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V_H gene-family representation in peripheral activated B cells from systemic lupus erythematosus (SLE) patients

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SUMMARY

A semiquantitative polymerase chain reaction (PCR) assay described in this study has been used to analyse the V_H1, V_H3 and V_H4 repertoire expressed by total IgM^+ and IgG^+ B cells from normal individuals and lupus patients. This approach consists of a combination of B cell selection, utilization of the anchored PCR technique to avoid technical bias in the amplification of different V_H gene family cDNA templates, and screening of the amplified IgM or IgG cDNA rearrangements by family-specific oligonucleotide probes. In four lupus patients, V_H family representation in IgM^+ and IgG^+ *in vivo* activated B cells, selected by anti-CD71 antibody, and in total $CD19^+$ B cells were compared. In all patients, V_H4 gene family segments were preferentially under-represented in IgM^+ activated B cells. In IgG^+ B cells the results suggest that V_H4 expression is variable, depending on the phase of the disease. Polyclonal B cell activation, which is usually considered as being the first event in autoantibody production in SLE, cannot explain our results. The data evoke the possible involvement of a V_H4-specific B cell superantigen in the onset or development of SLE. This hypothesis is also supported by the sequence conservation of the fourth β loop—a putative superantigen binding site—of functional V_H4 gene segments which are preferentially used by anti-dsDNA lupus antibodies of established clones and hybridomas.

Keywords lupus anti-DNA V gene family autoimmunity superantigen

INTRODUCTION

SLE, a prototypic systemic autoimmune disease, is characterized by B cell activation [1,2], hyperglobulinaemia and the presence of serum autoantibodies. These autoantibodies, which are present at strikingly high titres, bind to a limited number of self-antigens, including the constituents of chromatin [3]. Serum anti-dsDNA antibodies are generally regarded as markers of SLE. They are thought to be responsible for many clinical manifestations of SLE, including glomerulonephritis and arthritis. These autoantibodies differ from natural antisingle-stranded DNA autoantibodies found in healthy individuals, as they bind to DNA with high affinity, are often cationic in charge and do not cross-react with unrelated antigens [4].

The human V_H locus contains 51 functional V_H segments which can be classified into seven families, V_H1 to V_H7. In the $CD19^+$ IgM^+ B cell population of healthy adults, the V_H3 family is most frequently expressed (about 50%), while V_H4 and V H1 gene families represent about 20–30% each [5]. The molecular mechanism that drives autoantibody production in SLE remains enigmatic. Some studies suggest that polyclonal B cell activation is the early event which plays a key role in breaking self tolerance, followed by selection of autoreactive clonotypes [6]. We and others have previously shown that an antigen-driven B cell mechanism is also involved in the production of lupus pathogenic autoantibodies [7–10].

Using a new method described in this study we performed an analysis of V_H family representation including V_H1, V_H3 and V_H4 in four lupus patients. We found an underrepresentation of V_H4 gene family in IgM-activated B cells in all patients. V_H gene usage by IgG-activated B cells was found to depend on the stage of the disease. These data tend to minimize the role of B cell polyclonal activation in SLE. The possible involvement of a superantigen specific for B cells bearing V_H4 immunoglobulin is discussed.

MATERIALS AND METHODS

Blood samples and patients

Peripheral blood (10–40 ml) was obtained from five adult healthy donors (N-1 to N-5) (Dr G. Dighiero, Centre de Transfusion Sanguine, Institut Pasteur, Paris, France) and four SLE patients (Dr G. Fournié, Centre Hospitalier de Toulouse, France). All lupus patients fulfilled the 1982 revised criteria of the American Rheumatism Association for the classification of SLE. Patient L2 is a

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30-year-old woman with a 10-year history of SLE with cutaneous, arthritic and renal manifestations. Despite several different treatments, the disease was still active at the time of blood sampling. Patient L3 is a 26-year-woman with a 3-year history of multiorgan polyvisceral treatment-resistant SLE. Blood samples were obtained during the acute phase of the disease with renal, cardiac and neurological involvement. Patient L4 is a 26-year-old woman with a 6-year history of SLE associated with thyroiditis, in remission at the time of blood sampling. Blood from patient L5, a 35-year-old woman with a 2-year history of renal and haematological SLE associated with an anti-phospholipid syndrome, was obtained when there were no inflammatory symptoms of SLE.

B cell and activated mononuclear cell separation

$CD19^+$ B cell population from blood mononuclear cells, isolated by Ficoll–Isopaque density centrifugation, were positive-selected using anti-CD19 MoAb and indirect immunomagnetic cell separation using Dynal Magnetic Particle Technology (Dynabeads M450 goat anti-mouse IgG; Dynal AS, Oslo, Norway). Activated peripheral mononuclear cells, which are present in significant numbers in SLE, were positively selected with an antibody specific for the transferrin receptor (CD71 surface marker). Anti-CD19 antibodies and anti-CD71 antibodies were obtained from Immunotech S.A. (Marseille, France).

cDNA/anchor polymerase chain reaction approach to analyse V_H family representation

Magnetic beads covalently coupled with oligo (dT)25 (Dynabeads M-280 oligo(dT)25; Dynal) were used to isolate mRNA from $CD19^+$ or $CD71^+$ cells. Reverse transcription reaction was performed to generate a reusable pool of solid phase-coupled first-strand cDNA, as described previously [5]. We have used an anchored polmerase chain reaction (PCR) technique to avoid technical bias in the amplification of all V_H gene family members. The dG-tailing was performed on the hetero-duplex mRNA/cDNA in tailing buffer with 1 mM dGTP and 20 U terminal deoxynucleotidyl transferase for 30 min at 37°C. For synthesis of the second strand a dC15-anchor primer (dC1), 2 U of Taq polymerase (Perkin Elmer Roche, Branchburg, NJ), 200 mM dNTP and 3 mM Mg^{2+} in Cetus buffer were added and the following PCR cycle was performed five times: 30 s 94°C, 30 s 60°C and 10 min 72°C. Aliquots of the reaction mixture were then taken and amplification reactions performed, as described elsewhere [5]. The 5′ primer consisted of oligo(dC) nucleotide (dC1) and the 3′ primer was an oligonucleotide corresponding to the constant region $C\mu$ (5′CGGGATCCGAGACGAGGGGGAAAAGGGTT3′) or $C\gamma$ (consensus region of $C\gamma1$, $C\gamma2$, $C\gamma3$ and $C\gamma4$ isotypes) (5′ CGGGATCCCGCCAGGGGGAAGACCGATGGG 3′) cDNA PCR amplification was always successful with IgM mRNA from all samples but was successful in seven out of nine IgG.

The PCr products were size-fractionated on a 1·2% agarose gel and transferred onto Hybond-N^+ membranes (Amersham, Aylesbury, UK) and hybridized with J_H probes and either with V_H1, V_H3 or V_H4 family-specific oligonucleotide probes. J_H is present in all immunoglobulin variable regions and is used as an internal control. Hybridization was performed in 0·5 M Na_2HPO_4/NaH_2PO_4 Ph 7 and 7% SDS at −10°C; the filters were washed twice with 5 × SSC, 0·1% SDS for 15 min at room temperature. Gels were exposed for about 2 h on Kodak storage phosphor screens and the radioactive signal for each V_H was quantified with the J_H, as control, using a phosphorimager (Molecular Dynamics, Sunnyvale, CA). The V_{H/J_H} hybridization ratio was calculated from the data obtained with the phosphorimager.

Oligonucleotide probes used for V_H1, V_H3 and V_H4 analysis

Designed oligonucleotide probes were derived from the 5′ end of the highly conserved leader sequence within each V_H family. The probes used in this study designated HV_H1L (5′ ACCATGGACTGGACCTGGAG 3′), HV_H3L (5′ ACCATGGAGTTTGGGCTGAGCTGGGTTTTCCT 3′) and HV_H4L (5′ CTGG (T/A)GGCAGCTCCCAGATGGGTCCTG 3′), are highly homologous to conserved areas beginning near the ATG start codon of the leader sequences for each of the expressed human V_H families [11].

The specificity of our probes has been verified [5]. Using these probes, the representation of V_H gene family usage in libraries made from normal individuals [5] was not significantly different from previous results obtained by *in situ* hybridization [12] and in Epstein–Barr virus (EBV)-transformed B cells [13].

RESULTS

Analysis of V_H gene family representation in normal individuals

We have designed a molecular approach to examine the usage of V_H genes expressed by different B lymphocytes. This procedure consists of a combination of B cell selection with a MoAb against the CD19 molecule which is not expressed on plasma cells [14], the utilization of the anchored PCR technique to avoid technical bias in the amplification of different V_H gene family cDNA templates, and the screening of the amplified IgM or IgG cDNA rearrangements by V_H1, V_H3 and V_H4 family-specific oligonucleotide probes [5]. Using this approach, we first investigated V_H using in peripheral $CD19^+$ B cells of normal adults.

PCR products corresponding to IgM or IgG expressed by $CD19^+$ peripheral B cells of five healthy adults were hybridized by Southern blot analysis (Fig. 1) with a J_H probe which detected all amplified V_H-D-J_H rearrangements and with V_H gene family-specific oligonucleotides (HV_H1L, HV_H3L and HV_H4L) corresponding to the three main expressed families: V_H3, V_H1 and V_H4. Quantification of V_H family representation in IgM or IgG B cells was done by calculating the hybridization intensity ratio of V_H gene family-specific oligonucleotide to the J_H probe (Fig. 2).

No marked difference appeared in representation of V_H1, V_H3 or V_H4 family expression in IgM $CD19^+$ and IgG $CD19^+$ repertoires between the different individuals analysed (Fig. 2). Under our experimental conditions the $CD19^+$ cell is dominated by non-activated cells and can be considered as a representation of preimmune repertoire [5]. Moreover, the results correlate with previous findings [12].

V_H gene family representation in IgM in vivo *activated B cells of four lupus patients*

Increased numbers of peripheral *in vivo* activated B cells are characteristic of SLE [1,2]. They were positively selected with a MoAb against the transferrin receptor (CD71) expressed by activated lymphocytes. V_H-D-J_H rearrangements from IgM- or IgG-activated B cells were amplified by the anchor PCR approach described in Materials and Methods. The same approach was used to analyse the variable region of total peripheral $CD19^+$ cells of the corresponding patients. PCR products were hybridized by

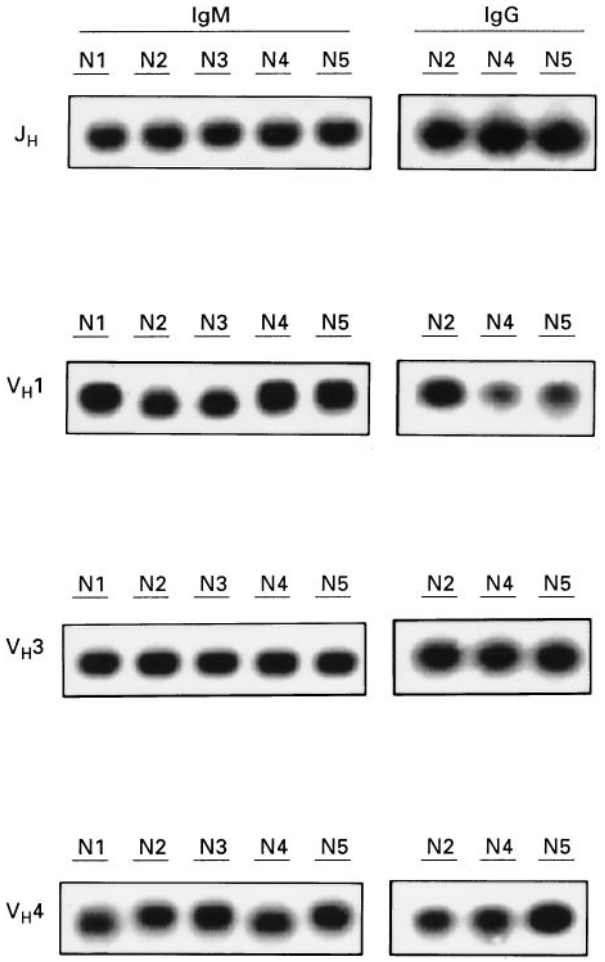

Fig. 1. Southern blot analysis of V_H gene family expression in healthy donors. Southern blot analysis of J_H gene segment and V_H gene families in $V_H-D-J_H-C\mu$ (IgM) and $V_H-D-J_H-C\gamma$ (IgG) rearrangements amplified from the peripheral blood $CD19^+$ B cells of five healthy adults. Signal intensities were obtained using the Phosphorimager Imag Quant software (Molecular Dynamics).

Southern blot (Fig. 3) with a J_H probe which detected all immunoglobulin-rearranged variable regions and with V_H gene family-specific oligonucleotides corresponding to the V_H3, V_H1 and V_H4 family.

Quantification of V_H family representation was done as described above. In all four patients, no significant differences ($P > 0{\cdot}05$; Student's *t*-test) were found in V_H1 and V_H3 gene family expression between the $CD71^+$ IgM^+ and the total $CD19^+$ IgM^+ subpopulations (Fig. 4). In contrast, V_H4 family members were significantly underrepresented ($P < 0{\cdot}001$) in activated B cells ($CD71^+$, IgM^+) in all lupus patients (Fig. 4).

V_H gene family representation in IgG-activated B cells

PCR products corresponding to $CD71^+$ IgG B cells or to total IgG B cells from the four lupus patients were hybridized by Southern blot with J_H probe and V_H1, V_H3 and V_H4-specific oligonucleotides (data not shown). Relative quantification of V_H family expression of V_H1, V_H3 and V_H4 was performed as described in Materials and Methods.

For all lupus patients, no marked differences appeared in V_H3 gene family expression of $CD71^+$ IgG^+ subpopulation in comparison with $CD19^+$ IgG^+ repertoire (Fig. 5). The V_H3 repertoires of IgG-activated B cells and of $CD19^+$ IgG B cells were statistically similar.

With the V_H4 repertoire expressed in IgG^+ B cells from lupus patients, results are very different from the results obtained with

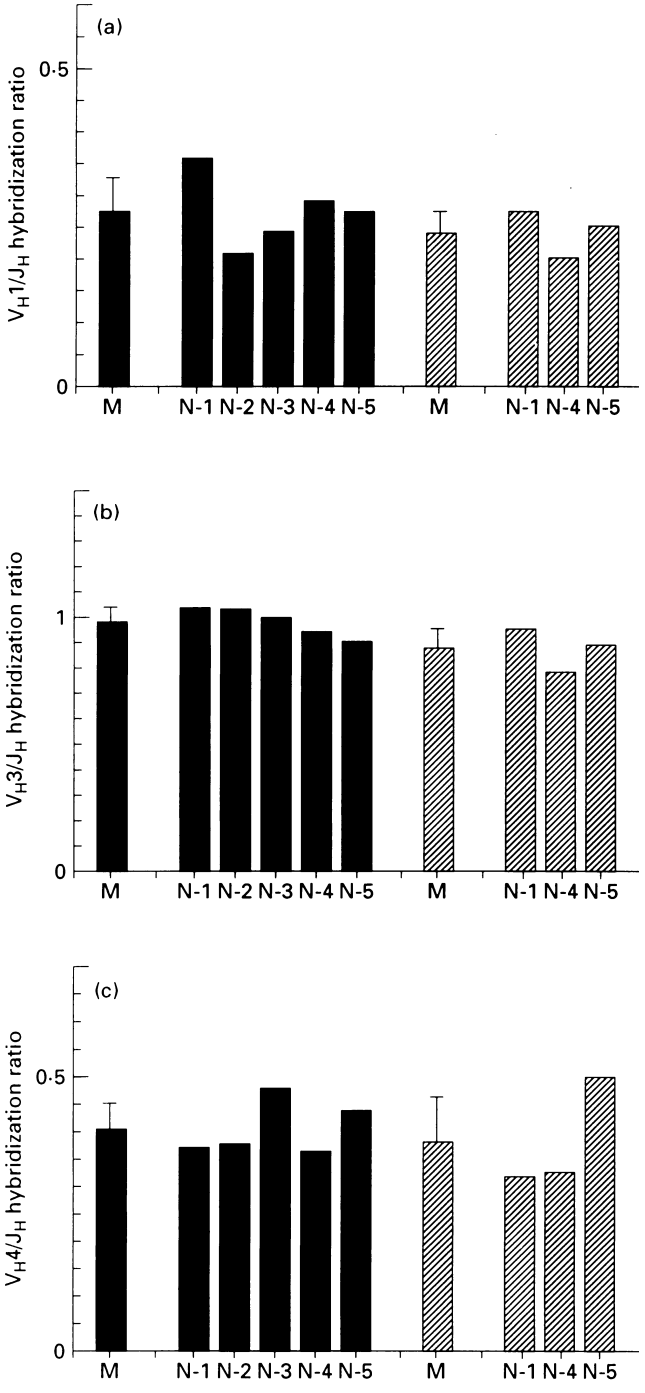

Fig. 2. V_H1, V_H3 and V_H4 expression in healthy donors. V_H1 (a), V_H3 (b) and V_H4 (c) gene family representation in IgM (■) and IgG (▨) $CD19^+$ B cells of five healthy adults (N-1–N-5) was analysed using the semiquantitative polymerase chain reaction as described in Materials and Methods. For IgG only three of five donors were analysed (N-1, N-4, N-5). Mean values of the ratio obtained with IgM and IgG-derived polymerase chain reaction (PCR) product are also shown (M).

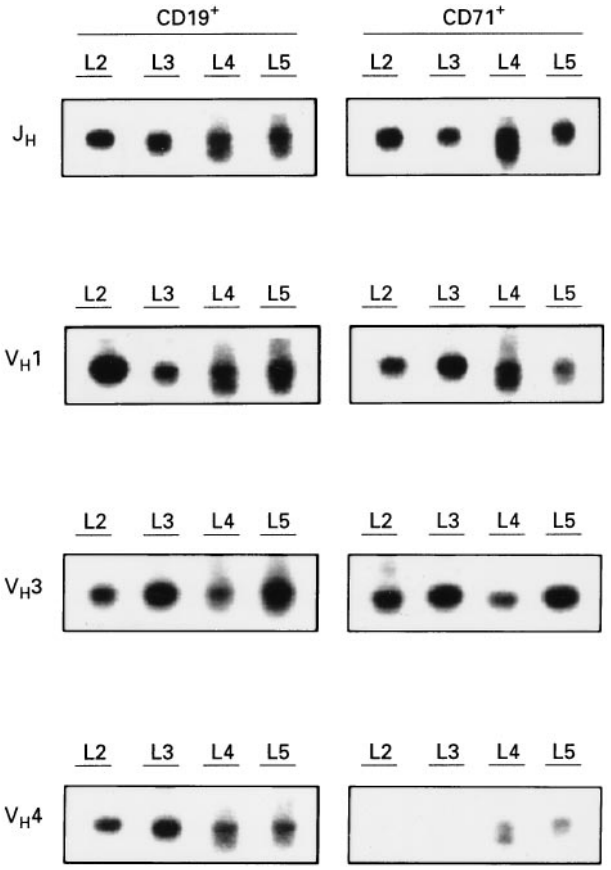

Fig. 3. Southern blot analysis of V_H gene family expression in IgM^+ B cells from lupus patients. Southern blot analysis with J_H and V_H gene family probes in $V_H-D-J_H-C\mu$ (IgM) rearrangements amplified from the peripheral $CD19^+$ or $CD71^+$ B cells of lupus patients. Signal intensities were obtained using the Phosphorimager Imag Quant software (Molecular Dynamics).

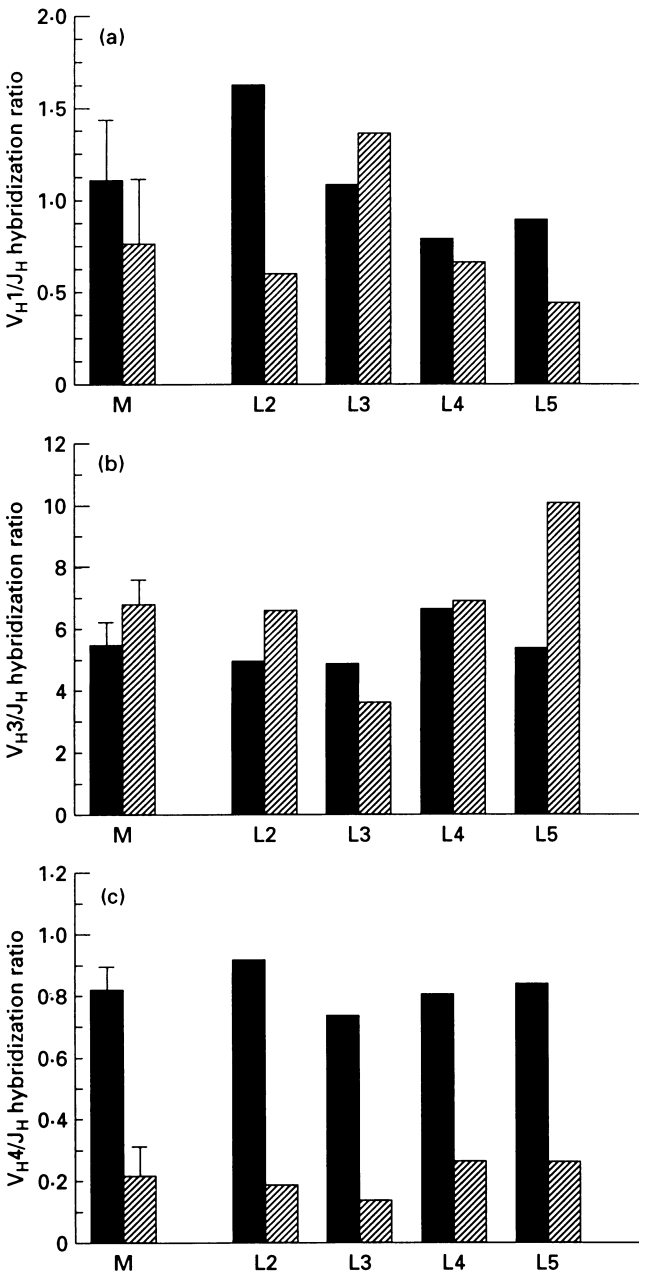

Fig. 4. V_H1 and V_H4 expression in IgM^+ B cells from lupus patients. The V_H1 (a) V_H3 (b) and V_H4 (c) gene family representation in CD19 (■) and activated $CD71^+$ (▨) IgM^+ B cells of four lupus patients (L) were analysed as described in Materials and Methods. Mean values of the ratio obtained with $CD19^+$ and $CD71^+$ B cells are also shown. V_H4 expression by $CD71^+$ B cells and total B cells is statistically different ($P < 0{\cdot}001$). The results obtained with V_H1 and V_H3 are not statistically different ($P > 0{\cdot}05$). The different V/J ratio obtained in Figs 3, 5 and 6 can be explained by the different specific activity of the radioactive probes used.

IgM^+ B cells. For patients in an inactive phase of the disease (L4 and L5), we noticed an underrrepresentation of V_H4 family expression in IgG circulating activated B cells (Fig. 5). However, for the two other patients in an active phase of the disease (L2 and L3), an over-representation of the V_H4 family was found. This suggests that in lupus, activated B cells which have undergone an IgG class switch either overexpress V_H4 genes during the crisis or behave like IgM^+ B cells which underexpress V_H4 when patients are in remission.

DISCUSSION

In SLE, we observed that V_H1 and V_H3 gene family representations are comparable in total $CD19^+$ B cells and in activated $CD71^+$ B cells (Figs 3, 4 and 5). Under our experimental conditions, the $CD19^+$ B cell population was dominated by non-activated cells and can be considered as a control [5]. Unlike V_H1 and V_H3, V_H4 gene representation was different in total $CD19^+$ and in $CD71^+$ IgM B cells. A strong under-representation of V_H4 expression was found in IgM-activated B cells from all patients. Because polyclonal activation implicates a mechanism not directly related to V_H gene family representation, this hypothesis cannot account for our results. The pattern of V_H4 representation found in IgG^+ $CD71^+$ B cells is also incompatible with the polyclonal activation hypothesis. Our data suggest a link between disease activity and over- or under-representation of V_H4 gene usage in IgG-activated B cells. However, the number of patients studied does not allow a firm conclusion on this point.

The role of a superantigen, binding to the heavy chain variable region on a solvent-exposed side face of the B cell receptor,

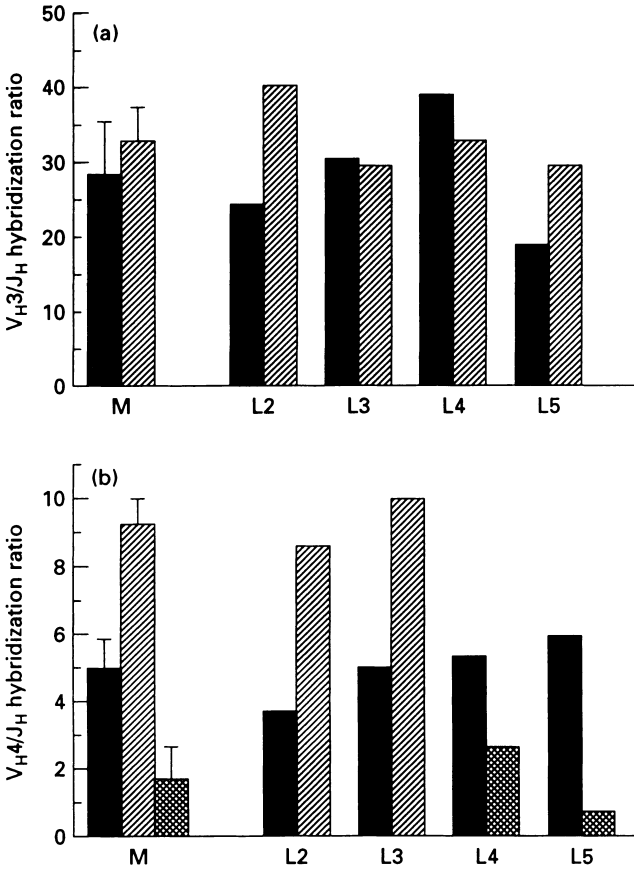

Fig. 5. V_H1, V_H3 and V_H4 expression in IgG^+ B cells from lupus patients. V_H3 (a) and V_H4 (b) gene family representation in CD19 (■) and $CD71^+$ (▨ and ▩ for patients with active and inactive disease, respectively) IgG^+ B cells of four lupus patients were analysed using the semiquantitative polymerase chain reaction (PCR) described in Materials and Methods. Mean value of the ratio obtained with $CD19^+$ and $CD71^+$ B cells is also shown with V_H3. The results obtained with V_H3 are not statistically different ($P > 0{\cdot}05$). Mean value of the ratio obtained after V_H4 hybridization is also shown with $CD19^+$ B cells. Mean value of the ratio obtained after V_H4 hybridization of PCR products from $CD71^+$ B cells from patients L2 and L3 and from $CD71^+$ B cells from patients L4 and L5 are also shown. The results obtained with $CD71^+$ B cells from patients L2, L3, L4 and L5 are statistically different from the results obtained with their $CD19^+$ counterpart.

distinct from that used by conventional antigens, may be considered to explain the present data. This hypothetical molecule, specific for the V_H4 gene encoded region, may therefore interact with up to 20% of naive B cells. By analogy to the T cell superantigen [15], the initial expansion of the target B cell will be followed by clonal deletion and/or anergy. This phenomenon may account for the underrepresentation of the V_H4 family in peripheral IgM^+-activated cells. The same explanation would be valid for activated IgG B cells from patients in remission.

As a consequence of this superantigenic stimulation, nuclear antigens will be generated and released *in vivo* by programmed cell death of the corresponding clones [16,17], explaining the high level of circulating nucleosomes in SLE [18]. These may provide a source of nuclear autoantigens and drive the expansion of some V_H4 activated B cells toward autoantibody production. The corresponding clones will escape anergy and/or deletion. This would explain the V_H4 over-representation in IgG B cells from patients in an active stage and in autoantibodies with anti-nuclear activity.

To document further the possible involvement of a B superantigen activation in the onset of SLE, we have analysed data from the literature to show V_H usage by the MoAb with anti-DNA activity published to date [7–10,19–22]. These auto-antibodies, from established clones and hybridomas, are class-switched isotypes and/or express a disease-related idiotypic marker that is not found under normal physiological conditions [7,9,20]. It is remarkable that 23/24 of the heavy chains of these autoantibodies are derived principally from the V_H4 (8) and V_H43 (15) gene families. Several other human anti-DNA-secreting cell lines from control individuals or SLE patients have been described (reviewed in [23]). However, these cell lines are all of the IgM isotype and often react with single-strand DNA. No V_H gene family restriction was found among these natural anti-DNA antibodies which reflect the natural autoantibody repertoire.

The frequent rearrangement of V_H3 gene family members among anti-dsDNA autoantibodies is not surprising, since this gene family, containing 21 functional genes [24], dominates the peripheral B cell population of normal adults and is also preferentially expressed in specific immune responses [25–29]. The preferential usage of V_H4 gene family members among these pathogenic autoantibodies does not correlate with their representation in the expressed repertoire of healthy adults [5] or in specified immune response [25–27]. This over-representation also contrasts with the absence of V_H1 gene family use. This last family is expressed as frequently as the V_H4 family in the preimmune repertoire.

It appears that this V_H4 gene family overrepresentation does not derive from the constitution of a particular antigen binding site. A large variety of the light variable region genes, from both $V\kappa$ and $V\lambda$ gene families, is also associated with this restricted V_H4 gene family (data not shown). In addition, the V_H4 gene family used by lupus anti-DNA autoantibodies seems not directly linked to their specificity, as an anti-Sm antibody ($IgG\kappa$), derived from a patient with active SLE [30], also uses a gene segment closely related to a V_H4 gene member (V4–34). Therefore the preferential usage of V_H4 gene segment implicated in SLE may be related to a multiclonal (family-specific) expansion derived from the involvement of a B cell superantigen.

Because amino acids of the fourth β loop determine superantigenic specificity for murine T cell superantigen [15] and for human B cell superantigen [31], we have analysed this region among V_H4 anti-DNA autoantibodies (Fig. 6). This solvent-exposed β loop subdomain corresponds to amino acid residues 67–82 of framework 3 (FR3). Interestingly, solvent-exposed amino acids of this fourth β loop are common to all immunoglobulin encoded by V_H4 functional germ-line genes [32]. During various antigen responses (including lupus anti-DNA response), somatic mutation processes occur. Residues corresponding to the solvent-exposed face of the fourth β loop are free to diverge without affecting the basic structure of the immunoglobulin fold. This is the case in high-affinity antibodies specific for various exoantigens (rabies virus; herpes simplex virus; hepatitis B virus; HIV) ($R/S = 2{\cdot}5$; the Replacement/Silent mutation ratio is used as an index to analyse the somatic mutation process that occurs during an antigen-driven response). On the other hand, in anti-DNA MoAb the common exposed residues of the fourth β loop show a low R/S ratio ($R/S = 1{\cdot}5$), suggesting that there is a selection pressure for

Name	Specificity	Isotype	67* * * **** *82
			V T I S V D T S K N Q F S L K L
NE-1	DNA	IgM	- - - - - - - - - - - - - - - - -
T20-24	DNA	IgM	- - - - - - - - - - **L** - - - - -
T33-1	DNA	IgM	- - - - - - - - - - - - - - - - **V**
T33-4	DNA	IgG	- **I** - - - - - - **E** - - - - - **R** -
T33-14	DNA	IgG	- **I** - - - - - - **E** - - - - - **R** -
33C9	DNA	IgG	**I I** - - - - **M** - - - - - - - - -
T14	DNA	IgG	- - - - - - - - - - - - - - - - -
D5	DNA	IgG	- **I M** - - - - - - - - - - - **N** -
H3	HSV	IgG	- - **V** - - - - - **T** - **Q** - - - **R** -
265-695	HBV	IgG	- - **V** - - - - - - - **K** - - - - **R** -
120-16	HIV	IgG	- **T L** - **A** - - - - - - - **I** - - **Q** -
98-6	HIV	IgG	**S** - - - - - - - - - **T K** - **F** - **R** -
268-D	HIV	IgG	**L** - - **T I D** - - **R K** - **L** - - **S** -
mAb58	RV	IgG	**L S** - - - **E R** - - - - - - - - -

Fig. 6. Molecular characteristics of the fourth β loop of V_H4 anti-DNA antibodies. Residues 67–82 (FR3) of V_H4 consensus sequence shown on the Figure derived from [32]. *Residues with exposed side chains. Comparison of amino acid sequences (positions 67–82 of FR3) of V_H4 anti-DNA antibodies ($n = 8$) characterized to date [7–10,19–22] is shown, V_H4 sequences from antibodies specific for various exo-antigens ($n = 6$), derived from references [25–27]. Specificity and isotype are indicated (RV, rabies virus; HSV, herpes simplex virus; HBV, hepatitis B virus). Dashes indicate amino acid identity with consensus sequence. (Replacement substitutions are shown in bold).

the conservation of this amino acid sequence of the fourth β loop. This is in agreement with the binding to a superantigen structure.

In conclusion, our results and the analysis of sequenced anti-DNA autoantibodies from the literature evoke the possible involvement of a B cell superantigenic in SLE. Since lupus is a multifactorial disease, the present finding may account for some aspects of its pathogenesis. B cells expressing V_H4 gene family immunoglobulins are clearly involved in the production of a significant proportion of pathogenic anti-DNA antibodies. However, the origin of this phenomenon remains to be explained at the molecular level.

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