Can isotype switch modulate antigen-binding affinity and influence clonal selection?

Otto Pritsch[1,4], Christian Magnac[1], Gérard Dumas[1], Jean-Pierre Bouvet[3], Pedro Alzari[2] and Guillaume Dighiero[1]

[1] Unité d'Immuno-Hématologie et d'Immunopathologie, Institut Pasteur, Paris, France
[2] Unité de Biochimie Structurale, Institut Pasteur, Paris, France
[3] INSERM U430 Hôpital Broussais, Paris, France
[4] Departamento de Bioquímica, Facultad de Medicina, Montevideo, Uruguay

Four different monoclonal Ig (MIg) (IgA1κ, IgG1κ, IgG2κ and IgG4κ) displaying anti-tubulin activity were detected in the serum from a lymphoma patient. The complete sequence of three of these MIg showed identical V_H and V_L domains and the presence of mutations compatible with an antigen-driven process. Surprisingly, despite complete homology in their variable domains, IgA1κ, IgG1κ, or their Fab fragments bound to a common motif recognized in β tubulin, with significant differences in affinity (IgA1κ 1.52×10^{-8} M, and IgG1κ 2.09×10^{-7} M). To substantiate these results, the V_H and V_L domains from IgA1κ were cloned and introduced into expression vectors containing the constant κ exon and either the μ or the γ1 constant exon, and complete recombinant IgMκ and IgG1κ were obtained. Like the IgA1κ, the IgMκ construction bound to the tubulin epitope with consistent affinity (7.7×10^{-9} M), whereas the IgG1κ construction displayed a significantly lower affinity (3.28×10^{-7} M). These results provide definitive evidence that isotype can influence binding affinity to antigen and suggest that malignant transformation occurred at the germinal center once the mutational process was achieved and the switch process was still active.

Key words: Isotype switch / Antigen-driven selection / Anti-tubulin activity / Immunocytic lymphoma / Multiple monoclonal Ig

Received	15/3/00
Revised	10/7/00
Accepted	21/8/00

1 Introduction

Immunoglobulins (Ig) are heterodimeric proteins composed of two heavy (H) and two light (L) chains. Each H and L chain contains a variable domain, which defines the antigen-binding site of the antibody, and a constant domain, which is assumed to play effector functions without intervening in the antigen-binding specificity of the antibody. Multisequence comparisons of variable domains have shown that each variable domain contains three regions of extensive sequence variability, termed complementary determining regions (CDR), and four regions of relative sequence stability, termed framework regions (FR). The three L chain CDR and the three H chain CDR are juxtaposed to form the antigen-binding site of the antibody, as classically defined. In turn the FR create a scaffold that surrounds, supports, and influences the conformation and structure of the CDR [1–4].

[I 20755]

Abbreviations: MIg: Monoclonal Ig **CDR:** Complementary determining regions **FR:** Framework regions

There are five major classes of H constant domains in a mammalian genome (μ, δ, γ, ε and α). B cells are endowed with the ability to switch from one set of constant domains to another [1]. This class switch allows the B cell to change the effector function of the antibody that it produces, while maintaining the antigen specificity of the original antibody [5]. The sequence and structure of the antigen-binding site can be altered through a process of somatic hypermutation that is targeted at the V domain, leading to a change in the binding affinity and specificity of the antibody [2, 3, 6, 7]. Both processes, class switching and somatic hypermutation of the variable domains, occur in the germinal center, where B cells undergo antigen selection and affinity maturation of their antibodies. However, except antibody multimerization leading to increased functional affinity, it is usually assumed that the constant domains do not play a role in the maturation of antigen-antibody interactions.

Molecular studies of Ig gene rearrangements in B cell tumors provides better understanding of the malignant transformation process, by allowing definition of the site where malignant transformation has occurred and assessing the role played by antigenic stimulation. We

© WILEY-VCH Verlag GmbH, D-69451 Weinheim, 2000 0014-2980/00/1212-3387$17.50+.50/0

ALXN2012
IPR2023-00999

have previously described [8, 9] the presence of four different clonally related monoclonal Ig (MIg) with anti-tubulin activity (IgA1κ^{PER}, IgG1κ^{PER}, IgG2κ^{PER} and IgG4κ^{PER}) in a single individual suffering from an immunocytic lymphoma, and shown that two of these human monoclonal Ig (IgA1κ and IgG1κ), sharing identical variable H and L sequences but expressing different isotypes, bound tubulin with significantly different affinities (2.85×10^{-8} M for IgA1κ^{PER} and 1.02×10^{-6} M for IgG1κ^{PER}) [9]. Since this difference was also observed at the Fab level, we have hypothesized that the C_H1 domain could play a role in modulating the binding affinity of these antibodies. To provide definitive evidence of this phenomenon in the present work, we have: (1) cloned the V_H and V_L domains of the IgA1 and transfected them into expression vectors where the V_H domain was associated with the human γ1 or μ isotypes and the V_L domain with the human κ constant domain; (2) expressed complete recombinant IgMκ and Igκ antibodies by transfecting V_H and V_L constructions into the X-63 myeloma line, (3) mapped a C-terminal epitope recognized by these antibodies in β tubulin, and (4) measured affinity constants of the interaction between these molecules and the synthetic peptide containing the epitope. Our results provide definitive evidence that the C_H1 domain can play an important role in the antigen-antibody interaction, and suggest that malignant transformation of this clone probably occurred at the level of the germinal center at a moment when the somatic mutation process was achieved and the switch process was still active. They also provide original evidence for an unusual antigen-selection mechanism, since predominance of the α1 clone in this patient is probably explained by the C_H1 domain playing a role in structuring the antigen-binding site into a more kinetically competent form.

2 Results

2.1 Characteristics of the four MIg present in serum PER

The total quantity of IgA1 in serum of the patient (PER) was 40 mg/ml (30 mg of dimer and 10 mg of monomer), whereas total IgG was 13.8 mg/ml (roughly 5.7 mg IgG1^{PER}, 5.7 mg IgG2^{PER} and 2.4 mg IgG4^{PER}). Isoelectric points of pH 8.9, 7.9 and 6.5 were found for IgG1^{PER}, IgG2^{PER} and IgG4^{PER}, respectively, whereas for both IgA1^{PER} monomer and dimer this value was of 4.9. In addition, the previously described murine monoclonal IgG1κ anti-idiotypic antibody 3.2/9B11 [8], was found to bind a private idiotope located in the κ chains of the four MIg (Table 1). We have previously reported that IgA1κ^{PER} and IgG1κ^{PER} bound to tubulin with significantly different affinities (2.85×10^{-8} M for IgA1κ^{PER}, 1.02×10^{-6} M for IgG1κ) when studied by surface plasmon resonance measurements. Interestingly, despite this difference in the binding to tubulin, affinity studies of IgA1κ^{PER} and IgG1κ^{PER} against anti-idiotypic antibody 3.2/9B11 carried out by plasmon resonance did not show significant differences between these two antibodies. Dissociation constants were $K_D = 0.99\times10^{-9}$ M ($k_{ASS} = 2.60\times10^5$ $M^{-1}s^{-1}$ and $k_{DISS} = 2.57\times10^{-4}$ s^{-1}) for IgA1κ^{PER}, and $K_D = 1.09\times10^{-9}$ M ($k_{ASS} = 3.11\times10^5$ $M^{-1}s^{-1}$ and $k_{DISS} = 3.39\times10^{-4}$ s^{-1}) for IgG1κ^{PER}.

Amino acid and nucleotide sequences were determined for IgA1κ^{PER}, IgG1κ^{PER} and IgG2κ^{PER}, and were found to be identical at the variable regions (Fig. 1; Gene Bank accession nos: AF184762; AF184763; AF184764; AF184765; AF184766; AF184767). In addition, when compared to their germinal counterparts they displayed numerous somatic mutations, predominating in CDR regions and leading to an important change in the hydropathicity profile, compatible with an antigen-driven process (Fig. 2A, B).

Table 1. Characteristics of MIg from patient PER

MIg	Molecular mass (kDa)	Serum level mg/ml	Ig (H+L) (pI)	L chains (pI)	Binding to anti-id 9B11
IgG1κ^{PER}	150	5.7	8.9	8.5	+
IgG2κ^{PER}	150	5.7	7.9	8.5	+
IgG4κ^{PER}	150	2.4	6.5	8.5	+
IgA1κ^{PER}	160 330	10 30	4.9	8.5	+

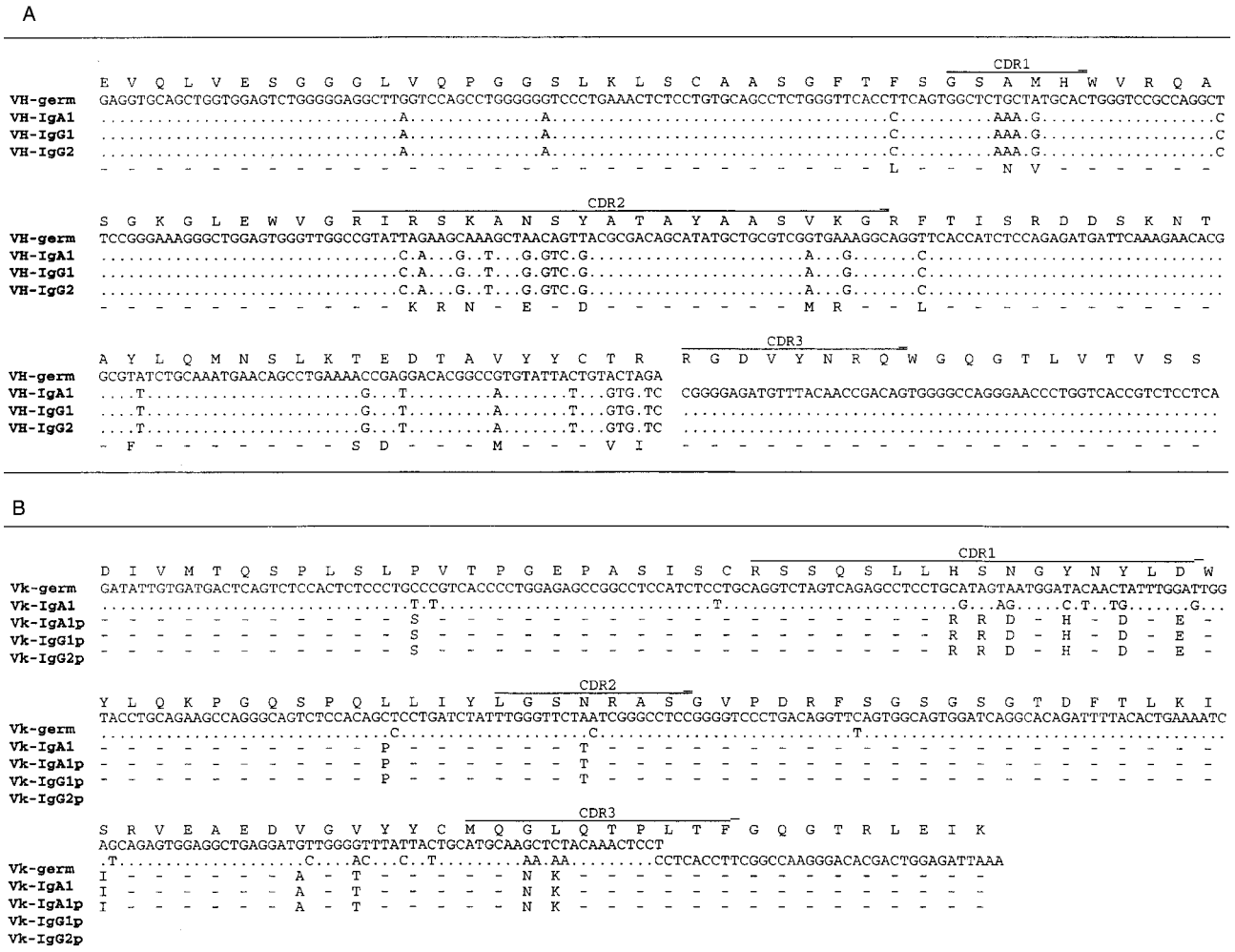

Fig. 1. Determination of V_H (A) and V_L (B) nucleotide and amino acid sequences from IgA1κ^{PER}, IgG1κ^{PER} and IgG2κ^{PER}. For V_H sequences nucleotide and amino acid sequences were obtained. Since nucleotide sequencing does not allow to assign the V_L corresponding to each isotype, V_L sequences were determined by amino acid sequence from purified IgA1κ^{PER}, IgG1κ^{PER} and IgG1κ^{PER}. Nucleotide sequence and amino acid identities are, respectively, indicated by dots and dashes. Replacements are depicted by the letters beneath the germ-line counterpart; CDR1, 2 and 3 are indicated.

2.2 Definition of the epitope recognized on β tubulin by PER antibodies

In a previous study, Houdayer et al. [8] demonstrated, after limited enzymatic proteolysis of purified tubulin, that IgA1κ^{PER} recognized C-terminal peptides of tubulin. For a better definition of the epitope recognized on tubulin by this antibody, we amplified and expressed a complete β tubulin chain, as well as a C-terminal fragment described in Sect. 4. Results of these experiments are depicted in Fig. 3A. It is shown that IgA1κ^{PER} recognizes the complete (lane 3) and C-terminal fragments of the β tubulin chain (lane 1).

To further define the epitope recognized by these antibodies, we sequenced the 450-bp C-terminal fragment from β tubulin (data not shown) recognized by both IgA1κ^{PER} and commercial anti-β tubulin antibody. On the basis of sequence data and using a peptide structure program, we designed eight tentative immunogenic peptides of 15 amino acids each corresponding to the C-terminal region of β tubulin. Fig. 3B depicts the results of these experiments. IgA1κ^{PER} (lane A) and commercial anti-β tubulin antibody (lane B) both bound to peptide 7. However, IgA1κ^{PER} was also found to bind to peptide 8. On the basis of these results, overlapping peptides were synthesized and tested for reactivity with these two monoclonal antibodies. As shown in Fig. 3C, IgA1κ^{PER}

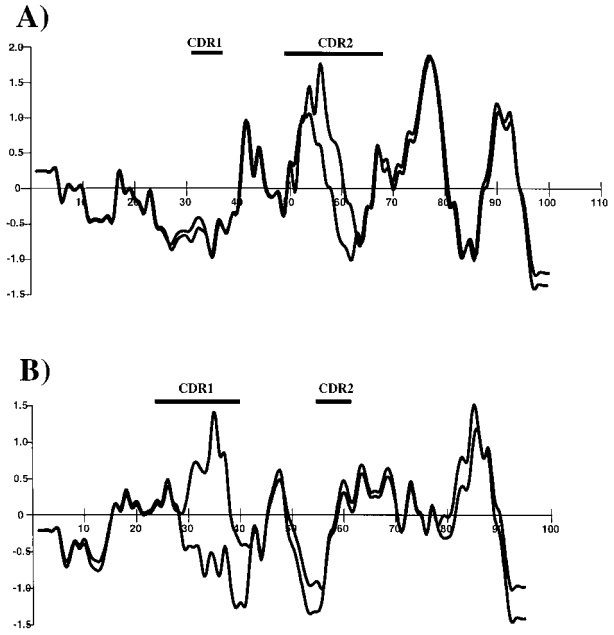

Fig. 2. Hopp and Woods hydropathicity plot of VH^{PER} and $V\kappa^{PER}$. Comparison of the V_H (A) and V_L (B) amino acid sequence of $IgA1\kappa^{PER}$ with their germinal counterparts [9] show the presence of high numbers of replacement mutations predominating in the V_H CDR2 region and V_L CDR1, which induce important changes in the Hopp and Woods hydropathicity plot.

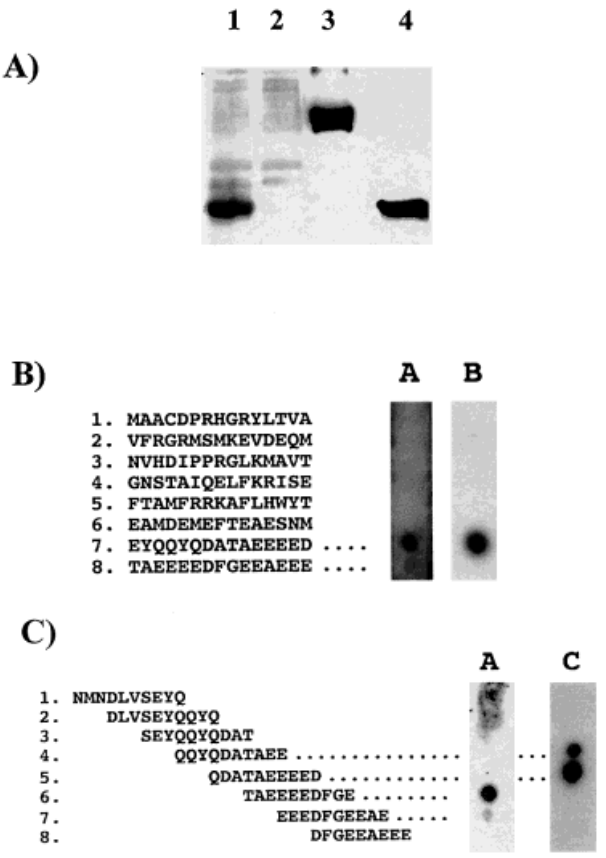

Fig. 3. Mapping of β-tubulin epitope. (A) Western blot experiments comparing the reactivities of anti-tubulin antibodies with the different expressed β tubulin fragments: binding of $IgA1\kappa^{PER}$ with the expressed C-terminal peptide derived from the 450-bp C-terminal fragment of β tubulin (lane 1) and the absence of binding with the control derived from the same bacteria not induced by IPTG (lane 2), or with brain tubulin (lane 3). Lane 4 represents the binding of commercial anti-β tubulin antibody to the 450-bp C-terminal fragment of β tubulin. (B) Both $IgA1\kappa^{PER}$ (lane A) and commercial anti-β-tubulin antibody (lane B) bind to peptide 7; $IgA1\kappa^{PER}$ also binds to peptide 8 (lane A). (C) $IgA1\kappa^{PER}$ recognizes a TAEEEEDFGE motif (lane A), whereas commercial anti-β tubulin binds to a QDATAEEEED motif (lane C).

recognized a TAEEEEDFGE peptide number 6 (lane A) in the C-terminal region of β tubulin. In contrast, commercial anti-β tubulin bound to peptides 4 and 5 (lane C) located immediately upstream of the β tubulin chain (Fig. 3C). The same epitope was also recognized by IgG1κ. Interestingly, a closely related acidic motif was present in the C-terminal region of α tubulin, which could explain recognition of both α and β tubulin by these antibodies.

2.3 Construction of two recombinant IgG1κ and IgMκ transfectomes containing the heavy and light variable domains of $IgA1\kappa^{PER}$

Our previous results showed that $IgA1\kappa^{PER}$ and $IgG1\kappa^{PER}$, sharing identical V_H and V_L domains but expressing different constant regions, bound tubulin with significant differences in affinity [9]. The observation that even Fab fragments from these antibodies reproduced differences in binding excluded a role for Fc interactions (including dimerization) and suggested that structural differences in the C_H1 domain accounted for the differences in the affinity constants.

To obtain definitive evidence, we cloned the V_H and V_L domains of $IgA1\kappa^{PER}$ and expressed them associated to human μ and γ1 heavy chain constant domains and human κ chain domains. The expression vectors for human μ (pRTM1μ-PER), γ1 (pcDNA1γ1-PER) and κ (pSVκ-PER) chains were constructed and co-transfected into the X63 cell line [10, 11]. Transfected clones were selected on the basis of their resistance to neomycin, and supernatants were tested by ELISA and Western blot (data not shown) for secretion of complete IgMκ and IgG1κ. Tranfectomas secreting complete human IgMκ and IgG1κ were expanded; culture supernatants were

Table 2. Equilibrium and kinetic constants for serum (S) and recombinant (R) MIgPER binding to synthetic peptide

	k_{DISS} (s^{-1})	k_{ASS} ($M^{-1}s^{-1}$)	K_D (M)
IgA1-PER (S)	3.00×10–4	1.98×104	1.52×10–8
IgG1-PER (S)	6.32×10–4	3.02×103	2.09×10–7
IgG1-PER (R)	8.99×10–4	2.74×103	3.28×10–7
IgMκ^{PER} (R)	4.23×10–4	5.50×10^4	7.70×10–9

concentrated and purified by affinity chromatography. As analyzed by gel filtration HPLC, secreted IgG1κ corresponded to monomeric and IgMκ corresponded to pentameric molecules (data not shown).

2.4 Affinity and kinetic constants determination by surface plasmon resonance

To investigate the role of the isotype in the binding to tubulin, we tested the binding affinity against the peptide motif of serum monomeric IgA1κ^{PER}, serum IgG1κ^{PER} and recombinant IgMκ^{PER}, and IgG1κ^{PER} against the TAEEEEDFGE peptide motif by surface plasmon resonance. Affinity constants were 1.52×10^{-8} M for serum IgA1κ^{PER}, 2.09×10^{-7} M for serum IgG1κ^{PER}, whereas it was, respectively of 7.7×10^{-9} M for recombinant IgMκ^{PER} and of 3.28×10^{-7} M for recombinant IgG1κ^{PER} (Table 2). Although the BIAcore system takes into account the valency of the molecule to calculate affinity constants, the possibility exists that the density of the peptide in the surface is so high that it allows multipoint binding. We examined this last eventuality by studying affinity constants on Sensor Chips with decreasing concentrations of the peptide. Results showed a decrease in bound IgM to the Sensor chip consistent with a decrease in peptide density, without revealing differences in affinity constants (data not shown).

3 Discussion

In this work we have cloned the Vκ domain and the V_H domain of the PER IgA1κ anti-tubulin antibody and transfected them into expression vectors allowing the expression of the Vκ and V_H domains from IgA1κ^{PER} associated to γ1 and μ C_H regions. Next, we have obtained the expression of the complete antibody by transfecting H and L chain constructions into the X-63 myeloma line. A C-terminal epitope recognized on tubulin by IgA1κ^{PER} could be defined for β tubulin. A motif close to this epitope also exists in α tubulin. Finally, we demonstrated that the recombinant antibodies resulting from the association of the V_H from IgA1κ^{PER} with γ1 and μ C_H domains bind this epitope with significantly different affinities.

These results provide additional evidence indicating that changes of the isotype can significantly influence the binding affinity interactions of the antigen-antibody complex. Our previous work, indicating that Fab fragments from these antibodies reproduce differences in binding, suggests that structural differences in the C_H1 domain underlie the differences in the affinity constants, and thus the differences in antigen binding.

It has long been assumed that the constant domains do not play a role in the maturation of antigen-antibody interactions. Indeed, Fab are pseudosymetric dimers where V_H contacts with C_H1, whereas no contact is observed between V_L and C_L [12]. Although crystallographic studies have shown that amino acids ensuring the link between Fv and C_H1 and C_L are flexible and allow spatial arrangements facilitating binding of epitopes by Fab, there is controversy concerning the possibility that elbow bending could facilitate signal transduction from the antigen-binding site to the Fc domain [13–16]. However, recent work has demonstrated that changes in the C_H domains can affect the functional affinity and specificity of antibody, through a complex mechanism involving antibody flexibility and cooperative interactions in the case of antibodies bound to multivalent antigen [17, 18]. Schneider et al. [19] showed that segmental flexibility is controlled by the C_H1 domain and hinge regions of antibodies, and Horgan et al. [20] reported that removal of the hinge region of an IgG4 resulted in a gain of relative affinity that approximated to that observed with the same antibody expressing an IgG1 isotype. Concerning the functional activity of antibodies, Cavacini et al. [21] found that antibody isotype can strongly influence virion neutralization of HIV-1 virus. One difficulty in substantiating this using standard crystallography may arise from the fact that the role of molecular mobile components in the interaction with another molecule may not be fully captured by this method [22].

The observed differences in antigen-binding affinities for antibodies with identical V_H/V_L domains may arise in part from multivalent antigen binding (isotype-dependent avidity), since IgM and IgA have a larger number of antigen-binding sites than the IgG subclasses. However, multivalency cannot explain our observations, since we have carried out our BIAcore studies with monomeric IgA1κ^{PER} isolated from the 0.030 M fraction and also IgA1κ^{PER} Fab fragments. Both monomeric IgA1κ^{PER} and its Fab fragments displayed comparable binding affinities for tubulin [9] and significant affinity differences when compared to IgG1κ^{PER} ([9] and present work). From

a structural point of view, two possible hypothesis spring to mind: (1) conformational signaling through the elbow angle, and (2) distinct antigen-binding sites in each isotype. The second hypothesis appears less plausible, since it implies that the high-affinity binding sites in the IgA and IgM molecules are not defined by the CDR of the variable domains from the light and heavy chains (otherwise, IgG should also bind the antigen with high affinity). Instead, we favor a mechanism by which specific differences in the C_H1 domains could impose structural and/or kinetic constraints on the antigen-binding site through the extended contacts at the V_H/C_H1 interface. Some structural evidence is compatible with this mechanism. In their comparisons of murine C_H1 domains from five different isotypes, Sheriff et al. [23] noted that the IgA C_H1 domain displays significant conformational changes and specific insertions/deletions with respect to IgG subclasses. We have constructed a model of the V_H-C_H1 interface of IgA1 and IgG1, using crystallographic data derived from homologous proteins. It is found that one of the C_H1 loops directly involved in V_H-C_H1 contacts displays a significantly different conformation for each isotype (Fig. 4). Such a different pattern of contacts could affect the relative orientation of the variable domains, which in turn might modulate antigen-antibody affinity.

Although, there is a consistent body of evidences on antigen-antibody interactions, demonstrating that the high-affinity antigen-binding sites are defined by the CDR of the variable domains, the results presented in this work provide evidence favoring the hypothesis that specific C_H1 differences can allosterically affect the antigen-combining site of an antibody. Whatever the frequency with which this phenomenon may occur, these results favor the view that affinity maturation of this particular tumoral clone could be achieved through class switching in the absence of further somatic mutations, probably because the C_H1 domain plays a role in structuring the antigen-binding site into a more kinetically competent form.

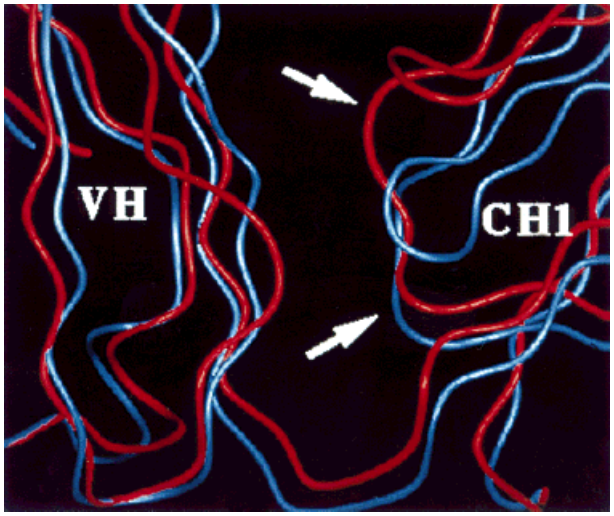

Fig. 4. Model of V_H-C_H1 interface. Schematic model of the V_H-C_H1 interface. The backbone models are shown for IgG1 (in blue) and IgA1 (in red). Note the different conformation of a C_H1 loop (indicated with arrows) that is directly involved in V_H-C_H1 contacts.

The mutation profile observed for the PER clone is compatible with a selective process driven by the antigen. Although in this work we have studied the pentameric soluble form of recombinant IgM and membrane IgM is monomeric, it can be hypothesized that the μ isotype allows good binding to the antigen. At this stage this clone may have been positively selected during the switch process.

Since all the four different clones isolated from PER (γ1, γ2, γ4 and α1) shared identical V_H and V_L sequences, it can be assumed that malignant transformation occurred once the mutational process was achieved, as previously reported in chronic lymphocytic leukemia B cells [24]. It is plausible to assume that the malignant transformation probably took place at the moment when the switch process was still active and probably rescued the three IgG clones, which, owing to the low affinity of their binding to tubulin, should in normal conditions be negatively selected. In contrast, the α1 clone predominated probably because the C_H1 domain of IgA allows the B cell receptor to establish higher affinity interactions with the antigen.

4 Materials and methods

4.1 Purification of MIg

Patient PER, affected by an immunocytic lymphoma expressed four different clonally related MIg in his serum [8]. MIg^{PER} were purified from serum by ammonium sulfate precipitation, followed by anionic-exchange chromatography on a DEAE-Sepharose column. Two different IgG of the γ1 and γ2 subclasses were eluted from the DEAE-Sepharose at the same molarity (0.005 M), and were further isolated by cationic-exchange chromatography on a CM-Sepharose column. $IgG4\kappa^{PER}$, monomeric and dimeric $IgA1\kappa^{PER}$ were eluted in the 0.015 M, 0.03 M and 0.05 M fractions, respectively. Purity, homogeneity and M_r of the $IgG1\kappa^{PER}$ and $IgA1\kappa^{PER}$ fractions were then assessed by ELISA and by HPLC, as described [8]. The isoelectric charge (pI) of the different MIg was measured by IEF.

4.2 V_H and V_L nucleotide sequence of IgA1κ^{PER}, IgG1κ^{PER} and IgG2κ^{PER}

4.2.1 cDNA preparation

RNA was isolated from 10^6 PBMC by the guanidinium thiocyanate-phenol-chloroform method [9]. cDNA synthesis was performed in a 20-μl reaction volume at 37°C for 60 min using an oligo(dT) primer 15-mer (Boehringer, Mannheim, Germany), 200 U Moloney murine leukemia virus reverse transcriptase (Gibco, BRL, Bethesda, MD), 40 U RNase inhibitor (Boehringer) and 0.5 mM of each deoxyribonucleotide.

4.2.2 V_H and V_L nucleotide sequence determination

PCR amplification was carried out from 1 μg of single-stranded cDNA, using Taq DNA polymerase (Cetus, Perkin Elmer, Emeryville, CA). According to the amino acid sequence the 5'primer used for V_H gene amplification was designed to anneal to the leader region of V_H3 family (5' L_H3 foward: GTA TCG ATG GAG TTT GGG CTG AGC TGG), and antisense oligonucleotides specific to C_H1 domain of Cα region, hinge of γ1 and γ2 regions were used, respectively, as 3' primers (3' Cα-reverse: ATC TGG CTG GGT GCT GCA GAG GCT; 3' hinge γ1: GGC ACG GTG GGC ATG TGT GAG and 3' hinge γ2: TGG GCA CGG TGG GCA CTC GAC). The 5' primer used for $V_κ$II gene amplification was designed to anneal specifically to the framework 1 (FR1) region of this κL chain family (5' $V_κ$II-foward: CTA GAG TCG ACG ATA TTG TGA TGA CTC AGT CTC C), and in 3', the consensus $C_κ$ primer (3' $C_κ$-reverse: CAG ATG GCG GGA AGA TG) was used. In all cases, 30 cycles of amplification were carried out. V_H and V_L cloning and sequencing were carried out as previously reported [9].

4.3 Amino acid sequence determination

Since nucleotide sequencing does not allow the assignment of the VL corresponding to each isotype, V_L and V_H sequences were determined by amino acid sequence followed by mass spectroscopy analysis from purified IgA1κ^{PER}, IgG1κ^{PER} and IgG2κ^{PER}, as reported previously [9].

4.4 Construction of two recombinant IgG1κ and IgMκ expressing lines displaying IgA1κ^{PER} V_H and $V_κ$ domains

4.4.1 PBMC and genomic DNA isolation

Genomic DNA was isolated from patients' IgAκ expressing lymphocytes [9].

4.4.2 Construction of μ chain expressing vector (pRTM1μ-PER)

Genomic DNA was amplified using a 5' primer annealing to the leader region (5´L_H3-*Cla*I: ATG CAT CGA TAA TCA CCA TGG AGT TTG GGC TGA GCT GG) and 3´consensus primer annealing to J_H region (J_H-*Sal*I: TGT GTC GAC TCA CCT GAG GAG ACG GTG ACC AGG G) primers. The amplified fragment was cloned in a pKS plasmid and sequenced to discard Taq infidelities and to confirm identity with original PER sequence. Next, it was cloned into a plasmid containing μ constant exons (pRTM1) kindly provided by T. Kipps [10, 25].

4.4.3 Construction of γ1 chain expressing vector (pcDNA1γ1-PER)

An L_H-J_H fragment was obtained by PCR amplification from pRTM1μ-PER plasmid by using 5' leader specific L_H3 *Hind*III (TGC AAG CTT ATC ACC ATG GAG TTT GGG CTG) and 3' J_H-*Xho*I (GAG CTC GAG ACT CAC CTG AGG AGA CGG TGA CC). Following a cloning step as above mentioned, nucleotide sequence was confirmed and the restriction fragment was ligated into the pcDNA1γ1 vector kindly provided by L. Silberstein [26].

4.4.4 Construction of κ chain expressing vector (pSVκ-PER)

An Lκ-Jκ PER fragment was obtained by genomic DNA amplification with 5' leader specific LκII-*Cla*I (CAT ATC GAT TTC TCA CAA TGA GGC TCC CTG CTC AGC) and 3' Jκ5-*Not*I (AGA GCG GCC GCA AAT GCT TAC GTT TAA TCT CCA GTC GTG TCC C) primers. A fragment containing a confirmed Vκ sequence was ligated into pSVκ vector [10].

4.4.5 Construction of IgG1κ^{PER} and IgMκ^{PER} secreting transfectomas

Both H and L chain expression vectors were transfected into the non-secreting murine myeloma P3X63Ag8.653 cell line by electroporation. Briefly, 5×10^6 X-63 cells in RPMI medium without FCS were co-electroporated with 10 μg of linearized DNA from pRTM1μ-PER or pcDNA1γ1-PER and pSVκ-PER, using a 250 V/1,500 μF pulse in an EasyJect+ device (Eurogentec, Belgium). Transfected cells were cultured in RPMI medium containing 20% FCS, selected with neomycin (G418, Gibco, France) at 1 mg/ml final concentration, and then, cloned by limit dilution. Expression of complete engineered IgMκ^{PER} and IgG1κ^{PER} molecules was analyzed by Western blot and ELISA, as previously reported [10]. Purification of recombinant IgM and IgG molecules was proceeded by affinity chromatography on an activated anti-human κ CNBr immunoadsorbent.

4.5 Cloning and expression of β tubulin fragments

The complete β-tubulin chain was amplified from murine cDNA, following sequence data from Villasante et al. [26] by: a 5' *Hind*III (GAC AAG CTT ATG AGG GAA ATC GTG CAC ATC CAG GCC GG) and a *Not*1 (CTT GCG GCC GCG GCC TCC TCT TCT GCC TCC TCT CCG AAA TCC) in 3'. In addition the 450-bp C-terminal fragment was amplified using: a 5' *Hind*III (GAC AAG CTT GAT GCC AAG AAC ATG ATG GCC G) and a *Not*1 (CTT GCG GCC GCG GCC TCC TCT TCT GCC TCC TCT CCG AAA TCC) in 3'. Amplified DNA fragments were cloned into the pET-22b(+) (Novagen, R&D System, Europe Ltd., Abington, GB) vector and transformation was carried out on NovaBlue (DE3) competent cells (Novagen, R&D System). Transformed clones were selected and expanded. Protein expression was induced by IPTG and the produced peptides were tested by Western blot for reactivity with IgA1κ^{PER} and with a commercial anti-β tubulin monoclonal antibody (Sigma-Aldrich chimie, St Quentin Fallavier, France). Blots were developed using the enhanced chemiluminescence detection system (ECL-Amersham International plc, GB) [27].

4.6 Peptides

Solid-phase synthesis of peptides was performed using SPOTs technology (Genosys Biotechnologies, Cambridgeshire, GB) on derived cellulose membranes and subsequently assayed for immunological reactivity [28]. Sequences of peptides are depicted in Fig. 4.

4.7 Affinity and kinetic constants determination by surface plasmon resonance

Equilibrium and kinetic constants for the interactions were determined by the BIAcore™ system (Pharmacia Biosensor AB Uppsala, Sweden). Immobilization of proteins via primary amines to the Sensor Chip CM5 was performed using an amine coupling kit. Activation of the carboxymethylated dextran matrix was obtained by injecting a mixture of N-ethyl-N'-(dimethyl-aminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS). A biotinylated-peptide: Biot-Q-19-E (biotinyl QQYQDATAEEEEDFGEEAE) was synthesized (Neosystem Laboratoire, Strasbourg, France) for affinity measurements. Immobilization of biotinylated peptide was achieved using a Sensor Chip SA containing streptavidin covalently coupled to a carboxymethylated dextran surface. Kinetic rate constants (k_{ASS} and k_{DISS}) as well as apparent equilibrium affinity constants ($K_D = k_{DISS}/k_{ASS}$) were determined using Bialogue™ Kinetics Evaluation software [29].

References

1 **Kabat, E. A., Wu, T. T., Reid-Miller, M., Perry, H. M. and Gottesman, K. S.,** Sequences of Proteins of Immunological Interest. *US Governement Printing Office, Bethesda* 1991.

2 **Tonegawa, S.,** Somatic generation of antibody diversity. *Nature* 1983. **302:** 575–581.

3 **Yancopoulos, G. D. and Alt, F. W.,** Regulation of the assembly and expression of variable-region genes. *Annu. Rev. Immunol.* 1986. **4:** 339–368.

4 **Kirkham, P. M. and Schroeder, H. W., Jr.,** Antibody structure and the evolution of immunoglobulin V gene segments. *Semin. Immunol.* 1994. **6:** 347–360.

5 **Cebra, J. J., Komisar, J. L. and Schweitzer, P. A.,** CH isotype 'switching' during normal B-lymphocyte development. *Annu. Rev. Immunol.* 1984. **2:** 493–548.

6 **Griffiths, G. M., Berek, C., Kaartinen, M. and Milstein, C.,** Somatic mutation and the maturation of immune response to 2-phenyl oxazolone. *Nature* 1984. **312:** 271–275.

7 **Weigert, M. and Riblet, R.,** Genetic control of antibody variable regions. *Cold Spring Harb. Symp. Quant. Biol.* 1977. **41:** 837–846.

8 **Houdayer, M., Bouvet, J. P., Wolff, A., Magnac, C., Guillemot, J. C., Borche, L. and Dighiero, G.,** Simultaneous presence, in one serum, of four monoclonal antibodies that might correspond to different steps in a clonal evolution from polyreactive to monoreactive antibodies. *J. Immunol.* 1993. **150:** 311–319.

9 **Pritsch, O., Hudry-Clergeon, G., Buckle, M., Petillot, Y., Bouvet, J. P., Gagnon, J. and Dighiero, G.,** Can immunoglobulin C(H)1 constant region domain modulate antigen binding affinity of antibodies? *J. Clin. Invest.* 1996. **98:** 2235–2243.

10 **Dumas, G., Pritsch, O., Pereira, A., Gallart, T. and Dighiero, G.,** A murine model of human cold agglutinin disease. *Br. J. Haematol.* 1997. **98:** 589–596.

11 **Oppezzo, P., Osinaga, E., Tello, D., Bay, S., Cantacuzene, D., Irigoin, F., Ferreira, A., Roseto, A., Cayota, A., Alzari, P. and Pritsch, O.** Hybridoma 2000, **in press**.

12 **Carayannapoulos, L. and Capra, J. D.,** In **Paul, W. E.** (Ed.), *Fundamental Immunology,* 3rd edn. Raven Press, New York 1993, p. 283.

13 **Strong, R. K., Petsko, G. A., Sharon, J. and Margolies, M. N.,** Three-dimensional structure of murine anti-p-azophenylarsonate Fab 36- 71. II. Structural basis of hapten binding and idiotypy. *Biochemistry* 1991. **30:** 3749–3757.

14 **Davies, D. R., Sheriff, S. and Padlan, E.,** Comparative study of two Fab-lysozyme crystal structures. *Cold Spring Harb. Symp. Quant. Biol.* 1989. **54:** 233–238.

15 **Lesk, A. M. and Chothia, C.,** Elbow motion in the immunoglobulins involves a molecular ball-and- socket joint. *Nature* 1988. **335:** 188–190.

16 **Wilson, I. A. and Stanfield, R. L.,** Antibody-antigen interactions: new structures and new conformational changes. *Curr. Opin. Struct. Biol.* 1994. **4:** 857–867.

17 **Cooper, L. J., Robertson, D., Granzow, R. and Greenspan, N. S.,** Variable domain-identical antibodies exhibit IgG subclass-related differences in affinity and kinetic constants as determined by surface plasmon resonance. *Mol. Immunol.* 1994. **31:** 577–584.

18 **Cooper, L. J., Shikhman, A. R., Glass, D. D., Kangisser, D., Cunningham, M. W. and Greenspan, N. S.,** Role of heavy chain

constant domains in antibody-antigen interaction. Apparent specificity differences among streptococcal IgG antibodies expressing identical variable domains. *J. Immunol.* 1993. **150:** 2231–2242.

19 **Schneider, W. P., Wensel, T. G., Stryer, L. and Oi, V. T.,** Genetically engineered immunoglobulins reveal structural features controlling segmental flexibility. *Proc. Natl. Acad. Sci. USA* 1988. **85:** 2509–2513.

20 **Horgan, C., Brown, K. and Pincus, S. H.,** Studies on antigen binding by intact and hinge-deleted chimeric antibodies. *J. Immunol.* 1993. **150:** 5400–5407.

21 **Cavacini, L. A., Emes, C. L., Power, J., Duval, M. and Posner, M. R.,** Effect of antibody valency on interaction with cell-surface expressed HIV-1 and viral neutralization. *J. Immunol.* 1994. **152:** 2538–2545.

22 **Van Regenmortel, M. H.,** Structural and functional approaches to the study of protein antigenicity. *Immunol. Today* 1989. **10:** 266–272.

23 **Sheriff, S., Jeffrey, P. D. and Bajorath, J.,** Comparison of CH1 domains in different classes of murine antibodies. *J. Mol. Biol.* 1996. **263:** 385–389.

24 **Fais, F., Sellars, B., Ghiotto, F., Yan, X. J., Dono, M., Allen, S. L., Budman, D., Dittmar, K., Kolitz, J., Lichtman, S. M., Schulman, P., Schuster, M., Vinciguerra, V. P., Rai, K., Stevenson, F. K., Gregersen, P. K., Ferrarini, M. and Chiorazzi, N.,** Examples of in vivo isotype class switching in IgM^+ chronic lymphocytic leukemia B cells. *J. Clin. Invest.* 1996. **98:** 1659–1666.

25 **Martin, T., Duffy, S. F., Carson, D. A. and Kipps, T. J.,** Evidence for somatic selection of natural autoantibodies. *J. Exp. Med.* 1992. **175:** 983–991.

26 **Villasante, A., Wang, D., Dobner, P., Dolph, P., Lewis, S. A. and Cowan, N. J.,** Six mouse alpha-tubulin mRNAs encode five distinct isotypes: testis-specific expression of two sister genes. *Mol. Cell. Biol.* 1986. **6:** 2409–2419.

27 **Cayota, A., Vuillier, F., Siciliano, J. and Dighiero, G.,** Defective protein tyrosine phosphorylation and altered levels of p59fyn and p56lck in CD4 T cells from HIV-1 infected patients. *Int. Immunol.* 1994. **6:** 611–621.

28 **Frank, R. and Doring, R.,** Simultaneous multiple peptide synthesis under continuous flow conditions on cellulose paper discs as segmental solid synthesis. *Tetrahedron* 1988. **44:** 6031.

29 **Malmqvist, M.,** Biospecific interaction analysis using biosensor technology. *Nature* 1993. **361:** 186–187.

Correspondence: Guillaume Dighiero, Unité d'Immuno-Hématologie et d'Immmunopathologie, Institut Pasteur, 28, rue du Dr. Roux, F-75724 Paris Cedex 15, France
Fax: +33-1-45-688951
e-mail: dighiero@pasteur.fr