Intraclonal Variability of VH Genes in Follicular Lymphoma Patients Who Have Received Anti-Idiotypic Immunotherapy

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Summary: Subclonal heterogeneity can affect idiotypic determinants present in the clonotypic immunoglobulin of B-cell follicular lymphomas (FLs) and may limit the effect of antilymphoma treatments performed by immunization of patients with their own tumor-associated idiotypic immunoglobulin. Idiotype-secreting hybridomas were obtained by fusion of tumor cells from 5 patients with FL, and the K6H6/B5 human heteromyeloma and rearranged VH genes from tumor samples and hybridomas were amplified, cloned, and sequenced. Sequences were aligned with germline genes and somatic mutations, intraclonal heterogeneity and genealogic relations of the B-cell clones in the different biopsy specimens were determined. The VH sequence of the progenitor clone was determined in samples of the tumoral population. Further diversification resulted in the presence of 2 to 6 subclones in 4 of the 5 samples studied. Only in 1 patient did the hypermutation mechanism introduce differences among most of the potential idiotopes present in individual subclones. The VH sequence of the hybridoma that provided the idiotypic-vaccine was identified in one of the tumor subclones in all cases. No relapse has been demonstrated in 3 of the 4 vaccinated patients (follow-up: 29–103 months). We conclude that despite potential differences in the idiotypic region expressed by individual tumor cells, at least some potential idiotopes may be preserved among all the tumor subclones in most cases studied. All vaccinated patients developed immune responses against the autologous tumor idiotypic immunoglobulin. Polyclonal anti-idiotypic immune responses induced with a vaccine obtained from 1 hybridoma may be effective against all the idiotypic variants present in the tumor population.

Key Words: non-Hodgkin lymphoma immunotherapy, subclones, lymphoma, idiotypes, tumour vaccines

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Follicular lymphomas (FLs) arise from the clonal expansion of B lymphocytes and are endowed with a "clonal signature" provided by the unique idiotypic region of the immunoglobulin expressed on the surface of the tumor cells.[1] Idiotypes are the epitopes (idiotopes) created by the hypervariable segments of the V regions of the immunoglobulin heavy and light chains. FL tumor cells arise from germinal center cells and can somatically hypermutate the immunoglobulin V genes,[2,3] as do their normal B-cell counterparts during antigen-driven selection and memory B-cell production.[4] The original tumor clone retains the activated mechanism of hypermutation, resulting in a further accumulation of immunoglobulin V mutations and the presence of different subclones within the tumor population.[3] These mutations could affect the idiotypic region of the immunoglobulin molecule and lead to differences in the antigenic idiotopes of the individual tumor subclones.

Although low-grade FLs are generally associated with reasonably long survival even if treatment results in only a partial response, they are usually not curable with conventional chemotherapy (QT)/stem cell transplantation. Based on consideration of the idiotype of the B-cell tumors as a tumor-specific antigen, Kwak et al[5] devised a different therapeutic approach to the treatment of B-cell lymphomas. This involved the induction in the patient of an immunologic response using a vaccine prepared with the idiotypic immunoglobulin expressed by the patient's own tumor cell. Anti-idiotypic immunization demonstrated antitumor effects in a small series of FL patients.[5–7] This kind of immunotherapy by idiotypic vaccination requires the preparation of a tumor-derived immunoglobulin protein, which is usually accomplished by hybridoma technology after the fusion of a sample of the patient's tumor cells and a heteromyeloma cell line. The fusion of a heterogeneous tumor population would lead to a mixture of hybrid cells secreting the different idiotypic variants of the original tumor. The identification of the idiotypic tumoral immunoglobulin secreted by the hybridoma cells is performed by low-resolution methods, including polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) restriction analysis of *HaeIII* digests.[7] Because for practical reasons, only 1 hybridoma clone is chosen for the growth and preparation of the idiotypic vaccine (Id-vaccine), it is possible that immune responses elicited against the Id-vaccine may not be effective against other tumor subclones expressing idiotypic variants that differ substantially from the Id-vaccine. Here, the clonal heterogeneity of the tumor samples of 5 lymphoma patients enrolled in a protocol of idiotypic vaccination has been studied, and the impact that the clonal variability of the tumor population can have on the outcome of the treatment is discussed.

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MATERIALS AND METHODS

Five patients previously diagnosed with follicular, B-cell, non-Hodgkin lymphoma, type II (Revised European American Lymphoma (REAL) classification) were entered into the present study. They were part of a protocol of active idiotypic vaccination carried out at our center that included 12 patients. All participants (patients 1–5) received several chemotherapy lines, and patients 1 and 4 also received autologous peripheral blood stem transplantation. Patients 1, 3, 4, and 5 were vaccinated after achieving a complete remission or minimal disease state. A tumor-infiltrated lymph node was taken from each patient during the last relapse, and a cell suspension obtained by mechanical disruption was fused with the K6H6/B5 heteromyeloma. One of the cloned hybridoma cells expressing the same VDJ rearrangements as the original tumor sample was chosen for producing the Id-vaccine. Details concerning the study design, approval of authorities, general methods, and preliminary results have been published elsewhere.[7]

Immune responses against the immunizing idiotype were studied in samples taken 1 month after completion of the treatment. Anti-idiotypic antibodies were detected in sera of immunized patients by enzyme-linked immunosorbent assay (ELISA). Pre- and postimmunization sera were diluted 1:16 in phosphate-buffered saline (PBS) and dispensed in wells of microtiter plates coated with the autologous tumor immunoglobulin protein or irrelevant isotype matched immunoglobulin. Bound antibodies were detected with horseradish peroxidase (HRP)–conjugated goat anti-human antiserum (Sigma-Aldrich, St. Louis, MO) against the light chain isotype not present in the corresponding idiotype. Antigen-specific proliferation and cytokine production were studied in pre- or postvaccine cryopreserved peripheral blood mononuclear cells (PBMCs). A total of 2×10^5 cells were resuspended in 200 μL AIMV medium (InVitrogen, Carlsbad, CA) and cultured at 37°C for 6 days in the presence of 50 μg/mL tumor immunoglobulin or an irrelevant isotype matched immunoglobulin. At day 3, 10 U recombinant interleukin (rIL)-2 (Endogen, Woburn, MA) was added. Proliferative response was measured by [^{3}H]thymidine incorporation during the last 18 hours of culture. Interferon (IFN)-γ released by activated cells was measured in supernatants collected after day 5 by a cytokine ELISA (Endogen).

Total RNA was isolated from cells of tumor lymph nodes or hybridomas using guanidine isothiocyanate–containing buffer to lyse the cells and was purified by selective binding onto a silica gel–based membrane (RNeasy Mini Kit; Qiagen, Valencia, CA) after treatment with deoxyribonuclease (DNase) to remove genomic material. Complementary DNA (cDNA) was synthesized from 20 μL total RNA using a first-strand cDNA synthesis kit (Amersham Biosciences, Uppsala, Sweden) and oligo (dT) primer according to the manufacturer's instructions.

A total of 5 μL cDNA was amplified by Taq polymerase with a specific 5′ primer, which corresponds to one of the human variable H chain family leaders (VH_1–VH_6), and a 3′ antisense JH consensus primer.[8]

In all cases, PCR was performed at a final volume of 50 μL with 20 pmol of each primer, 50 μmol deoxynucleotide triphosphate, and 1.25 U *Taq* DNA polymerase. Amplification consisted of an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles at 94°C for 1 minute, 56°C for 1 minute, and 72°C for 1 minute, with a final extension step of 5 minutes at 72°C. Amplified variable regions were analyzed on a 2% low-melting-point agarose/Tris acetate-ethylenediamine tetraacetic acid (EDTA) gel and visualized with ethidium bromide. The band of the expected size was excised and purified by adsorption to a silica matrix (QIAquick columns; Qiagen).

The PCR products were subcloned into pCR4-TOPO using the TOPO TA cloning system (InVitrogen) and introduced into chemically competent bacteria. Plasmids from randomly picked colonies were checked for correct insert size by PCR analysis. Plasmid DNA was isolated from bacterial cultures using the QIAGEN Plasmid Mini Kit (Qiagen), and the sequence of the inserts was determined using the Cy5 AutoCycle Sequencing Kit (Pharmacia Biotech, Uppsala, Sweden) on a Pharmacia Biotech ALFexpress Automated DNA Sequencer.

Sequences were aligned with germline sequences derived from the Vbase database and DNA plot on the Internet (http://mcr.cpe.cam.ac.uk/imt-doc). Different V_H regions were identified according to the Kabat numbering. The V_H gene sequences were compared with the germline genes with the highest homology, and the number of somatic mutations was calculated accordingly.

To determine the presence of ongoing mutations in FL, tumor samples were examined by repeated cloning and sequencing of at least 8 clones from each patient. Subclonal evolution was depicted in the form of genealogic trees.[9] GenBank accession numbers are AY954972 for patient 1, AY960198–202 for patient 2, AY952644–6 for patient 3, AY962562–7 for patient 4, and AY952641–3 for patient 5.

For the evaluation of intraclonal heterogeneity, only those point mutations in a particular position observed in more than 1 PCR clone were considered as evidence of intraclonal heterogeneity (confirmed mutations). The accuracy of *Taq* DNA polymerase was estimated to be 1540nt after 35 PCR cycles; thus, sporadic mutations found in only 1 PCR clone were disregarded.

RESULTS

Table 1 shows the characteristics of the 5 patients included in this study together with their current clinical situation and the time elapsed since they received the Id-vaccine. At the time of preparing this report, patient 2 has not yet received idiotypic therapy. Follow-up of vaccinated patients ranged from 29 to 103 months from the initiation of vaccination. Two participants (patients 1 and 4) were in complete remission, and 2 (patients 3 and 5) had residual disease before vaccination. The first 2 patients remain in remission at the time of this report. Patient 3 experienced disease progression, whereas patient 5 achieved complete remission after vaccination.

Humoral and cellular immunologic responses against the immunizing idiotype were studied in blood samples collected before and 1 month after the last treatment (Table 2). Controls included an isotype-matched nonrelated idiotype. All patients developed specific antibody responses against the autologous idiotype. In patient 1, a substantial level of anti-idiotypic activity was found in the prevaccination serum.

TABLE 1. General Characteristics and Clinical Data of the Patients

Patient No.	Tumor Immunoglobulin	Age*/Sex	Diagnosis	Stage	Previous Therapy	Clinical Situation		Follow-Up (mo)
						Before Vaccine	At Present	
1	IgG,λ	43/M	FL type II	IV	SCL + ASCT	CR	CR	103
2	IgM,λ	50/M	FL type II	IV	Untreated		NV	
3	IgG,λ	31/M	FL type II	IV	SCL	PR	DP	60
4	IgM,κ	41/M	FL type II	IV	SCL + ASCT	CR	CR	29
5	IgG,λ	38/M	FL type II	IV	SCL	PR	CR	62

*At diagnosis.
ASCT indicates autologous peripheral blood stem cell transplant; CR, complete remission; DP, disease progression; NV, not vaccinated; PR, partial remission; SCL, several chemotherapy lines.

To study cellular responses, we stimulated pre- and postvaccine PBMCs with purified tumoral idiotypes. As found in previous reports,[6] T-cell responses were highly variable in different patients (range stimulation index: 1.7–4.8 for proliferative responses and 2.6–7.3 for IFNγ release), but all patients demonstrated responses to the autologous idiotype after but not before vaccination.

cDNA was extracted from the lymph node sample used to prepare the hybridomas and cloned in competent bacteria, and the plasmids isolated from at least 8 bacterial colonies were sequenced. The repetition of a single predominant VDH sequence with shared mutations identified the common ancestral lymphoma progenitor from which all the other subclones were derived. Table 3 shows the VH gene families and the D and J gene segments used by each progenitor cell lymphoma. The VH genes were derived from VH families 3 (3–21*01, 15*07, and 48*03) and 4 (4–59*01), which belong to the largest families of VH genes expressed in human beings[10] and have been reported to be preferentially expressed in FLs.[2,3]

The length of CDR3 varied between 6 and 23 amino acids (see Table 3). The N plus P addition was a consistent finding in all analyzed sequences. When compared with the most homologous germline sequence, VH sequences from patients 1 and 2 showed an in-frame insertion of 3 and 9 nucleotides in the CDR2 region, respectively. During the process of hypermutation in normal B cells, insertions or deletions of nucleotides can be introduced in the V region genes of immunoglobulins[11] and have also been detected in FL tumor cells.[3,12] All sequences were potentially functional and predicted to encode a normal polypeptide chain. It is worth noting that all sequences derived from patient 4 and the sequence of subclone 2 from patient 5 had a W36R and a W36G amino acid exchange, respectively. Trp 36 is an invariant residue located at the center of all immunoglobulin domains and has been predicted to be required for correct packing of the immunoglobulin fold.[13] Those mutant forms are readily detected in the tumor cell membrane, however, and the corresponding hybridomas secreted immunoglobulin material containing H and L chains with normal apparent molecular weight.

Analysis of mutations present in the VH sequences expressed by each ancestral progenitor disclosed mutated VH genes in all cases studied. The number of mutations was variable, from 10 in patient 4 to 34 in patient 3, representing 96.5% and 88.3% homology to the closest germline match, respectively (see Table 3). Mutations were mainly concentrated in the gene segments encoding the framework regions of the variable regions.

Cloning of the PCR products representing tumor VH sequences permits the study of clonal heterogeneity and the disclosure of the genealogic relations of the VH gene sequences

TABLE 2. Cellular ([^{3}H]thymidine Incorporation and IFNγ Secretion) and Humoral (immunoglobulin) Immune Responses of Patients 1 and 3 Through 5

	Patient 1		Patient 3		Patient 4		Patient 5	
Antigen→	Id-1	NR-Id	Id-3	NR-Id	Id-4	NR-Id	Id-5	NR-Id
^{3}H								
Pre-V	1.10*	0.90	4.2	4.2	10	10	3.1	2.9
Post-V	4.10	0.95	21	4.3	17	10	6.4	3.1
IFNγ								
Pre-V	0.23†	0.12	0.25	0.25	0.4	0.4	0.15	0.15
Post-V	2.75	0.23	1.91	0.26	1.2	0.4	0.45	0.17
Immunoglobulin								
Pre-V	0.66‡	0.12	0.21	0.19	0.21	0.21	0.2	0.2
Post-V	1.1	0.12	1.75	0.20	1.66	0.22	1.3	0.2

Results are expressed as the arithmetic mean of triplicate wells. Standard deviations were less than 15% of the mean value.
*Counts per minute $\times 10^{-3}$.
†ELISA (A_{405}).
‡ELISA (A_{492}).
Id indicates autologous idiotype; NR-Id, nonrelated idiotype; Pre-V, prevaccination samples; Post-V, postvaccination samples.

TABLE 3. Immunoglobulin Heavy Chain Gene Expression and Mutational Analysis of the Rearranged VH Genes in Tumor Samples of Patients 1 Through 5

Patient No.	Rearranged H Chain Gene		CDR3 Length	VH Gene Homology	FR/CDR	Observed Mutations†	
	VH Gene*	DH Gene/JH Gene*				R	S
1	VH3–21*01	D2–21*02/JH6*02	23	88.88%	FR	12	9
					CDR	10	1
2	VH4–59*01	D1–7*01in/JH5*02	10	93.19%	FR	8	7
					CDR	3	2
3	VH4–59*01	D1–1*01inv/JH6*01	6	88.30%	FR	17	9
					CDR	6	2
4	VH3–48*03	D5–12*01/JH6*03	14	96.50%	FR	8	0
					CDR	2	0
5	VH3–15*07	D2–21*01/JH4*02	14	93.60%	FR	8	4
					CDR	3	2

*Most similar germline gene.
†In progenitor clone.
R indicates replacement mutations; S, silent mutations.

derived from individual lymphomas. For each patient, a minimum of 8 bacterial colonies was sequenced. As an example, Figure 1 shows the nucleotide sequence of the rearranged VH gene expressed by tumor cells from patient 5. The study of 9 PCR clones disclosed the presence of 3 distinct sequences, each comprising 3 PCR clones. Comparison with the closest germline gene (VH3–15*07) revealed that one sequence contained only shared mutations and defined the progenitor clone, whereas the presence of individual point mutations in the other sequences revealed the existence of 2 independent subclones derived from the progenitor clone.

The genealogic trees raised from each progenitor tumoral clone constructed with the sequences of patients 2, 3, 4, and 5 are shown in Figure 2. The biopsy specimen from patient 1 contained only a single sequence showing no apparent intraclonal heterogeneity, and it is not included in this analysis. The individual mutations that differentiate each subclone from the common progenitor are indicated. Mutations affecting several subclones are indicated along the corresponding common branch. In each patient, the VH sequence expressed by the hybridoma used for preparing the vaccine material was identical to the sequence of one of the predicted tumor subclones (labeled * in Fig. 2).

Only 2 independent subclones were derived from the ancestral progenitor clone in patients 3 and 5. In each case, only 1 replacement mutation affected the CDR region (A193T in subclone 1 of patient 3 and T187A in subclone 1 in patient 5). Lineage relations in patients 2 and 4 gave a bushier tree, with 2 independent branches departing from the common progenitor that gave rise to several secondary branches. For patient 2, up to 5 different sequences could be identified, one of which corresponded to the sequence of the original ancestral

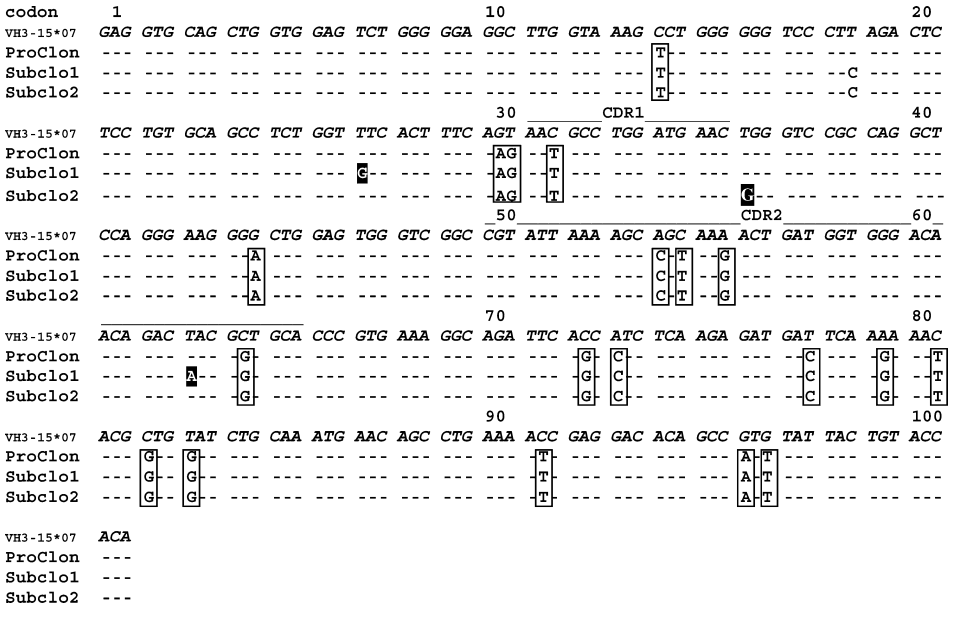

FIGURE 1. Related VH sequences isolated from biopsy specimens of patient 5. Nine PCR clones were studied. Each sequence was found in 3 independent PCR clones. Sequence identity to germline (upper row: VH3–5*07) is indicated by dashes, with each mutation by the appropriate nucleotide. Shared mutations (boxed) permitted the identification of the progenitor clone (ProClon). Individual mutations that could give rise to 2 independent subclones (subclo1 and 2) are shown as black squares. The 3 sequences can be fitted into a genealogic tree (Fig. 2, 5).

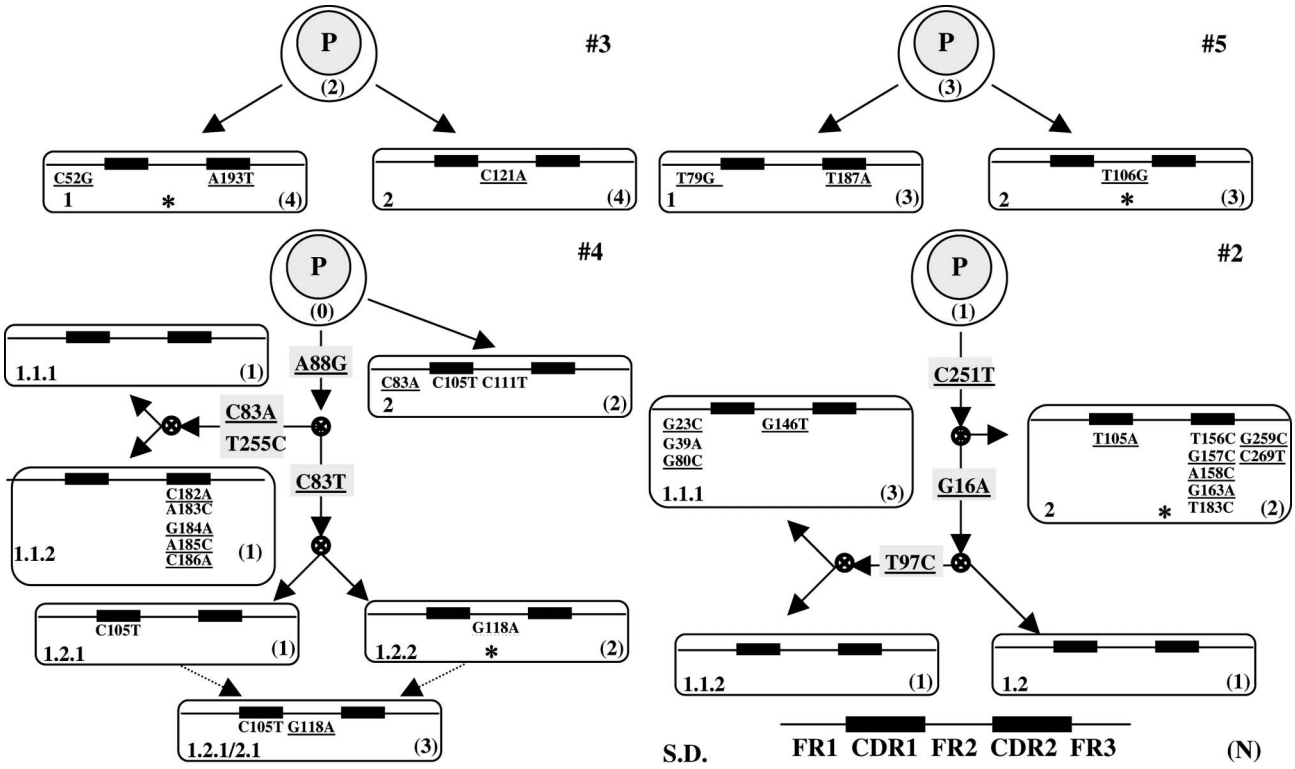

FIGURE 2. Genealogic trees constructed using VH gene sequences of tumor cells from 4 patients with FL. Point mutations are indicated below the corresponding CDR or FR region in individual subclones by the nature of the base change and their codon number. The positions of shared nucleotide differences are indicated alongside the arrows. Mutations that result in amino acid exchange are underlined. N indicates number of (bacterial) sequences representative of each subclone; P, progenitor clone; SD, subclone designation. *Subclone expressing a VH sequence identical to that found in the hybridoma used in vaccine preparation.

progenitor. The VH sequence of subclone 2 showed 1 T105A replacement mutation in CDR1 and 3 (G157C/A158C, both in codon 53, and G163A) in CDR2, whereas subclones 1.1.1 and 1.1.2 differ from the progenitor sequence in only 1 CDR1 replacement mutation (T97C).

Six different derived sequences were identified for patient 4. In this case, the sequence of the original progenitor was not found in any of the bacterial clones studied and was deduced from the mutations shared by all the inspected clones. This could be a case of extreme clonal diversification in which the original progenitor tumor cell was no longer present in the most advanced stages of the evolution of the lymphoma. For patient 4, subclone 2 showed no replacement mutations in the CDR region, whereas all the other 5 subclones shared a single A88G transition change affecting the CDR1 region. The subclone 1.1.2 accumulates up to 4 additional mutations that affect 2 codons in the CDR2 region (C182A/A183C and G184A/A185C/C186A, corresponding to codons 61 and 62, respectively). Although some of the described mutations were observed in only 1 PCR clone (n = 1), they can define a particular tumor subclone. For instance, subclone 1.1.2 (n = 1) of patient 2 shares the C251T/G16A/T79C mutations with the 1.1.1 clone (n = 3), but it does not have the 4 additional mutations that are present in the FR1 and FR2 regions of the latter.

Only 8 (patients 1 and 2) to 10 (patients 3 and 4) PCR clones were studied in each lymphoma; therefore, it is possible that some VH sequences present in the tumor population were not picked up during the process of PCR cloning. The fact that the sequence of the hybridoma used in the Id-vaccine preparation could be also recognized in one of the sequenced tumor subclones (labeled * in Fig. 2) in all cases suggests that clonal heterogeneity is not much higher than that described in this report.

DISCUSSION

The effectiveness of immunotherapeutic treatments that use purified preparations of tumoral antigens can be severely compromised by the heterogeneity of the tumoral population. This is particularly important for the vaccination of FL patients with purified preparations of the idiotypic immunoglobulin. FLs are prototypic germinal center tumors, and most of them acquire mutations that affect the expressed V immunoglobulin genes. We have studied the VH sequence expressed by the original progenitor in 5 FL tumors and found in all an accumulation of point mutations in relation to the corresponding germline sequence in agreement with previous reports.[3]

Different levels of heterogeneity can be found in FLs. First, up to 10% of diagnosed FLs[14,15] can be primary biclonal or even triclonal tumors, probably reflecting independent neoplastic events. In these cases, anti-idiotypic immunotherapy requires the isolation of the individual idiotypes of each clone to prepare an effective vaccine, as has already been published.[7,15] A second level of heterogeneity is provided by the intraclonal diversification derived from the accumulation

of V gene mutations after the initial neoplastic event. These mutations can introduce changes in the hypervariable region and affect the idiotypic region of the immunoglobulin molecule targeted in the idiotypic vaccination. It is worth noting that the initial heterogeneity is maintained or even narrowed in late-stage disease after multiple rounds of chemotherapy.[16,17]

The idiotype of an immunoglobulin molecule can be viewed as an antigenically complex region that comprises several distinct epitopes (idiotopes). The amino acids encoded by the 6 hypervariable CDR regions are involved in the formation of the different idiotopes of the immunoglobulin molecule, and it is commonly assumed that sequence identity in the CDR regions means idiotype identity. Immunogenic peptides derived from the framework region may induce cytotoxic T lymphocyte (CTL) responses after in vitro immunization,[18] but anti-idiotypic T-cell–mediated immune responses of idiotypic-vaccinated FL patients are directed exclusively against peptides encoded by the CDR segments of the immunoglobulin molecule.[19]

Because the hybridoma used to prepare the Id-vaccine is the fusion product of one of the subclones present in the tumoral sample, the existence and degree of intraclonal diversification must be taken into account when considering idiotypic vaccination. It is possible that some of the other subclones show changes affecting the idiotopes of the molecule and could escape the induced immune response to the vaccine idiotype. In the 5 lymphoma tumors studied here, no intraclonal diversification was found in 1 case (patient 1), whereas in patients 3 and 5, only 1 amino acid change in CDR2 differentiates the subclones. This implies that in those 3 patients, the idiotype used in the preparation of the vaccine probably contains all (patient 1) or most of the idiotopes present in the whole tumor. Despite the higher heterogeneity of the lymphoma cells in patient 4, differences between the vaccine idiotype (subclone 1.2.2) and the most distant VH subclone (subclone 1.1.2) affect only one of the CDR regions (Ala61Asp and Asp62Thr). Finally, for patient 2, differences between the Id-vaccine (subclone 2) and the subclone 1.1 affect the CDR1 (T97C→Ser33Pro and T105A→Asn35Lys) and CDR2 (Asp53Pro and Gly55Arg) regions, and it is possible that they do not share a significant number of idiotopes.

Although an idiotype-specific immune response was evidenced in all patients after vaccination, disease progressed in patient 3. Discrepancies between anti-idiotypic immune responses and molecular remissions have been reported previously,[6] indicating that at least in some cases, circulating antibodies and in vitro induction of T-cell activation may not truly reflect the efficacy of the antitumor immune responses. For ethical reasons, a sample of the relapsed tumor in patient 3 could not be obtained, precluding the search for an idiotypic escape variant at the time of disease progression.

Although obtained from a small number of patients, our results suggest that in most cases (patients 1, 3, 4, and 5), some of the idiotopes expressed by the original tumor progenitor are preserved after intraclonal diversification. Only patient 3 showed signs of disease progression (see Table 1), whereas patients 1 and 4 maintained their initial clinical situation and patient 5 underwent tumor regression. The lack of tumor relapses in these patients may reflect the presence of shared epitopes among the tumor subclones and validate the production of the vaccine with the idiotype secreted by 1 hybridoma. Polyclonal immune responses induced by the Id-vaccine would lead to the rejection of all the subclones present in the tumor population. In some cases, however, exemplified here by unvaccinated patient 2, the coexistence of highly different subclones within the tumoral samples may suggest the preparation of the Id-vaccine using material isolated from more than 1 hybridoma.

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