Recent reports have shown that human B-cell malignancies are characterized by an extremely variable incidence of acquired, potential N-glycosylation sites (AGS) in their tumor-specific immunoglobulin (Ig) variable region sequences. In particular, most if not all cases of follicular lymphoma (FL) present with this feature, whereas other B-cell malignancies, including those that, like FL, originate from the germinal center, show a substantially lower occurrence of this phenomenon. This molecular difference is unlikely to be solely of a stochastic nature, but so far no explanation has been provided for it. Furthermore, it is unclear whether the presence of AGS has prognostic and/or therapeutic implications. Here, we describe our findings on the incidence and peculiar presentation patterns of these AGS in first-relapse B-cell malignancies including, for the first time, data on mantle cell lymphoma (MCL).

**Background and Objectives.** Among B-cell malignancies, follicular lymphomas (FL) more frequently show acquired, potential N-glycosylation sites (AGS) within tumor-specific immunoglobulin. The aim of this study was to extend this observation and to evaluate the pattern of presentation of AGS within five different forms of B-cell lymphoma.

**Design and Methods.** We sequenced the tumor-specific immunoglobulin heavy chain variable region fragment, including complementarity-determining regions 2 and 3, of forty-seven consecutive patients with a B-cell malignancy enrolled in idiotype vaccine clinical trials. This sequencing approach is known to allow the identification of most AGS. We then statistically analyzed differences in presentation pattern, in terms of tumor histology, immunoglobulin isotype, AGS location and amino acid composition.

**Results.** All twenty-four FL cases presented with at least one AGS, whereas the vast majority of four B-cell lymphoma types other than FL did not. The non-FL group of tumors included four cases of Burkitt’s lymphoma, six of diffuse large cell lymphoma, seven mantle cell lymphomas and six small lymphocytic lymphomas. Most IgM-bearing follicular lymphoma cases featured their AGS within complementarity-determining region 2, as opposed to those bearing an IgG, which mostly displayed the AGS within complementarity-determining region 3. The vast majority of AGS located within either complementarity-determining region ended with a serine residue, whereas those located within framework regions mostly featured threonine as the last amino acid residue.

**Interpretation and Conclusions.** In our series, all cases of FL had AGS within their tumor-specific immunoglobulin heavy chain variable regions. In contrast, most B-cell malignancies other than FL did not. Further studies are warranted in order to establish the possible meaning of these findings in terms of disease pathogenesis, their diagnostic value in doubtful cases and their potential implications for immunotherapy.

**Key words:** glycosylation sites, immunoglobulin, B-cell malignancies, isotype.
mined independently without knowledge of the presence or absence of AGS.

**Identification of the surface tumor-specific Ig isotype**

Fresh tumor cells were washed twice in phosphate-buffered saline (PBS), resuspended and incubated for 15 minutes at room temperature with goat anti-human IgM-FITC, goat anti-human IgG-FITC and goat anti-human IgA-FITC (Biosource International, Camarillo, CA, USA). Finally, following a further wash in PBS, flow cytometry analysis was carried out in a FACScan (BD Immunocytometry System, Mountain View, CA, USA) using the CellQuest software.

**Sequencing of V_{\gamma} CDR2 and CDR3**

All methodological details concerning the identification process of the V_{\gamma} CDR2 and CDR3 sequences on which this study is based have been previously published.8-9

Briefly, following total RNA extraction and first cDNA synthesis, polymerase chain reaction (PCR) amplifications were performed using both consensus primers7 and the PCR conditions7-8 previously described.

PCR products were subsequently purified using the Qiaquick PCR purification kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions.

Cycle sequencing was carried out using the BigDye terminator kit (Applied Biosystems, Foster City, CA, USA), and a 377 ABI-PRISM sequencer (Applied Biosystems) was used for the automated sequencing.

All PCR product-related electropherograms were aligned to the closest germ line counterpart9 using the Sequence Navigator software (Applied Biosystems) and the final sequence analyses were performed utilizing the IgBLAST program at the NCBI web site (http://www.ncbi.nlm.nih.gov/igblast).

**Statistical analysis**

Fisher’s exact test was used for all group comparisons: a 2-tail \( p < 0.05 \) was considered statistically significant.

**Results**

Acquired, potential N-glycosylation sites were found in all 24 (100%) FL cases, but in only 2/23 (9%) cases of B-cell malignancy other than FL. Among the 23 non-FL cases, six cases had no somatic mutations and were, thus, unlikely to have AGS. Therefore, the incidence of AGS among non-FL B-cell malignancies in which some somatic mutations had occurred was 2/17 (12%; \( p < 0.0005 \)) (Table 1). Within the FL cases, histologic grade\(^{10,11} \) did not influence the number of AGS (Table 1). Furthermore, in six of the FL cases, tumor-specific IgGs involved a germ line gene with a natural, potential N-glycosylation site (NGS): V4–34 in all cases. However, in all six cases, the NGS was lost and at least one novel AGS had been acquired (Figure 1). All six cases of primary diffuse large B-cell lymphoma (DLCL) were characterized by a completely different pattern. In no case did the tumor-specific Ig derive from a germ line bearing an NGS (Figure 2) or develop any AGS, despite containing somatic mutations (Figure 2). Moreover, the germ line genes from which DLCL tumor-specific IgGs were derived curiously always differed from those related to FL-specific IgGs (Figure 1 and 2).

Regarding MCL, it is known that, although the vast majority of tumor-specific IgGs do not have somatic mutations, a limited number of such IgGs may have somatic mutations as a consequence of being follicular or post-follicular origin.12-14 Among our seven cases of MCL, four had no mutations (Figure 2), including one case (MCL5, which only showed one possible mutation involving the last amino acid before the N-terminus of CDR3) derived from V4–34 and, as such, bearing one NGS. On the other hand, 1 of 3 cases of somatically mutated MCL had an AGS (Figure 2). Among our six cases of SLL, four were characterized by a tumor-specific Ig featuring at least a few somatic mutations. However, none of them had an AGS (Figure 2).

Finally, only one of our four cases of sporadic Burkitt’s lymphoma (BL) had an AGS despite the presence of somatic mutations in all four cases (Figure 2).

All thirty-seven AGS detected in our study were analyzed for possible occurrence within a hotspot for somatic mutations according to the definition of the RGYW motif.15 Only one out of four AGS located within a framework region (FR) presented this feature. In contrast, AGS located in any CDR occurred within such a hotspot in 8/16 (50%) and in 7/9 (77%) cases, respectively, depending on whether or not the AGS present in

<table>
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<tr>
<th>Histology</th>
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<tr>
<td>FL</td>
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<td>BL</td>
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Table 1. Frequency of acquired, potential N-glycosylation sites (AGS) within the V_{\gamma} FR2-CDR3 sequence fragment of B-cell tumor-specific IgGs.
Figure 1. Location of AGS within the V<sub>h</sub>-FR2-CDR3 sequence fragment of B-cell tumor-specific Igs. Dots represent identity with the corresponding germ line sequence, whereas the novel sites are highlighted. Boxes indicate AGS occurring within somatic mutation hotspots according to the definition of the RGYW motif.

Figure 2. V<sub>h</sub>-FR2-CDR3 sequence fragments belonging to B-cell tumor-specific Igs not presenting AGS. Dots represent identity with the corresponding germ line sequence.
sequences derived from the NGS-bearing V4-34 germ line gene were taken into account (Figure 1).

The location of somatic mutation-induced AGS is different in IgM and IgG (Figure 3). In particular, 7/9 AGS-containing, IgM-bearing FL displayed an AGS at or near the N-terminus of the CDR2, whereas the remaining cases (2/9) featured the AGS near or at the N-terminus of the CDR3. In contrast, 10/14 AGS-containing, IgG-bearing FL displayed an AGS at or near the N-terminus of the CDR3, whereas 4/14 featured the AGS at or near the N-terminus of the CDR2. However, the location of the AGS correlated only with isotype. Two IgG-expressing cases had AGS in both CDR2 and CDR3 (FL2, FL24) and in one of these cases (FL24), neither AGS was near the N-terminus of the region. All in all, the likelihood of finding an AGS at or near the N-terminus of the CDR2 was markedly higher in clonal IgM-bearing FL (p=0.036), whereas the likelihood of finding an AGS at or near the N-terminus of the CDR3 was significantly higher in clonal IgG-bearing FL (p=0.036). Moreover, none of the twenty-four FL cases had an AGS only outside the CDR2 and the CDR3 in contrast to the non-FL cases, in which the only identified AGS was found within FR3 (p=0.003) (Figure 3). Finally, among B-cell malignancies bearing no AGS, the presence of a tumor-specific Ig of IgM isotype was demonstrated in most BL, MCL and SLL cases, whereas the same trend did not emerge among DLCL cases (Figure 4).

The amino acid sequences of individual AGS differed when the site was in a CDR rather than a FR (Figure 3). Eighty-six percent (25/29) of AGS located within any CDR ended with serine, whereas 3/4 of the AGS found within FR3, had threonine as the last amino acid (p=0.023).

Discussion

Acquired, potential N-glycosylation sites are a feature of the immunoglobulin genes on the surface of follicular lymphoma cells that have undergone somatic mutation. With this study, we extend and confirm prior reports on this phenomenon, show a correlation between the location of the AGS and the FL Ig isotype, identify amino acid sequence differences between AGS in CDR versus FR, and assess the frequency of AGS in other B-cell tumors.

An obvious limitation of our data on the acquisition of AGS by the tumor-specific Igs of first-relapse B-cell malignancies is that they are still numerically very limited. Moreover, although it appears ever more evident that the vast majority of acquired sites lie within VH CDR2 and CDR3,1-2 some AGS do not. Therefore, since our data refer only to these two CDRs, a comprehensive assessment of AGS has not yet been performed.

On the other hand, a few features are becoming clear. For instance, AGS may be useful to distinguish authentic FL from other types of lymphoma. In our series, 2 cases originally classified as FL were found to lack AGS and central review found that the diagnosis in both cases was SLL. Of course, this is not sufficient to consider the presence of AGS as an element capa-
ble of defining cases of FL. However, the analyses of samples from larger groups of patients might help to determine whether AGS could become a defining feature of FL.

It is striking that no AGS were detected within the mutated sequences of DLCL cases. Many FL under histologic progression to DLCL and a substantial fraction of de novo DLCL cases are thought to be derived from follicular center cells. It will be important to compare AGS in de novo DLCL and in DLCL that has progressed from FL to see whether the processes by which these diseases develop are similar or different.

It is also interesting that the acquisition of AGS occurs in different places as a function of Ig isotype: preferentially within CDR2 for IgM and within CDR3 for IgG. Even when the tumor Ig contains an NGS (V4-34 germ line) in the CDR2 (Figure 1), IgM-expressing FL lose the NGS and acquire an AGS in the CDR2, whereas an IgG-expressing FL (FL20), lost the CDR2 NGS and acquired an AGS within CDR3. These data argue that the AGS location is affected by the Ig isotype.

The role of AGS is undefined, but it may be both of value to enhance antigen binding and exploitable in our clinical efforts to make a selective and specific tumor vaccine. Asn, Trp, Tyr and Ser, but not Thr, are amino acids within the combining sites known to improve the affinity of antibody for antigen. Similarly, an Ig with these somatic mutations may be more strongly antigenic when used as a vaccine rather than as an antibody. In this respect, the presence of AGS, particularly in FL-specific Igs, might per se enhance both the specificity and the efficacy of any vaccine-induced, Id-specific, polyclonal, humoral response, possibly targeting these portions of the Id-containing Ig amino acid sequence. Whether similar considerations may also apply more broadly to vaccine-induced, Id-specific, T-cell responses remains speculative, as the exact location and distribution of the single idiotopes within the lg variable regions has yet to be elucidated.

The role of AGS in the immunogenicity of tumor immunoglobulin has not yet been defined. Structural studies are needed to assess whether these sites are actually glycosylated in tumor cells or are merely potential sites for glycosylation. In addition, this variable may need to be examined in the framework of idiotype rescue in the generation of idiotype vaccines. If important idiotypes were glycosylated on the tumor-specific immunoglobulin, it would be reasonable to suggest that the immunogen used to elicit an immune response to the tumor should also be glycosylated. To date, the influence of this variable on the outcome of idiotypic vaccination has not been assessed. It may be necessary to undertake comparisons of glycosylated and non-glycosylated idiotype vaccines to assess whether the AGS may enhance the effectiveness of idiotype-directed therapies.

Natalia Zabalegui, contributed to the study design, carried out a number of experiments and wrote the manuscript. Ascensión López de Cerio, Susana Inogés and Mercedes Rodrigoz-Calvillo carried out the remaining experiments. Javier Pérez-Calvo performed the statistical analysis. Milagros Hernández, Jesus García-Foncillas, Salvador Martin and Eduardo Rocha contributed to the data interpretation. Maurizio Bendandi contributed to both study design and data interpretation, supervised the whole study and revised the last version of the manuscript, giving the authorization for its publication. The authors reported no potential conflicts of interest.

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Figure 4. Distribution of Vh, FR2-CDR3 sequence fragments not bearing AGS according to histology and Ig isotype.
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