 present in all of the isoforms (clone PT1; Oncogene, Cambridge, MA). After washing the blot three times with Tris-buffered saline/0.05% Tween, the peroxidase-coupled second antibody [rabbit-antimouse horseradish peroxidase (1:2,000); DAKO, Hamburg, Germany; or goat antirabbit horseradish peroxidase (1:2,000); DAKO] was added. Blots were developed using the enhanced chemiluminescence system (Amersham-Pharmacia) according to the manufacturer's recommendations.

RT-PCR. Poly(A)⁺ RNA was extracted from the cell lines using the MicroMACS mRNA Isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Subsequently, cDNA was generated using an oligodeoxythymidylate oligonucleotide and Superscript reverse transcriptase (Life Technologies, Inc., Karlsruhe, Germany). An oligonucleotide hybridizing to the 5' coding region (Oct2.S 5'-agccctcaaggcagccac t-3') and two oligonucleotides hybridizing to the 3' untranslated region of the two known human Oct-2 isoforms (Oct2.AS 5'-tcaagaagagcggcgaggt-3' and Oct2.AS-trun 5'-cctgcttctcccaccgat-3') were used for amplification of Oct-2. For amplification of the alternatively spliced COOH-terminal end of Oct-2B, an oligonucleotide hybridizing to the 3' end of a known Oct-2 isoform (GenBank accession no. NM002698) was used (Oct2.AS-R4 5'-caggcaagggaagcagga-3'). Primers for amplification of Bob-1 hybridized to exon 2 (Bob1.S 5'-tgtgaagaagccagtgagg-3') and exon 4 (Bob1.AS 5'-aacactgaggaggcccca-3'), respectively. RT-PCR was performed in a 50- μ l reaction mixture containing 50 mM KCl, 2.5 mM MgCl₂, 200 μ M of each deoxynucleotide triphosphate, and 25 pmol of each oligonucleotide. All of the amplifications (35 cycles) were done at an annealing temperature of 57°C. For sequence analysis, PCR products were purified from agarose gels (1%) using the Jetsorb kit (Genomed, Bad Oeynhausen, Germany) and subsequently cloned into pGEMeasy constructs (Promega). Ligation, heat shock transformation of the *Escherichia coli* strain DH5 α , and DNA preparation were done according to standard protocols. Sequencing of plasmid DNA was done using the oligonucleotides M13/pUC Forward and Reverse (Life Technologies, Inc.) and the Ready Reaction Dye Terminator cycle sequencing kit (Perkin-Elmer, Weiterstadt, Germany). Sequence reactions were separated and analyzed using a sequencing unit (ABI 377; Applied Biosystems/Perkin-Elmer) following instructions of the manufacturer.

Immunohistochemistry. Immunostaining was performed on paraffin-embedded formalin-fixed lymph nodes from all of the patients. Staining of a nonmalignant reactive lymph node was used as positive control. Anti-Oct-2 antibodies (clone PT1; Oncogene) and anti-Bob-1 antibodies (sc955; Santa Cruz) were used for these experiments. Sections (6 μ m) were mounted on standard slides, deparaffinized in xylene, rehydrated in graded alcohol, and washed in water. Staining was performed according to standard procedures. Antibody reactions were detected with avidin-biotin-coupled alkaline phosphatase (DAKO) and FastRed as chromogen (DAKO). Subsequently, slides were counterstained with hemalaun (Merck, Darmstadt, Germany). The percentage of Oct-2 and Bob-1 positive H-RS cells of a given case was evaluated, and staining was classified in four categories: strong (+), weak (+/-), absent (-), or not informative.

RESULTS

Detection of Oct-2 Transcripts in HD-derived Cell Lines. To amplify human Oct-2 isoforms differing at their COOH terminus, we used two sets of oligonucleotides hybridizing to the different isoforms. We detected transcripts for Oct-2A and Oct-2B in all of the H-RS cell lines as well as in the BJA-B cell line. The amount of amplified products was similar in all of the HD-derived cell lines but much lower than in the B-cell line BJA-B. Because cell lines L1236, L428, KM-H2, and L591 harbor rearrangements of immunoglobulin genes and cell line DEV expresses IgA2, these cell lines are considered to derive from B cells. By comparison, cell lines L540 and HdIm-2 harbor clonal rearrangements of the T-cell receptor genes and, thus, are considered to derive from T cells. Because both the B-cellular and the T-cellular HD-derived cell lines express the same low level of the B-cell-specific Oct-2 transcripts, this indicates a non-B-cell-specific transcription background in H-RS cell lines (Fig. 1).

Lack of Oct-2 Expression in HD-derived Cell Lines. Using a polyclonal antibody raised against a peptide mapping at the COOH terminus of Oct-2A, we analyzed the expression of this protein in the three EBV negative B-cellular HD-derived cell lines L1236, L428, and KM-H2. Western blot analysis showed a strong signal for BJA-B cells used as positive control, whereas Oct-2A expression was missing in the HD-derived cell lines (Fig. 2). The Oct-2A signal does not reflect expression of a single protein but describes several human isoforms (24–26) that comigrate at M_r 55,000–60,000, whereas Oct-2B (24, 27) migrates at M_r 75,000. The amino acids recognized by the antibody used in this experiment are encoded by the 3' part of

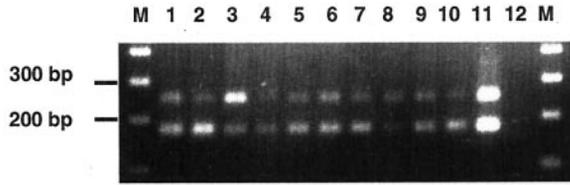


Fig. 1. RT-PCR analysis of the 3' end of Oct-2 shows transcription of different isoforms. Analysis of Oct2A and Oct2B transcription in naïve B cells (IgD⁺CD27⁻) and GC B cells (CD38⁺CD77⁺; Lanes 1 and 2, respectively); in HD-derived cell lines L1236, L428, KM-H2, DEV, L591, L540, and HDLM-2 (Lanes 3–9, respectively); and in the lymphoblastoid B-cell lines IARC277 and BJA-B (Lanes 10–11, respectively). Lane 12, no template; M, molecular size marker (100-bp ladder). Oligonucleotides Oct2.S and Oct2.AS were used for amplification of Oct-2 cDNA.

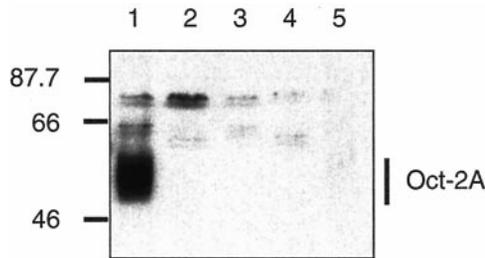


Fig. 2. Western blot for Oct-2 expression using a polyclonal antibody mapping to the COOH terminus of Oct-2. Analysis of the lymphoblastoid B-cell line BJA-B (Lane 1), the fibroblast cell line NIH3T3 (Lane 2), and the HD-derived cell lines L1236, L428, and KM-H2 (Lanes 3–5, respectively). The fragment size of the molecular weight marker is given on the left side. The position of the Oct-2A complex is indicated on the right side.

exon 12 (8 amino acids) and the 5' part of exon 14 (12 amino acids). Therefore, Oct-2B cannot be detected using this antibody, because the antibody-binding site is disrupted, attributable to the splicing of exon 13. To elucidate whether Oct-2B protein expression is present in HD-derived cell lines, we performed a Western blot experiment using an antibody raised against the NH₂ terminus of all of the Oct-2 isoforms. However, also using this NH₂-terminal-binding antibody, Oct-2A and Oct-2B expression was absent in the three HD-derived cell lines (Fig. 3).

Absence of Bob-1 Transcription in HD-derived Cell Lines. Oct-2 and Bob-1 are both necessary for activation of immunoglobulin promoters. Therefore, we tested the HD-derived cell lines also for transcription of Bob-1. Bob-1 transcripts are completely absent in all of the HD-derived cell lines with the exception of DEV (Fig. 4). Notably, DEV is derived from a case of lymphocyte-predominant HD⁴ and expresses surface immunoglobulin. This is in line with surface immunoglobulin expression commonly observed in lymphocyte-predominant HD as opposed to classical HD. Bob-1 mRNA was expressed by naïve and GC B cells and was also detected in two B-cell lines (BJA-B and IARC277). B-cell specificity of Bob-1 transcription is demonstrated by the absence of transcripts from Jurkat cells.

Lack of Oct-2 and Bob-1 Protein Expression in H-RS Cells. Staining for Oct-2 and Bob-1 was performed in 20 cases of classical HD at primary diagnosis and in one reactive lymph node. For immunohistochemistry, staining of a nonmalignant reactive lymph node specimen was used as a positive control for Oct-2 and Bob-1 expression. Staining revealed nuclear expression of both Oct-2 and Bob-1 in GC lymphocytes (Fig. 5, A and B). In addition to these controls, epithelial cells and, if present, residual GCs were used as an internal negative and positive control, respectively. Analysis of 20 cases of classical HD showed absence of Oct-2 and Bob-1 expression in 18 and 20 cases, respectively (Table 1; Fig. 5, C and D). Two cases of

HD were not evaluated for Bob-1 expression because of an unspecific staining of epithelial cells. It is concluded that in all of the informative cases of classical HD, H-RS cells fail to express Oct-2 and Bob-1.

DISCUSSION

Lack of immunoglobulin gene expression has been shown by *in situ* hybridization in primary cases of classical HD, despite the detection of potentially functional *Ig VDJ* gene rearrangements in most of these cases (3). Therefore, we investigated a potential mechanism of transcriptional deregulation of immunoglobulin genes in classical HD, namely expression of Oct-2 and Bob-1.

Results from RT-PCR indicate a low-level transcription of Oct-2 in the HD-derived cell lines. Because the number of transcripts is similar in HD-derived cell lines of both T- and B-cell origin, this may indicate a non-B-cell-specific baseline *Oct-2* gene activity. That view is in line with the fact that we, in contrast to others (28), were unable to detect Oct-2 protein in B-cellular HD-derived cell lines in Western blot experiments using two different Oct-2 specific antibodies.

Bob-1 recently has been identified as a B-cell-specific cofactor that is necessary for promoter-proximal activity together with either Oct-1 or Oct-2 (14). These data strongly support the idea that Bob-1 contributes to the transcriptional regulation in B cells. In fact, up-regulation of Bob-1 has been detected in GC B cells, whereas there was little expression of Bob-1 in naïve cells. In a study by Greiner *et al.* (29), GC-derived B-cell lymphomas including Burkitt lymphoma, follicular lymphoma, and diffuse large-cell B-cell lymphoma showed an up-regulation of Bob-1 similar to their physiological counterpart, the GC B cell.

H-RS cells are derived from GC B cells (16) and, thus, are expected to express Bob-1. We addressed the question whether Bob-1 is transcribed in HD-derived cell lines using RT-PCR. Our results show transcription of Bob-1 in GC cells and in naïve B cells, consistent with previous studies (29). Bob-1 mRNA was detectable in two lymphoblastoid cell lines but not in a T-cell line (Jurkat). Surprisingly, in four B-cell lines from patients with classical HD, a complete lack of Bob-1

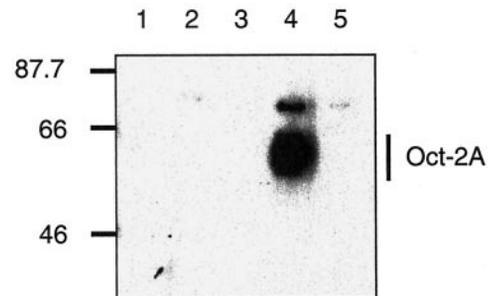


Fig. 3. Western blot for Oct-2 expression using a monoclonal antibody raised against the NH₂ terminus of Oct-2. Analysis of the HD-derived cell lines L428, L1236, and KM-H2 (Lanes 1–3, respectively), the lymphoblastoid B-cell line BJA-B (Lane 4), and the fibroblast cell line NIH3T3 (Lane 5). The fragment size of the molecular weight marker is given on the left side. The position of the Oct-2A complex on the right side.

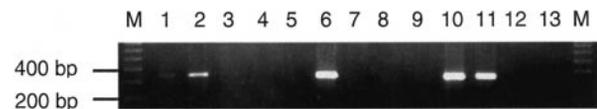


Fig. 4. RT-PCR analysis of Bob-1 shows the lack of transcription in six of seven HD-derived cell lines. Analysis of Bob-1 transcription in naïve B cells (IgD⁺CD27⁻) and GC B cells (CD38⁺CD77⁺; Lanes 1 and 2, respectively); in the B-lineage HD-derived cell lines L1236, L428, KM-H2, DEV, and L591 (Lanes 3–7, respectively); in the T-lineage HD-derived cell lines L540 and HDLM-2 (Lanes 8–9, respectively); in the lymphoblastoid B-cell lines IARC277 and BJA-B; and in Jurkat (Lanes 10–12, respectively). Lane 13, no template; M, molecular size marker (100-bp ladder).

⁴ S. Poppema and N. L. Groningen, personal communication.

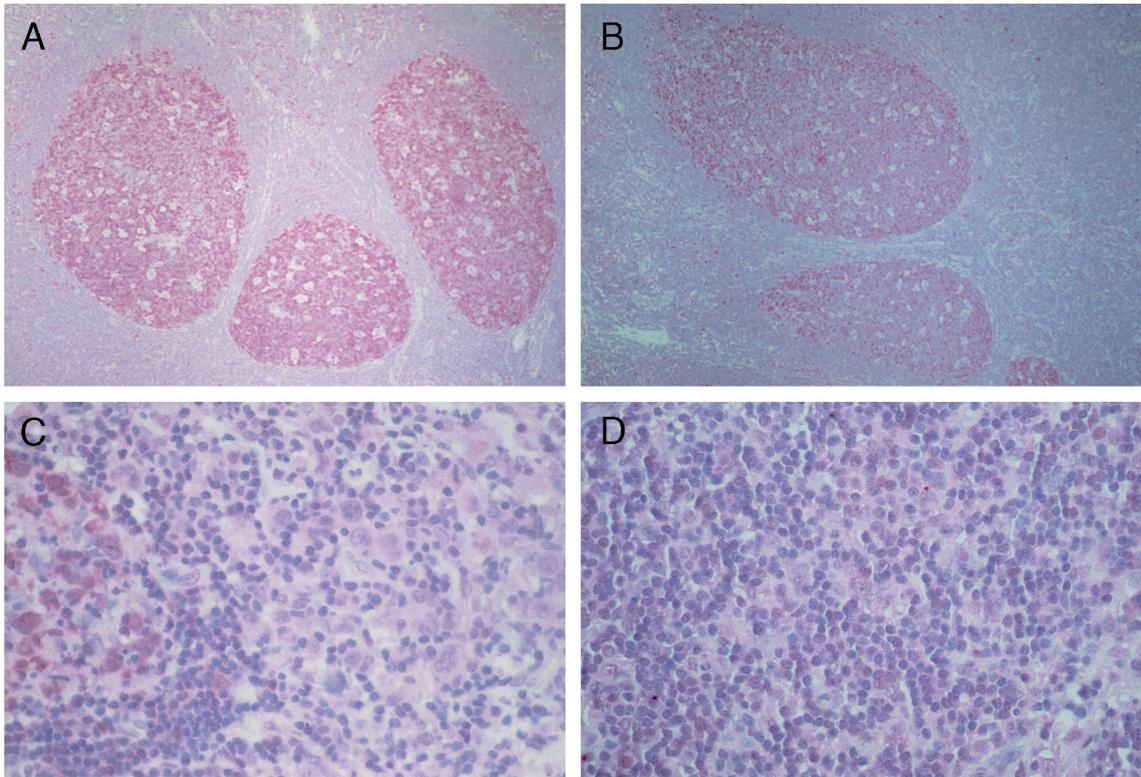


Fig. 5. Immunohistochemistry of the Oct-2 and Bob-1 protein using an anti-Oct-2 antibody and an anti-Bob-1 antibody. Pathological specimens were prepared as described in "Material and Methods." Shown is a section from a nonmalignant reactive lymph node, stained with (A) anti-Oct-2 antibody and (B) anti-Bob-1 antibody showing GCs, and an example of a case of classical HD, stained with (C) anti-Oct-2 antibody and (D) anti-Bob-1 antibody. A and B, $\times 170$; C and D, $\times 600$.

transcription was observed. In contrast, transcription of Bob-1 was present in the cell line (DEV) derived from a patient with lymphocyte-predominant HD. Because the malignant clone in lymphocyte-predominant HD but not in classical HD expresses immunoglobulin genes, absence of Bob-1 expression in classical HD may reflect transcriptional deregulation of immunoglobulin genes specific for classical HD.

Previous studies (1–5, 30) demonstrated that the lack of immunoglobulin gene expression in a substantial proportion of classical HD cases is because of obviously destructive mutations of the rearranged immunoglobulin genes. Because transcription is important for somatic hypermutation (31) and H-RS cells harbor somatic mutations within their rearranged immunoglobulin genes, immunoglobulin gene transcription should have taken place in these cells. Thus, it is likely that in cases harboring nonfunctional immunoglobulin gene rearrangements the deregulation of transcription factors as presented here is a secondary event after the destructive immunoglobulin gene mutations.

Because three of the four analyzed HD-derived cell lines of B-cell origin [L1236 (5), L428 (32), and L591⁵] harbor destructive mutations within their immunoglobulin genes, in these cell lines the down-regulation of Oct-2 and Bob-1 seems to be a secondary event regarding absence of immunoglobulin gene expression. For the B-cell-derived cell line KM-H2, data are lacking with regard to the *IgH* and *IgL* genes and, therefore, the relevance of the down-regulation of both transcription factors as a primary event in the deregulation of immunoglobulin gene transcription is unclear.

To test the hypothesis that the lack of Oct-2 and Bob-1 is a common feature in H-RS cells of primary cases of classical HD, we performed immunohistochemistry for Oct-2 and Bob-1 in 20 cases of classical HD. In contrast to GC B cells, which showed strong nuclear staining

for both Oct-2 and Bob-1, H-RS cells stained negative for both proteins in all of the informative cases. Results show that the lack of both transcription factors is a peculiar feature of H-RS cells. Because it is known that in a proportion of cases of classical HD the H-RS cells harbor potentially functional immunoglobulin gene rearrangements (3), one might conclude that in these cases, absence of Oct-2 and Bob-1 may represent the main cause for absence of immunoglobulin gene expression.

In summary, both Oct-2 and Bob-1 are absent in classical HD-derived cell lines and in all of the primary cases of classical HD tested thus far. Given that Oct-2 and Bob-1 expression is present in GC B cells, lack of Oct-2 and Bob-1 expression in classical HD-derived B-cell lines and in primary H-RS cells was an unexpected finding and may represent a novel mechanism involved in transcriptional deregulation of immunoglobulin genes in classical HD.

ACKNOWLEDGMENTS

We thank Julia Jesdinsky and Nadia Massoudi for excellent technical assistance.

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