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Among B cell non-Hodgkin's lymphomas, MALT lymphomas express a unique antibody repertoire with frequent rheumatoid factor reactivity

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We analyzed the structure of antigen receptors of a comprehensive panel of mature B non-Hodgkin's lymphomas (B-NHLs) by comparing, at the amino acid level, their immunoglobulin (Ig)V_H-CDR3s with CDR3 sequences present in GenBank. Follicular lymphomas, diffuse large B cell lymphomas, Burkitt's lymphomas, and myelomas expressed a CDR3 repertoire comparable to that of normal B cells. Mantle cell lymphomas and B cell chronic lymphocytic leukemias (B-CLLs) expressed clearly restricted albeit different CDR3 repertoires. Lymphomas of mucosa-associated lymphoid tissues (MALTs) were unique as 8 out of 45 (18%) of gastric- and 13 out of 32 (41%) of salivary gland–MALT lymphomas expressed B cell antigen receptors with strong CDR3 homology to rheumatoid factors (RFs). Of note, the RF-CDR3 homology without exception included N-region–encoded residues in the hypermutated *IgV$_H$* genes, indicating that they were stringently selected for reactivity with auto-IgG. By in vitro binding studies with 10 MALT lymphoma–derived antibodies, we showed that seven of these cases, of which four with RF-CDR3 homology, indeed possessed strong RF reactivity. Of one MALT lymphoma, functional proof for selection of subclones with high RF affinity was obtained. Interestingly, RF-CDR3 homology and t(11;18) appeared to be mutually exclusive features and RF-CDR3 homology was not encountered in any of the 19 pulmonary MALT lymphomas studied.

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Abbreviations used: Ag, antigen; B-CLL, B cell chronic lymphocytic leukemia; B-NHL, B non-Hodgkin's lymphoma; BCR, B cell antigen receptor; BL, Burkitt's lymphoma; D, diversity; DLBCL, diffuse large B cell lymphoma; FL, follicular lymphoma; FR, framework region; GC, germinal center; HCV, hepatitis C virus; HID, healthy immunized donor; ICV, intraclonal sequence variation; J, joining; LIDA lymphoma-idiotype-derived Ab; MALT, mucosa-associated lymphoid tissue; MCL, mantle cell lymphoma; MZBCL, marginal zone B cell lymphoma; RF, rheumatoid factor; V, variable.

B cell non-Hodgkin's lymphomas (B-NHLs) comprise >85% of malignant lymphomas worldwide. They are in majority of germinal center (GC) or post-GC phenotype and often harbor chromosomal translocations typically involving immunoglobulin (Ig) loci (1). In spite of their genetic defects, most B-NHLs do not replicate spontaneously in vitro, indicating that they still depend on environmental stimuli for their growth. To date, these external factors are ill defined. Evidence exists that B cell antigen receptor (BCR) ligands have, as in normal B cell development, a pivotal role in the pathogenesis of at least some B-NHLs. For example, the architecture and cellular composition of follicular lymphomas (FLs) is highly reminiscent of normal GCs. Furthermore, extranodal marginal zone B cell lymphomas (MZBCLs) of mucosa-associated lymphoid tissue (MALT) arise at sites of antigenic stimulation due to organ-specific autoimmunity; e.g., Sjögren's sialadenitis (2) and Hashimoto's thyroiditis (3), or infection like *Helicobacter pylori* gastritis (4, 5) and *Borrelia burgdorferi* dermatitis (6, 7), respectively. Similarly, a role of hepatitis C virus (HCV) infection has been inferred in the development of malignant B cell proliferation, including splenic MZBCL and MALT lymphoma (8, 9). Most recently, it has been claimed that ocular adnexal MALT lymphoma and immunoproliferative small intestinal disease (also known as α-heavy chain disease) are associated with *Chlamydia psittaci* and *Campylobacter jejuni* infections, respectively (10, 11). The low tendency of MALT lymphomas to spread beyond the environment in which they evolve may be related to the expression of certain homing and chemokine receptors, such as α4β7 and CXCR3 (12, 13). In addition, it has been proposed that even during the tumor

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stage, antigen (Ag) plays a growth-sustaining role. This notion is strongly supported by the observation that a proportion of low grade gastric MALT lymphomas (14, 15) and skin MZBCLs (7) are curable by bacterial eradication alone, while interferon α-2b treatment can cause regression of HCV-associated MZBCLs (16, 17).

Analysis of the Ig variable (*IgV*) genes supported the concept of Ag-driven lymphomagenesis in FL and MALT lymphoma. The IgV heavy (IgV_H) and IgV light (IgV_L) chain genes of these malignancies are heavily mutated, compatible with a GC or post-GC derivation (18–22). The mutation patterns unequivocally indicate that Ag-based selection occurs at some stage of their development; despite high mutation loads, the overall structure of the Ig is generally being preserved in these lymphomas often during years of disease. Apparently, selective forces prevent the outgrowth of BCR^- lymphoma mutants.

Although many studies on FL and MALT lymphomas allude to a role for Ag in the pathogenesis of these lymphomas, only sporadic data exist on the exact ligands that these B cell neoplasms might recognize. Although the obvious candidate ligand for gastric MALT lymphoma was *H. pylori,* in vitro cultures revealed that the tumor B cells were not directly stimulated by *H. pylori*, but indirectly by CD40/CD40L-mediated help of intratumoral, *H. pylori*–specific T cells (23). Hussell et al. (24) and Greiner et al. (25) observed reactivity of MALT lymphoma–derived Abs with follicular DCs (FDCs), various epithelia, or postcapillary venules of Peyer's patches, but no specific Ags were molecularly defined. It is well documented that a significant proportion of B cell chronic lymphocytic leukemias (B-CLLs) express Ig (poly) reactive with a diversity of autoantigens (26–31). In contrast, with other B-NHL entities, autoreactivity has only sporadically been reported (32–34). Finally, viral antigens of HTLV-I and HCV have been implicated as BCR ligands of individual cases of B-CLL (35) and diffuse large B cell lymphoma (DLBCL; reference 36), respectively.

To address the issue of antigen-receptor specificity of B-NHL, we performed a systematic analysis of the antigen binding sites of 132 extranodal MZBCL (24 from our laboratory and 108 from literature) and, for comparison, from a comprehensive panel of 478 other B-NHL. We provide ev-

Table I. Clinical presentation, immunohistochemistry, and genetics of 24 MALT lymphomas

					Immunohistochemistry				PCR
Patient	Sex	Age	Location	Clinical presentation	Ig isotype	Light chain	CXCR3	α4β7	t(11;18)
M4	f	60	thyroid gland	Hashimoto's thyroiditis	NC	κ	−	−	−
M5	f	64	parotid gland	Sjögren's syndrome	IgM	κ	+	+	−
M6'95	f	55	stomach	gastritis	ND	κ	+	+	−
M6'96	f	56	stomach	gastritis	IgM	κ	+	+	ND
M8	m	44	parotid gland	unknown	IgM	κ	−	+	−
M9	f	58	tonsil	unknown	IgM	κ	−	+	−
M11	m	38	parotid gland	unknown	IgM	κ	+	+	−
M13	m	63	ileum	unknown	NC	NC	+	+	−
M14	f	70	parotid gland	Sjögren's syndrome	IgA	κ	+	+	−
M15	f	45	lacrymal gland	Sjögren's syndrome	IgA	κ	+	+	−
M19	f	74	lung	unknown	IgM	λ	+	+	+
M20	m	39	lung	unknown	IgM/IgA	λ	+	+	+
M21	f	64	parotid gland	Sjögren's syndrome	IgM	κ	+	+	−
M22[a]	m	71	groin lymph node	unknown	IgA	κ	+	+	−
M23	m	52	lung	unknown	IgG	κ	+	+	+
M30	f	60	stomach	gastritis	NC	κ	ND	ND	−
M41	f	55	stomach	gastritis	ND	ND	ND	ND	−
M45	m	81	stomach	gastritis	ND	ND	ND	ND	−
M46	m	65	stomach	gastritis	ND	ND	ND	ND	−
M55	m	41	lung	unknown	ND	ND	ND	ND	−
M56	m	78	stomach	gastritis	IgM	κ	+	+	+
M57	m	81	stomach	gastritis	IgM	κ	+	+	+
M58	m	74	stomach	gastritis	IgM	NC	+	+	+
M60	m	71	stomach	gastritis	IgM	λ	+	+	+
M61	m	38	stomach	gastritis	IgM	λ	+	−	+

[a]Initially located in salivary gland.
NC, not clear.

idence that among the various B cell neoplasms, gastric- and salivary gland-MALT lymphomas express a distinctive Ig repertoire and frequently originate from precursor B cells clonally selected for auto-IgG binding capacity. The fact that B-NHL entities express qualitatively different Igs points toward different roles of the BCRs in their pathogenesis.

RESULTS

IgV_H and IgV_L sequence analysis of MALT lymphomas

A panel of 24 MALT lymphomas was analyzed (Table I). All lymphomas were $CD20^+$ and virtually all expressed the chemokine receptor CXCR3 and the mucosal homing integrin $\alpha4\beta7$. The pulmonary lymphomas *M19, M20,* and *M23* and the gastric lymphomas *M56, M57, M58, M60,* and *M61* carried the t(11;18), involving the *API2* and *MALT1* genes (Table I). Except for *M60*, all IgV_H and $IgV\kappa$ genes analyzed were somatically mutated with means of 21 (range 0–68) and 18 mutations (range 1–58) per IgV gene, respectively (Tables II and III). Of *M6,* biopsies were available of two time points spanning a 9 mo interval. At relapse (*M6′96*), 15 somatic mutations were found in the expressed V3-7 IgV_H gene while at presentation (*M6′95*) an additional replacement mutation in codon 13 had been present (Table II). Immunohistochemistry (Table I) and RT-PCR (Table II) indicated that the lung lymphoma *M20* contained both IgM- and IgA-expressing tumor cells. The IgM- and the IgA-related IgV_H sequences were identical and contained 5 mutations (Table II). In 17 of the 24 lymphomas (71%), the replacement versus silent (R/S) mutation ratios in the framework regions (FRs) of the IgV_H genes, were significantly <1.5, implying that, in spite of the high mutation frequencies, selective forces had preserved the BCR in these lymphomas (unpublished data). Intraclonal variation (ICV) was determined for 16 IgV_H genes and for 8 $IgV\kappa$ genes (18). In 10 out of the 16 (63%) MALT lympho-

Table II. Immunoglobulin variable heavy chain genes of 24 MALT lymphomas

Patient	Ig isotype (RT-PCR)	VH family	Closest VH germline gene	No. of mutations (%)	D gene	JH gene	Intraclonal variation[a]
M4	γ	VH3	V3-23 (DP47)	68 (23)	NA	JH4b	0.8 (5)
M5	μ	VH3	V3-7 (DP54)	17 (5.8)	D3-3	JH3b	0.8 (5)
M6'95	μ	VH3	V3-7 (DP54)	16 (5.4)	D3-22	JH3b	ND
M6'96	μ	VH3	V3-7 (DP54)	15 (5.1)	D3-22	JH3b	1.0 (5)
M8	μ,δ[b]	VH3	V3-30 (DP49)	26 (8.8)	D5-24	JH5	ND
M9	μ,δ[b]	VH1	V1-69 (DP10)	8 (2.7)	NA	JH4b	ND
M11	μ	VH1	V1-69 (DP10)	11 (3.7)	D6-13	JH4b	3.0 (7)
M13	μ	VH4	V4-31 (DP65)	14 (4.7)	D5-24	JH4	1.8 (5)
M14	α	VH3	V3-23 (DP47)	43 (15.0)	NA	JH6	2.3 (6)
M15	α	VH1	V1-18 (DP14)	46 (16.0)	NA	JH4b	<0.4 (5)
M19	μ,δ[b]	VH3	V3-53	29 (10.0)	NA	JH4b	ND
M20	μ,α	VH4	V4-30.4 (DP78)	5 (1.7)	NA	JH3b	ND
M21	μ,δ[b]	VH3	V3-23 (DP47)	23 (7.8)	D2-2	JH4b	0.7 (11)
M22	α	VH1	V1-69 (DP88)	14 (4.8)	D4-17	JH4b	<0.4 (15)
M23	γ	VH1	V1-69 (DP88)	7 (2.4)	NA	JH4b	<0.4 (7)
M30	γ	VH2	V2-5 (VII-5)	33 (11.1)	NA	JH1	0.7 (7)
M41	ND	VH1	V1-69 (DP10)	19 (6.5)	D3-22	JH4b	<0.4 (7)[c]
M45	ND	VH1	V1-3 (DP25)	22 (7.5)	D3-10	JH4b	ND
M46	ND	VH3	V3-30/30.5 (DP49)	12 (4.1)	D5-12	JH6b	ND
M55	ND	VH3	V3-7 (DP54)	24 (8.1)	D3-22	JH4b	ND
M56	μ	VH1	V1-69 (DP10)	7 (2.4)	D1-14	JH4b	<0.4 (11)
M57	μ	VH1	V1-18 (DP14)	14 (4.8)	NA	JH6b	1.9 (9)
M58	μ	VH3	V3-53	23 (7.9)	D3-22	JH4b	ND
M60	μ	VH1	V1-69 (DP10)	0 (0.0)	D2-15	JH5b	<0.4 (9)
M61	μ	VH1	V1-18 (DP14)	19[d] (6.5)	D2-15	JH4b	0.8 (4)

[a]The intraclonal variation is indicated as the mean number of nucleotide differences observed per clone, as compared with the consensus sequence. Numbers in parentheses indicate the number of clones that were sequenced.
[b]IgD was not detected immunohistochemically.
[c]M41 contained distinct subclones (Fig. 1).
[d]M61 had a deletion of the three nucleotides of codon 29.
NA, the germline D gene could not definitely be assigned.

Table III. Immunoglobulin variable light chain κ genes of the MALT lymphomas

Patient	Vκ family	Closest Vκ germline gene	No. of mutations (%)	Jκ gene	Intraclonal variation[a]
M4	Vκ1	L9 (Ve+)	58 (20.0)	Jκ4	0.5 (4)
M5	Vκ3	L2 (kv328, DPK21)	9 (3.2)	Jκ1	1.0 (5)
M6'96	Vκ3	L2 (kv328, DPK21)	5 (1.8)	Jκ1	0.4 (5)
M8	Vκ1	O12/O2 (DPK9)	27 (9.5)	Jκ4	ND
M9	Vκ3	A27 (kv325, DPK22)	2 (0.69)	Jκ1	ND
M11	Vκ3	A27 (kv325, DPK22)	1 (0.35)	Jκ1	1.5 (6)
M14	Vκ2	A19/A3 (DPK15)	51 (17.0)	Jκ4	1.2 (6)
M15	Vκ4	B3 (DPK24)	17 (5.6)	Jκ4	9.3 (6)
M21	Vκ3	A27 (kv325, DPK22)	13 (4.5)	Jκ1	0.5 (4)
M22	Vκ3	A27 (kv325, DPK22)	6 (2.1)	Jκ2	ND
M23	Vκ1	O12/O2 (DPK9)	6 (2.1)	Jκ1	0.8 (8)

[a]The intraclonal variation is indicated as the mean number of nucleotide differences observed per clone, as compared to the consensus sequence. Numbers in parentheses indicate the number of clones that were sequenced.

mas, significant ICV was found in IgV_H (Table II). Except for *M6*, all lymphomas with ICV in IgV_H genes also displayed ICV in the $IgV\kappa$ genes, generally of a lower degree (Table III). In *M15*, lacking ICV in the IgV_H gene, an exceptionally high degree of ICV was observed in its $IgV\kappa$ gene (Tables II and III). Interestingly, *M41* appeared a somatically diversified, V1-69/D3-22/JH4b-expressing lymphoma, harboring distinct subclones (*M41-A, B, C, and D*) with 19, 19, 20, and 2 mutations, respectively (Fig. 1).

IgV_H–CDR3 amino acid sequences of MALT lymphomas and splenic MZBCL

We compared the IgV_H-CDR3 amino acid (aa) sequences of our panel of MALT lymphomas to IgV_H-CDR3 aa sequences present on GenBank using the NCBI Protein-BLAST program with the option "search for short nearly exact matches" (BLASTP 2.2.6; reference 37). CDR3 regions consisting of at least 7 aa (all except *M20*) were analyzed (Table IV). A CDR3 sequence was considered to be homologous to previously reported CDR3 sequences on GenBank (a) if sharing at least 75% aa sequence homology and (b) a length difference between the CDR3 sequences not exceeding 3 aa (maximum gap of 3 aa). Applying these homology criteria, we found that the CDR3 of 4 MALT lymphomas (*M23, M46, M56,* and *M60*) displayed homology to different B cell clones analyzed previously in repertoire studies. Interestingly, 4 cases (*M5,*

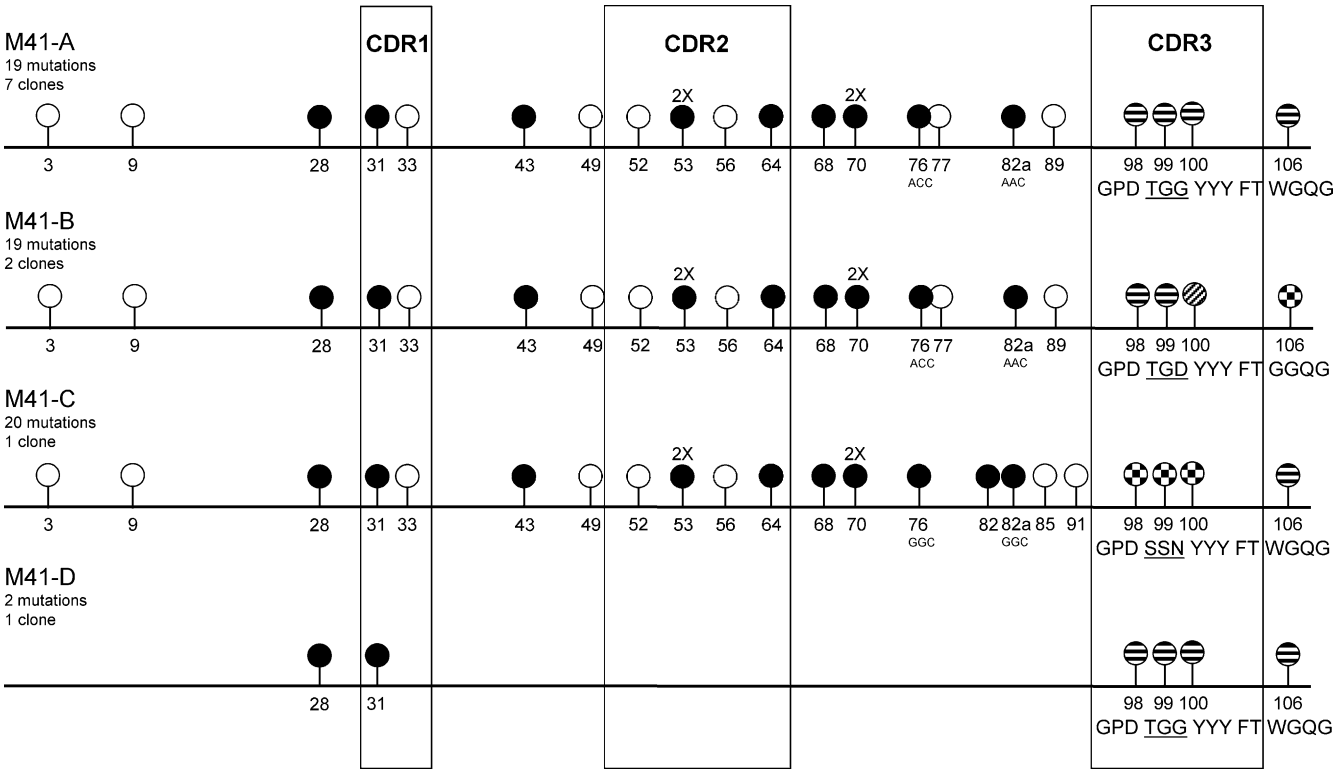

Figure 1. Schematic representation of the IgV_H clones identified in *M41*. The lollipop-shaped symbols indicate nucleotide differences as compared with the V1-69 (DP10) germline IgV_H gene. Except for the CDR3 region, replacement and silent mutations are indicated with closed and open circles, respectively, with codon numbering according to V-base indicated underneath. 2×, two mutations in the indicated codon. The mutations in codons 76 and 82a are different between *M41-A/B* and *M41-C*, respectively. In the CDR3 and in codon 106, interclonal differences are indicated by different filling patterns of the circles. In the CDR3, the deduced aa sequence is depicted in the one-letter code underneath. The CDR3 of *M41-A/D, M41-B* and *M41-C* displayed, respectively, 73, 82, and 91% homology to the CDR3 of *RF-WOL*. *M41-D* has only two mutations, shared with *M41-A, B,* and *C*.

Table IV. Amino acid sequences of the variable heavy chain CDR3 of the 24 MALT lymphomas

Patient	V_H-D-J_H rearrangement	CDR3 amino acid sequence[a]	CDR3 length
			aa
M4	V3-23 (DP47)/JH4b	*CTK* AHVPYFDGLSPSNV *WGQG*	14
M5	V3-7 (DP54)/D3-3/JH3b	*CAR* GD FWSGDY ID AFDI *WGQG*	14
M6	V3-7 (DP54)/D3-22/JH3b	*CAR* GD YFDSSGSF ID AFDI *WGQG*	16
M8	V3-30 (DP49)/D5-24/JH5	*CAK* DGSEFRLIY WFDS *WGRG*	13
M9	V1-69 (DP10)/JH4b	*CAR* DWAHQGETRSNFLYY *WGQG*	15
M11	V1-69 (DP10)/D6-13/JH4b	*CAR* E GIAAA VNP FDY *WGQG*	12
M13	V4-31 (DP65)/D5-24/JH4	*CAG* D RGGYN LL DC *WGHG*	10
M14	V3-23 (DP47)/JH6	*CAK* QMGLAGTQR FYGLDV *WGKG*	15
M15	V1-18 (DP14)/JH4b	*CAR* ATLDLDGYM DF *WGQG*	11
M19	V3-53/JH4b	*CAT* PISGTYHLY Y *WGQG*	10
M20	V4-30.4 (DP78)/JH3b	*CAR* DQ AFDI *WGQG*	6
M21	V3-23 (DP47)/D2-2/JH4b	*CAK* DLFFV GYCTTTGC NT FDY *WGQG*	18
M22	V1-69 (DP88)/D4-17/JH4b	*CAR* GSN DYGDN VPVQPH Y *WGQG*	15
M23	V1-69 (DP88)/JH4b	*CAR* VSGNSH FDY *WGQG*	9
M30	V2-5 (VII-5)/JH1	*CAQ* RGGFYDSSLGFYIAPFP H *WGQG*	18
M41-A	V1-69 (DP10)/D3-22/JH4b	*CAR* GP DTGGYYY F Y *WGQG*	11
M41-B	V1-69 (DP10)/D3-22/JH4b	*CAR* GP DTGDYYY F Y *GGQG*	11
M41-C	V1-69 (DP10)/D3-22/JH4b	*CAR* GP DSSNYYY F Y *WGQG*	11
M45	V1-3 (DP25)/D3-10/JH4b	*CAR* GTKIRGIVKPFP DY *WGQG*	14
M46	V3-30/30.5 (DP49)/D5-12/JH6b	*CAK* DSGYVNFYYT MDV *WGQG*	13
M55	V3-7 (DP54)/D3-22/JH4b	*CAK* WDYENSAYFLH Y *WGQE*	12
M56	V1-69 (DP10)/D1-14/JH4b	*CAR* DT GN H YFDY *WGQG*	9
M57	V1-18 (DP14)/JH6b	*CAT* PPPRAGDGP YYYYGMDV *WGQG*	17
M58	V3-53/D3-22/JH4b	*CAR* HSYDNNAY DF *WGQG*	10
M60	V1-69 (DP10)/D2-15/JH5b	*CAR* DPVD CSGGSCY LS WFDP *WGQG*	17
M61	V1-18 (DP14)/D2-15/JH4b	*CAR* D YCSGGICY GG DY *WGQG*	13

[a]The FR3 and FR4 are indicated in italics. The assignable nontemplated region-encoded amino acids are underlined.

M6, *M11*, and *M41*) showed strong homology with CDR3 sequences of other MALT lymphomas, as well as with CDR3 sequences of rheumatoid factors (RF). Salivary gland lymphoma *M5* and gastric lymphoma *M6*, both expressing a V3-7/JH3 rearrangement, were homologous to a plethora of IgV_H-CDR3 aa sequences originating from salivary gland MALT lymphomas, gastric MALT lymphomas, HCV-associated lymphomas, B cell clones derived of benign myoepithelial sialadenitis, one DLBCL and two genuine V3-7–encoded RFs (Table S1, available at http://www.jem.org/cgi/content/full/jem.20050068/DC1). Salivary gland lymphoma *M11*, which expressed a V1-69/JH4 rearrangement, was homologous to five normal B cell clones, one salivary gland MALT lymphoma *Isolate-4* (38), and three V1-69-encoded RFs (Table S1). Interestingly, *Isolate-4* is one of the five cases described by Miklos et al. (38) having distinct CDR3 characteristics, indicating that *M11* belongs to this group which is characterized by distinct aa motifs (ERG and NP) at the V_H-D_H- and the D_H-J_H-junctions, respectively. Gastric MALT lymphoma *M41*, which also expressed V1-69/JH4, was homologous to HCV-associated lymphomas of different histological subtypes, one splenic MZBCL, one salivary gland MALT lymphoma, one gastric MALT lymphoma and finally to one RF termed *RF-WOL* (reference 39 and Table S1).

We then, additionally, examined CDR3 of 35 gastric-, 26 salivary gland- and 15 pulmonary-MALT lymphomas as well as 32 splenic MZBCL from literature and/or GenBank (Table V). This revealed that overall 8 out of 45 (18%) gastric- and 13 out of 32 (41%) salivary gland-MALT lymphomas expressed IgV_H-CDR3 with RF homology. Three major RF homology groups could be distinguished, i.e., nine encoded by V1-69/JH4 (V1-69-RF), eight by V3-7/JH3 (V3-7-RF) and three by V1-69/JH4 (RF-WOL) rearrangements, respectively (Table V). Intriguingly, in all cases homology areas included aa encoded by the nontemplated nucleotide (N) regions (Fig. 2). In addition, gastric MALT lymphoma *ML15* (40) was homologous to another RF termed *RF-C93* (41). Noteworthy, none of the 19 pulmonary MALT lymphomas expressed RF-homologous CDR3 whereas among the splenic MZBCLs, only one case expressed a CDR3 homologous to *RF-WOL* (Table V).

Currently, 10 genuine V1-69/JH4-encoded RFs, isolated from rheumatoid arthritis patients or from healthy donors immunized with mismatched red blood cells (HID),

Table V. Comparison of IgV_H-CDR3 amino acid sequences of a panel of mature B-NHL with IgV_H-CDR3 amino acid sequences from GenBank

Type of lymphoma	Mean CDR3 length	N	No. of cases with CDR3 homology				
			Overall[a]	RF[b]	V1-69 RF[c]	V3-7 RF[c]	WOL-RF[c]
	aa		*(%)*	*(%)*			
Gastric MALT	13.6	45	16 (36)	8 (18)	1	4	2
Salivary gland MALT	14.5	32	15 (47)	13 (41)	8	4	1
Pulmonary MALT	12.7	19	2 (11)	0 (0)	0	0	0
Other MALT	NI	4	0 (0)	0 (0)	0	0	0
Splenic MZBCL	16.3	32	8 (25)	1 (3)	0	0	1
MCL	13.3	23	10 (44)	0 (0)	0	0	0
B-CLL IgV_H unmutated	17.4	165	73 (44)	2 (1)	0	0	0
B-CLL IgV_H mutated	13.7	143	24 (17)	0 (0)	0	0	0
FL	12.1	48	4 (8)	0 (0)	0	0	0
DLBCL	12.2	20	2 (10)	1 (5)	0	1	0
BL	12.6	48	5 (10)	0 (0)	0	0	0
Myeloma	13.0	31	5 (16)	0 (0)	0	0	0

The lymphomas used for the homology analyses are listed in the supplemental legend to this table (available at http://www.jem.org/cgi/content/full/jem.20050068/DC1).
[a]Indicates the number of lymphomas that show at least 75% homology, according to the criteria described, to at least one IgV_H-CDR3 sequence present in GenBank.
[b]Indicates the number of lymphomas that show homology to known rheumatoid factor (RF) IgV_H-CDR3 sequences.
[c]Indicates the number of lymphomas with homology to canonical V1-69-, V3-7-encoded RFs and to WOL-RF. All the B-NHL that expressed RF homologous CDR3 regions are as follows: Gastric MALT lymphomas: *ML13* homology to V1-69-RF; *ML25, ML27, ML39a,* and *M6* homology to V3-7-RF; *ML16* and *M41* homology to WOL-RF; and *ML15* homology to C93-RF. Salivary gland MALT lymphomas: *Isolate 1–5, PO-1, BA-2.2,* and *M11* homology to V1-69-RF; *G552, SH, Isolate 10,* and *M5* homology to V3-7-RF; and *JA-1* homology to WOL-RF. Splenic MZBCL: *Isolate 1* homology to WOL-RF. B-CLL unmutated: *CLL011* homology to TB-3-D13-RF and *ID-74* homology to SJ2-RF. DLBCL: *EJ* homology to V3-7-RF.
NI, not informative.

have been described (42). V1-69/JH4 RFs typically contain a 12–14 aa CDR3 and are combined with an A27(kv325)-encoded IgVκ chain. Among the V1-69-RF-homologous MALT lymphomas, only of salivary gland lymphoma *M11* the IgVκ is known, which indeed proved to be the canonical A27(kv325) IgVκ (Table III and Table S2, available at http://www.jem.org/cgi/content/full/jem.20050068/DC1). W*OL-RF* is also V1-69/JH4-encoded, but with a distinct 13 aa CDR3, again in combination with an A27(kv325)-encoded IgVκ chain. Five V3-7 RFs have been described, isolated from HIDs and an RA patient (42).V3-7-RFs are encoded by V3-7/D3-22/JH3 rearrangements, typically possessing a 16–17 aa CDR3 with the D3-22 in reading-frame 2, and in combination with an L2 (kv328)/Jκ1-encoded IgVκ chain. The V3-7/JH3-expressing salivary gland lymphomas *M5* and *SH* (43) and gastric MALT lymphoma *M6* indeed all co-expressed the canonical L2 (kv328)/Jκ1-encoded IgVκ chain (Table III and Table S2).

IgV_H-CDR3 amino acid sequences of other mature B-non Hodgkin's lymphomas

For comparison, IgV_H-CDR3 analyses were extended to a comprehensive panel of other mature B-NHLs available from literature and/or on GenBank. For most B-NHL entities, the average CDR3 aa length was comparable to that of mature naive B cells being 13,5 aa (44). Only splenic MZBCLs and the IgV_H-unmutated B-CLLs expressed CDR3 of higher mean lengths (Table V). Of 48 FLs, 20 DLBCLs, 48 Burkitt lymphomas (BL) and 31 myelomas, only 16 cases (11%) displayed CDR3 aa homology with other CDR3 sequences present in GenBank. Thirteen of the 16 cases resembled those of normal B cells analyzed in repertoire studies (45–47). As mentioned above, one DLBCL *EJ* (48) showed homology to several gastric- and salivary gland-MALT lymphomas as well as to V3-7-RFs (Table V).

Analyses of 23 mantle cell lymphomas (MCL), deposited on GenBank, revealed that their CDR3 displayed a high frequency of homology (10 out of 23, 44%), mostly (7 cases) with CDR3 regions of unmutated normal B cells.

We analyzed a panel of 308 B-CLLs, 165 (54%) and 143 (46%) of the *IgV*-unmutated and the *IgV*-mutated subsets, respectively (Table V). Overall, the CDR3s of 97 out of the 308 B-CLLs (31%) displayed CDR3 homology, 59 of which (19%) with CDR3s of B-CLLs (inter B-CLL homology). Of the group of 97 B-CLLs with any homology, 75% belonged to the *IgV*-unmutated subset. This relative overrepresentation was even more outspoken among the group with inter-B-CLL homology in which 50 out of the 59 cases (85%) were unmutated. In fact, applying our criteria for homology, we distinguished eight CDR3-homology groups within 37 of these 59 B-CLLs, each of which with at least three representatives (Table VI). Very recently, B-CLL homology groups, except for our group 8, have been identified by other investigators as well based on distinct homology criteria (references 49–52 and supplemental legend to Table VI, available at http://www.jem.org/cgi/content/full/jem.20050068/DC1).

RF-group			FR3	N	D	N	JH	FR4	CDR3 length	Hom.	Id.	Gap
V3-7	*M5*	:	CAR	GD	F-WS-GDY	ID	AFDI	WGQG	14aa	93%	86%	2
				||	† x| |||	||	||||					
	RF-M7	:	CAR	GD	YYDSGGDY	ID	AFDI	WGQG	16aa			
V3-7	*M6*	:	CAR	GD	YFDSSGSF	ID	AFDI	WGQG	16aa	88%	75%	0
				||	|†||x|x†	||	||||					
	RF-M7	:	CAR	GD	YYDSGGDY	ID	AFDI	WGQG	16aa			
V1-69	*M11*	:	CAR	E	GIAAA	VNP	FDY	WGQG	12aa	75%	67%	0
				|	|xxx|	†||	|||					
	RF-BOR	:	CAR	E	GRRMA	INP	FDY	WGQG	12aa			
WOL	*M41-C*	:	CAR	--GP	DSSNYYY	F	Y	WGQG	11aa	91%	64%	2
				|x	|†|†|||	†	|					
	RF-WOL	:	CAR	EYGF	DTSDYYY	Y	Y	WGQG	13aa			
V3-7	*ML27*	:	CAR	GD	YYDSSGHF	SD	AFDI	WGQG	16aa	88%	75%	0
				||	|||||x†	x|	|||x					
	RF-TT5	:	CAR	GD	YYDSSGNY	HD	AFDC	WGQG	16aa			
V1-69	*Iso-3*	:	CAR	E	GRGTEY	TNP	FDY	WGQG	13aa	92%	69%	0
				|	||x††|	†||	|||					
	RF-112/113	:	CAR	E	GRSSDY	SNP	FDY	WGQG	13aa			
WOL	*ML16*	:	CAR	EFSY	DRSGYYY	Y	Y	WGQG	13aa	77%	62%	0
				|†x†	|x|x|||	|	|					
	RF-WOL	:	CAR	EYGF	DTSDYYY	Y	Y	WGQG	13aa			

Figure 2. IgV_H-CDR3 amino acid sequences of selected cases of MALT lymphoma with homology to IgV_H-CDR3 of rheumatoid factors. MALT lymphomas *M5/M6*, *M11*, and *M41* share homology with V3-7-RF, V1-69-RF, and WOL-RF, respectively. In addition, RF CDR3 homology of three MALT lymphoma cases from literature (*ML27*, *Iso-3*, and *ML16*) with V3-7-RF, V1-69-RF, and WOL-RF, respectively, is depicted. The amino acid sequences are depicted by the single letter code. FR3 and FR4, framework region 3 and 4; N, amino acids encoded by the nontemplated nucleotides; D, gene segment; JH, gene segment; |, identical amino acids; +, similar amino acids; x, nonmatching amino acids; CDR3 length, length of the CDR3 region; Hom, percentage of homologous amino acids; Id, percentage of identical amino acids; Gap, length difference in amino acids of the compared IgV_H-CDR3 regions.

A significant fraction of B-CLLs derives from poly (auto) reactive B cells (26–30). In vitro RF reactivity has been proven for one representative of homology group 1 (*POR*) and one of group 2 (*AIG*) (30). Two B-CLLs, *CLL-011* (53) and *ID-74* (54) (*CLL-011* belonging to homology group 1), showed CDR3 homology to two different, IgV_H-unmutated, RFs termed *TB-3-D13* (55) and *RF-SJ2* (56), respectively (Table V). Poly-reactivity toward different auto-antigens, including IgG, has been demonstrated for homology group 4 member *SMI* (29). In addition, group 4 members share CDR3 homology with an anti-cardiolipine Ab (AF460965). It is noted that the B-CLLs that displayed in vitro RF reactivity (*POR, AIG,* and *SMI*) as well as the two B-CLLs (*CLL-011* and *ID-74*) that displayed CDR3 homology to two unmutated RFs, all belong to the *IgV*-unmutated B-CLL subset. This clearly contrasts with the MALT lymphomas with V1-69-RF, V3-7-RF or WOL-RF homology as these RFs as well as the MALT lymphoma Igs are encoded by heavily mutated IgV_H genes. Finally, none of the 308 B-CLLs showed CDR3 homology to V1-69-, V3-7-, or WOL-RFs.

RF activity of recombinant lymphoma-derived IgM antibodies

To prove that MALT lymphomas with RF-CDR3 homology possess IgG-binding activity, lymphoma-idiotype-derived Abs (LIDA) of IgM class were generated of *M5*, *M6*, *M11,* and *M41-A, C,* and *D*. Since, due to shortage of DNA, we could not resolve the IgVκ of *M41* we combined each of the IgV_H variants of the *M41-A, C,* and *D* subclones with the RF-canonical IgVκ chain of *M22*. Recombinant IgM LIDAs were also produced of 6 MALT lymphomas (*M8, M9, M14, M21, M22*, and *M23*), which are all devoid of RF-CDR3 homology. As additional controls, IgM LIDAs were generated of four follicular lymphomas (*FL1*, *FL6′94*, *FL8′92*, and *FL13*), one B-CLL, *B-CLL26*, and two anti-Rhesus(D) Ab producing B cell lines (*8D8* and *LOS3*; reference 57). The IgV_H and IgVκ sequences of these FLs and

Table VI. B-CLL IgV_H-CDR3 amino acid sequence homology groups

Group	Characteristic V_H–D–J_H	Mutation status	CDR3 length	No. of cases
			aa	
1	(VH1, VH5, VH7)/D6-19 (frame 3)/JH4	unmutated	11–12	9
2	(V1-69/V4-34)/D2-2 (frame 3)/JH6	unmutated	18–20	6
3	V4-34/D5-5 (frame 1)/JH6	mutated	18	3
4	V1-69/D3-16 (frame 3)/JH3	unmutated	19	3
5	(V1-2/V1-3)/D1-26 (frame 3)/JH6	unmutated	15	3
6	V4-39/D6-13 (frame 1)/JH5	unmutated	16-17	5
7	V1-69/D3-10 (frame 3)/JH6	unmutated	18	5
8	(V1-2/V1-46)/D3-22 (frame 2)/JH4	unmutated	17	3

The B-CLL cases of the eight IgV_H-CDR3 homology groups and the resemblance of homology groups 1–7 to earlier described B-CLL homology groups are in the supplemental legend of this table.

B-CLL have been previously reported by us (18, 58, 59). The lymphomas originally expressed IgM, with exception of *M14*, *M22* (both IgA^+), and *M23 FL6′94*, *FL8′92* (both IgG^+) (Tables I and II). RF-ELISA studies pointed out that the LIDA of *M5*, *M6*, and *M11* were indeed strongly reactive with human IgG (Fig. 3). The LIDA of *M9* and *M22*, both with a IgV_H-CDR3 region not completely fulfilling our homology criteria but both with the RF-canonical V1-69/JH4-A27(kv325) combination of IgV_H and IgVκ chains, also displayed strong RF activity (Tables II–IV and Fig. 3). Of note, also sera of patient *M22*, which contained high concentrations (22 mg/ml and 6.5 mg/ml) of lymphoma-related IgA paraprotein, displayed strong IgA-RF activity in ELISA (Fig. 3). This thus independently confirmed our finding with the *M22*-LIDA and underscored the validity of our approach of recombinant lymphoma Ab production in the eukaryotic expression system used. Moreover, the LIDA of *M21*, not even harboring an RF-canonical IgV_H rearrangement but with an A27(kv325)-encoded IgVκ chain, also possessed strong RF activity. In contrast, none of the LIDAs of MALT lymphomas *M8, M14, M23,* the four FLs, the B-CLL nor the anti-Rhesus(D) Abs, all, except *M23*, lacking canonical IgV_H RF rearrangements, bound to IgG (Fig. 3). Finally, LIDA *M41-A/M22* (the dominant subclone with 19 mutations) and *M41-C/M22* with 20 mutations bound

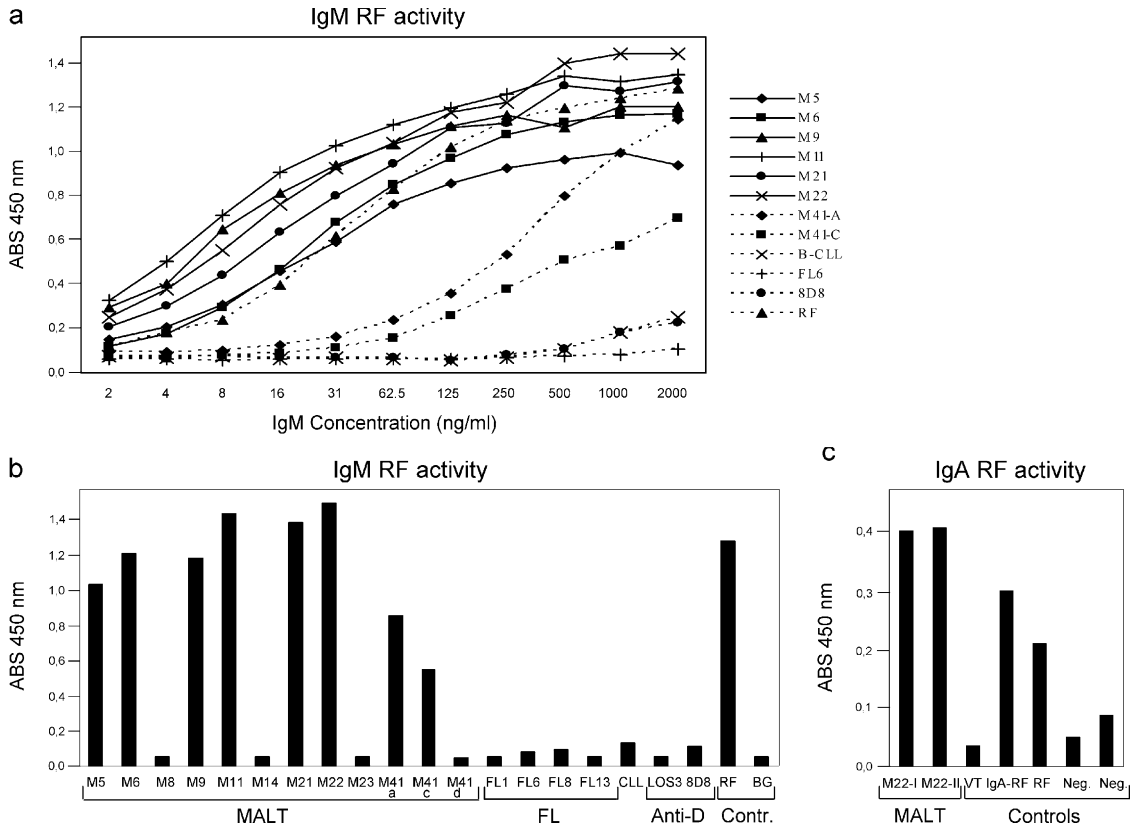

Figure 3. RF activity of lymphoma–idiotype–derived antibodies. (a) Titration of IgM LIDAs *M5, M6, M9, M11, M21, M22, M41-A, M41-B, FL6′94, B-CLL26, 8D8,* and RF control serum in the IgM-RF ELISA. The LIDAs *M8, M14, M23, M41-D, FL1, FL8′92, FL13,* and *LOS3* did not react with human IgG and showed a similar binding curve as that of *FL6′94.* (b) Binding activity of IgM LIDAs, RF control serum, and anti-Rh(D) control IgM Abs (*LOS3* and *8D8*) in the IgM-RF ELISA. All samples were tested at a stratified concentration of 500 ng/ml IgM. The ABS 450 nm is plotted without subtraction of the background (BG) ABS 450 nm. (c) IgA-RF ELISA of two serum samples of *M22* and as controls an IgA anti-Rh(D) Ab (*VT-7G3*), an IgA-RF-containing serum, the RF control serum that was also used in the IgM-RF ELISA, and two negative-control serum samples, respectively. All samples were tested at a stratified concentration of 20 μg/ml IgA. The net ABS 450 nm is plotted with subtraction of the background ABS 450 nm.

IgG in ELISA whereas the LIDA of *M41-D/M22* (a subclone with only two mutations) did not (Figs. 1–3).

We next tested the binding capacities of the RF^+ LIDAs of *M5, M6, M9,* and *M11* with recombinant IgG1 and IgG3 preparations. The LIDA of *M5, M6,* and *M9* reacted with IgG1 only. *M11* reacted with both IgG1 and IgG3, and may thus be a pan-IgG reacting RF (unpublished data). In chronic gastritis, RF-expressing B cells may theoretically be stimulated by IgG coated on *H. pylori* or due to existence of cross-reacting epitopes between *H. pylori* and IgG-Fc. Upon comparison, one *H. pylori* (strain 26695, GenBank/EMBL/DDBJ accession no. AE000511) peptide, of the gene product "virulence-associated protein homologue VacB" (GenBank/EMBL/DDBJ accession no. AAD08293), was found to share 68% homology with aa 336–354 of the IgG1 Fc at the CH2-CH3 junction. However, none of *M5, M6, M8, M9, M11,* or *M14* LIDA, with or without RF activity, reacted with a synthetic "336-354" peptide, nor did this peptide block the binding of the RF-LIDAs to IgG. In addition, no binding of any of these LIDAs to *H. pylori*–infected HM02 epithelial cells (strains 26695 and 1061) was observed. In addition, all LIDAs lacking RF-activity (*M8, M14, M23, FL1, FL6′94, FL8′92, FL13,* and *B-CLL26*) showed no antinuclear antibody (ANA) activity on Hep2 cells either (unpublished data).

To explore whether other BCR ligands are present within the tissue of MALT lymphomas or FLs, we also produced LIDAs of the IgG class of 3 (non-RF-CDR3-homologous) MALT lymphomas (*M8, M14,* and *M15*) and of 4 FLs (*FL1, FL3′93, FL6′94,* and *FL63*; reference 18). These LIDAs were FITC-labeled and tested immunohistochemically for reactivity on the corresponding lymphoma tissues. In none of these experiments however we detected reactivity with any tissue components (unpublished data).

DISCUSSION

We systematically analyzed the immunoglobulin repertoires of a comprehensive panel of mature B-NHLs. Unbiased comparison of IgV_H-CDR3–encoded aa sequences of individual B-NHLs with all IgV_H-CDR3 presently available in GenBank revealed distinct patterns of the various B-NHL entities. This provided interesting clues concerning their potential ligands, which was functionally confirmed for the group of MALT lymphomas.

FLs, DLBCLs, BLs, and myelomas all exhibited a low degree of overall IgV_H-CDR3 sequence homology (Table V). In none of these 147 B-NHLs, recurrent IgV_H-CDR3 motifs were found. The majority of the homologous lymphomas expressed CDR3 that resembled those present in normal B cells. A few, however, shared homology with B-CLL and MALT lymphomas. One DLBCL displayed homology with gastric- and salivary gland-MALT lymphomas as well as with V3-7-RFs (Table V). Thus, in general FL, DLBCL, BL, and myelomas, all carrying significantly hypermutated *IgV* genes, seem to recognize unique epitopes, suggesting that they arise randomly out of the pool of B cells selected for nonself-antigens, most likely during the germinal center reaction. This is in accordance with previous observations that the germline IgV_H gene usage of these B-NHLs is similar to that of normal peripheral B cells (18, 60). In contrast, B-CLL and MCL cases showed a high degree of overall CDR3 homology (31 and 44%, respectively) (Table V). Focusing on B-CLL that shared CDR3 homology with at least two other B-CLL (which held for 37 out of the 308 B-CLL analyzed), we distinguished eight CDR3-homology groups (Table VI). These homology groups in part overlap with B-CLL subgroups as reported by others (49–52). Inter-B-CLL homology was largely confined to the IgV_H-unmutated subset, which shows a strong bias toward V1-69 usage (53): 62 out of 165 (38%) IgV_H unmutated B-CLL expressed V1-69, most often (34/62, 55%) combined with JH6. In addition, the previously described poly-autoreactivity of a significant fraction of B-CLL was also clearly reflected in our study: Eighteen B-CLLs shared CDR3 homology with either of five B-CLL for which reactivity with auto-Ags such as IgG (RF), cardiolipin or myoglobulin has been reported (29, 30). Although the number of available MCL IgV_H-CDR3 sequences was limited, we observed overall homology for almost half of the cases with CDR3 of unmutated IgV_H genes of normal B cells. Still, MCLs are different from B-CLLs with respect to the IgV_H repertoire bias, i.e., with preferential usage of V3-21 and V4-34 IgV_H genes by MCLs (61, 62).

MALT lymphomas were found to express a highly distinctive IgV_H repertoire, confirming and extending earlier reports by the groups of Miklos et al. (38) and De Re et al. (63) on salivary gland MALT lymphomas and HCV-associated B cell lymphomas, respectively. Out of a total of 100 MALT lymphomas that we analyzed, 33 cases shared CDR3 aa homology with other, previously published, CDR3. Twenty-one of these 33 MALT lymphomas harbored, according to the criteria chosen, significant homology to RF-related CDR3 and, except for one case, could be classified into either of 3 canonical RF groups; V1-69-RFs, V3-7-RFs and WOL-RFs (Table V). In addition, 5 salivary gland MALT lymphomas were included, reported by Bahler et al. (43, 64) and Miklos et al. (38), which did not completely fulfill our stringent criteria for V1-69-RFs, but did express the typical V1-69/JH4 RF gene rearrangement. The RF-homology group solely involved gastric- and salivary gland-MALT lymphomas. The in vitro binding studies with the recombinant LIDA formally proved that MALT lymphomas with canonical RF IgV_H- and IgVκ-chain rearrangements and RF-CDR3 homology indeed posses strong RF activity (Fig. 3). Moreover, MALT lymphomas *M9*, *M21,* and *M22*, which did not match our criteria for RF homology but of which *M9* and *M22* expressed the canonical RF V1-69/JH4 rearrangement, also exhibited strong IgG-binding capacity in vitro (Fig. 3). Thus, the actual proportion of MALT lymphomas with specificity for human IgG is likely to be higher than calculated on basis of our arbitrary homology criteria.

The degree of RF-CDR3 homology found in the 21 MALT lymphomas is strikingly high, taking into account that it concerns heavily mutated IgV_H genes and that homology in-

cluded N-region encoded aa residues (Fig. 2). This suggests a distinct pathogenesis and indicates that these lymphomas originate from precursors strongly selected for auto-IgG specificity. The latter notion is well supported by the finding that the LIDA of the major subclone of *M41* (*M41-A*), with 19 somatic mutations, and *M41-C*, with 20 mutations, exhibited significant intrinsic IgG-binding activity of the expressed IgV_H chains, whereas this could not be measured of a presumed ancestral subclone, *M41-D*, with 2 mutations (Figs. 1 and 3).

MALT lymphomas typically evolve in a background of chronic inflammation due to infection or autoimmunity. Evidence exists that the tumor B cells in gastric MALT lymphoma are not *H. pylori*–specific but largely depend on CD40 stimulation by *H. pylori*–specific T helper cells (23). It has recently been reported, in a murine model, that RF-expressing B cells can be selectively activated in a T cell independent manner by IgG-chromatin complexes through the synergistic engagement of the BCR and toll-like receptor 9 (TLR9; reference 65). TLR9 is expressed in the endoplasmic compartment and serves as pathogen sensor that binds unmethylated CpG DNA motifs which are more common in bacterial than in mammalian DNA. In the human system, CpG-DNA was shown to trigger T cell independent proliferation of memory B cells, but not of naive B cells, which correlated with the levels of TLR9 expression of memory and naive B cells, respectively (66, 67). Stimulation of TLR9 may thus, parallel to the CD40/CD40L pathway, operate in lymphoproliferations of MALT. In gastric MALT, RF B cells may receive synergistic signals of the RF-BCR by IgG–*H. pylori* complexes and of TLR9 by *H. pylori* DNA. Also in inflamed salivary gland tissue in Sjögren's syndrome, as well as in other autoimmune diseases, RF B cells may receive these signals of the BCR and TLR9 by complexes of IgG and DNA released during normal or pathological cell death. This scenario clearly lends support from the fact that virtually all Sjögren's syndrome patients produce antinuclear antibodies (ANA), including anti-SS-A and SS-B antibodies.

The most frequent genetic alteration found in MALT lymphoma is the t(11;18)(q21;q21) encoding an API2-MALT1 fusion product that constitutively activates the NF-κB pathway (68). The t(11;18) is present in ~40 and ~25% of pulmonary- and gastric-MALT lymphomas respectively whereas it is virtually absent in MALT lymphomas of the salivary gland (~2%; references 69–71). We found that ~40% of the salivary gland- and ~20% of the gastric-MALT lymphomas express RF-like BCRs whereas we did not identify RF-like BCRs in any of the 19 pulmonary MALT lymphomas. In addition, none of the MALT lymphomas with a t(11;18) possessed RF-CDR3 homology (Table VII). Accordingly, the LIDA of the $t(11;18)^+$ lung lymphoma *M23* did not bind IgG in vitro (Fig. 3). This tentative inverse relation between RF-specificity and the t(11;18) suggests that MALT lymphomas containing t(11;18) do not depend for their expansion on BCR-, CD40-, or TLR9-mediated NF-κB activation (Table VII). The fact that $t(11;18)^+$ gastric lymphomas are resistant to *H. pylori* eradication therapy is in support of this hypothesis (14, 15). By contrast, the $t(11;18)^-$ gastric and salivary gland MALT lymphomas with RF BCR may need chronic stimulation by IgG in Ag-Ab complexes in gastric- and salivary gland-MALT lymphomas e.g. as IgG–*H. pylori* and IgG-ANA complexes, respectively. Finally, the different Ig repertoire of $t(11;18)^+$ MALT lymphomas, as compared with MALT lymphomas devoid of this translocation, indicates that this genetic alteration as such provides growth advantage and occurs before the selection process favoring RF-expressing clones.

Table VII. Relation between the presence of t(11;18) and RF-CDR3 homology and/or RF activity of MALT lymphoma immunoglobulins

	t(11;18) and RF homology/ activity of 24 MALT lymphomas			Frequencies of t(11;18) and RF homology among MALT lymphoma cohorts			
		t(11;18)[a]					
	n	+	−	*n*	t(11;18)[b]	*n*	RF-CDR3[c]
					(%)		*(%)*
Salivary gland	6	0 (0)	6 (4)	114	2 (2)	32	13 (41)
Gastric	10	5 (0)	5 (2)	209	50 (24)	45	8 (18)
Pulmonary	4	3 (0)	1 (0)	113	47 (42)	19	0 (0)
Other MALT	4	0 (0)	4 (1)	ND	NA	4	0 (0)

[a]Numbers in parentheses indicate quantity of cases with RF-CDR3 homology and/or in vitro RF activity.
[b]The data on t(11;18) and MALT lymphoma localization refer to combined data described previously (69–71).
[c]Data adapted from Table V.
NA, not applicable.

MATERIALS AND METHODS

Patient material and immunohistochemistry. Frozen or paraffin-embedded tissue of 23 low-grade and one large cell *(M22)* MALT lymphomas was obtained from the Westeinde Hospital, The Hague; the Free University Medical Center, Amsterdam; The Netherlands Cancer Institute, Amsterdam and the Academic Medical Center, Amsterdam, The Netherlands.

Tumor cell immunophenotypes were determined by immunohistochemical stainings on acetone-fixed cryostat sections and on formalin-fixed paraffin embedded sections using the highly sensitive Powervision+ detection system (ImmunoVision Technologies). Monoclonal antibodies used: IgM, κ- and λ-light chains (Becton Dickinson), IgG, IgA, CD20 (L26; DakoCytomation), CXCR3 (1C6; BD Biosciences), and α4β7 (Act-1).

M22 was a large cell lymphoma consisting of immunoblasts which had developed in a patient suffering from Sjögren's syndrome. The original diagnosis MALT lymphoma was not made in our hospital and unfortunately we were not able to recollect material from previous biopsies. This lymphoma most likely developed from a MALT-associated clone given the expression of IgA, CXCR3, the mucosa homing receptor α4β7 as well as the obvious plasmacytoid differentiation with the concurrent lymphoma-related paraproteinemia. These are characteristics highly compatible with extranodal marginal zone lymphomas but extraordinary for diffuse large B cell lymphomas.

This study was conducted in accordance with the ethical standards in our institutional medical ethical committee on human experimentation, as well as in agreement with the Helsinki Declaration of 1975, as revised in 1983.

DNA and RNA isolation; cDNA synthesis; and IgV_H, IgVκ, and t(11;18) RT-PCR. DNA was isolated from paraffin sections by over night proteinase K digestion. RNA was isolated from frozen sections using the

TRIzol reagent (Invitrogen) and cDNA was synthesized with $Pd(N)_6$ random primers. The *IgV_H* and *IgV*κ genes were amplified using IgV_H and IgVκ family-specific leader primers combined with the appropriate reverse primer being either JH, Cμ, Cγ, Cα, Jκ, or Cκ. To determine the clonally expressed *IgV_H* gene of the tumor B cells, the CDR3 region was also amplified, directly on cDNA and in nested PCRs on the IgV_H family-specific PCR products, using a forward primer specific for the framework region 3 (FR3) in combination with one of the different nested downstream primers specific for JH, Cμ, Cγ, Cα or Cδ. The PCR programs and primers sequences were described previously (18, 58). Translocation t(11;18) was determined using the primers and the PCR program as described by Liu et al. (15).

Cloning and sequencing. IgV RT-PCR products of MALT lymphomas were either directly sequenced or cloned into pTOPO-TA-vectors and transformed into TOP10 bacteria (Invitrogen), to generate molecular IgV clones. Sequencing on both strands was performed by an ABI sequencer (Applied Biosystems) using the big dye-terminator cycle-sequencing kit. To identify the *IgV* germline gene used and the somatic mutations therein, the consensus sequence of each MALT lymphoma was compared with published germline sequences, using the Vbase database (72) and DNAplot on internet (http://www.mrc-cpe.cam.ac.uk). The IgV sequences of the MALT lymphomas were deposited on GenBank/EMBL/DDBJ (accession nos. AY281324-AY281343, AY466502, AY466503, AY561708 and AY927657-AY927668). The degree of intraclonal variation (ICV) of *IgV_H* genes and *IgV*κ genes was calculated as the mean number of nucleotide differences of each molecular clone as compared with the consensus *IgV_H* or *IgV*κ sequences (18). ICV was considered significant if exceeding 0.4 mutations/clone.

Production of IgM antibodies derived of B-NHL. Recombinant IgMκ antibodies of the lymphomas (lymphoma-idiotype-derived Ab [LIDA]) of patients *M5, M6, M8, M9, M11, M14, M21, M22, M23, M41, FL1, FL6′94, FL8′92,* and *B-CLL26* were produced using the pIgH(μ) and pIgL(κ) expression vectors as described previously (59). In brief, the IgV_H and IgVκ sequences of each of these lymphomas, including one EBV B cell clone (*8D8*), which produces a human monoclonal antibody specific for the erythrocyte Rhesus(D) blood group antigen (57), were each cloned into the pIgH(μ) and pIgL(κ) vectors respectively. For production of recombinant antibody, 10 μg pIgH(μ) and 10 μg pIgL(κ) were linearized with PvuI and cotransfected into SP2/0 myeloma cells by electroporation. Subsequently, the transfected cells were selected in geneticin-containing medium. The IgV_H of *M41-A, C,* and *D* were expressed with the IgVκ of *M22*. An Ig-secreting heterohybridoma of an IgMλ expressing FL *FL13* (18), was produced by electrofusion with F3B6 (73) as described previously (74). *LOS3* and *VT-7G3* are an IgMκ and an IgAκ anti-Rhesus(D)-secreting EBV B cell clone, respectively (57). Supernatants were screened for IgMκ or IgMλ, using ELISAs as described previously (57). The pIgH(μ) and pIgL(κ) expression vectors were provided by J. van Es and T. Logtenberg (Utrecht Medical Center, Utrecht, The Netherlands).

LIDA reactivity in rheumatoid factor ELISA and on *H. pylori*–infected HM02 cells. LIDA reactivity with hIgG was determined using the IgM Rheumatoid Factor ELISA kit (Sanquin) according to the manufacturer's instructions. The plates were developed using TMB as substrate, as described previously (57). For IgA RF activity, a HRP-labeled rabbit anti-IgA Ab (DakoCytomation) was used. The "336-354" IgG1 Fc peptide was coated at 4 μg/ml, incubated with LIDA, followed by mAb anti-IgM-HRP (MH15/1-HRP; Sanquin), and developed as described previously (57). For blocking RF activity, LIDAs were tested in RF-ELISA in the presence of a 50–500 molar excess of the "336-354" IgG1 Fc peptide. To detect LIDA reactivity with *H. pylori*, ~80% confluent cultures of HM02 cells were incubated with $2*10^6$ CFU *H. pylori* (strains 1061 and 26695) and cultured for 1 wk. The cells were then fixed with methanol-aceton (1:1), immunocytochemically stained with LIDA, HRP labeled rabbit anti-IgM Ab (DakoCytomation) and developed with AEC as substrate.

Online supplemental material. Table S1 summarizes the IgV_H-CDR3 amino acid sequence homology of MALT lymphomas *M5, M6, M11,* and *M41* with other normal and malignant B cells as well as with RF-producing B cells. Table S2 gives an overview of the IgVκ-CDR3 amino acid sequences of the MALT lymphomas of Table III. The supplemental legend to Table V summarizes all lymphomas used in the IgV_H-CDR3 homology analysis. The supplemental legend to Table VI depicts the B-CLL cases belonging to the eight IgV_H-CDR3 homology groups and their resemblance to homology groups as described previously (49–52). Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20050068/DC1.

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