



Research paper

Detection of one V_H antibody sequence in both healthy donors and urticaria patients

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Abstract

We have previously isolated anti-FcεRIα autoantibodies from phage libraries of healthy donors and urticaria patients. Strikingly, the same antibody, LTMα15, was isolated from both libraries. Sequence analysis revealed a germline configuration of the LTMα15 variable heavy (V_H) chain with a slightly mutated variable light (V_L) chain supporting its classification as a natural autoantibody. Distribution analysis of anti-FcεRIα autoantibodies by functional or serological tests delivered conflicting data. For this reason we have developed a new real-time PCR to analyse the distribution of LTMα15V_H in healthy donors and urticaria patients. Our new bioinformatic program permitted the design of a minor groove binder (MGB) *TaqMan* probe that specifically detected the LTMα15V_H. We were able to demonstrate a broad range of rearranged V_H gene copy number without any correlation to the state of health. Monitoring LTMα15V_H gene copy number in a single donor over a period of 70 days revealed a time-related fluctuation of circulating B cells carrying LTMα15V_H. We propose that our real-time PCR may serve as a model for the quantification of natural antibody sequences at a monoclonal level.

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1. Introduction

Chronic idiopathic urticaria (CIU) is defined by the occurrence of daily wheals and itching for at least 6 weeks. The wheals, redness and itching of CIU are due

to the release of histamine and other mediators from dermal mast cells. Evidence of the involvement of functional autoantibodies against the α-chain of the high-affinity IgE receptor (FcεRIα) of dermal mast cells and basophils in CIU was first reported by [Hide et al. \(1993, 1994\)](#). Such anti-FcεRIα autoantibodies were suggested to be a selective marker for a subset of urticaria patients ([Fiebiger et al., 1995](#)). However, we have previously reported the presence of anti-FcεRIα autoantibodies in the serum of healthy donors ([Pachlounik et al., 2004](#)) as well as in multidonor intravenous IgG (IVIg) preparations ([Horn et al., 1999](#)). Furthermore we have isolated anti-FcεRIα autoantibodies (LTMα15, UMα16, UGα8) using phage display tech-

Abbreviations: V_H, variable heavy chain; V_L, variable light chain; CDR, complementary determining region; CIU, chronic idiopathic urticaria; FcεRIα, α-chain of the high-affinity IgE receptor; C_T, threshold cycle number; MGB, Minor groove binder; PBGD, porphobilinogen deaminase.

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nology (Horn et al., 2001; Miescher et al., 2001). LTM α 15 was isolated from an IgM library constructed from children's tonsillar B cells. UM α 16 and UG α 8 originated from an IgM and IgG library, respectively, that were constructed from the B cells of urticaria patients. Comparison with the known germline sequence of human variable heavy (V_H) and variable light (V_L) chain segments (Tomlinson et al., 1992, 1995; Williams et al., 1996) indicated that LTM α 15, UM α 16 and UG α 8 V_H chains showed 100% homology to the germline V_H 3–23 sequence, whereas the λ light chains were slightly mutated and most similar to the V_λ germline λ 6a. Furthermore, sequence analysis showed that LTM α 15 and UM α 16 are 100% identical. Due to the germline configuration of the heavy chains and the fact that the light chains are only slightly mutated, we suggest that LTM α 15, UM α 16 and UG α 8 represent natural autoantibodies, which may be present in all humans. This evidence points to the ubiquitous presence of natural anti-Fc ϵ RI α autoantibodies but their role as a selective marker for urticaria patients remains unclear. Therefore, our goal was to investigate further the distribution of anti-Fc ϵ RI α autoantibodies in healthy donors and urticaria patients.

Up to now the distribution of anti-Fc ϵ RI α autoantibodies was analysed by functional and serological tests. However, no significant relation was found between autologous serum skin test positivity and serological detection of anti-Fc ϵ RI α autoantibodies by enzyme immunoassays (Sabroe et al., 2002; Pachlounik et al., 2004). This discrepancy may occur due to the sensitivity of the performed tests. Here we describe the analysis of the distribution of the LTM α 15 V_H sequence by semi-nested RT-PCR which was shown to be two times more sensitive than a serological test to detect infectious agents (Pacheco et al., 2001). Additionally we have performed a real-time PCR. Quantitative analysis of the real-time PCR results enabled us to compare the relative gene copy number of LTM α 15 V_H sequence in healthy donors and urticaria patients. Because of the presence of V sequences highly similar to the LTM α 15 V_H and V_L sequence in the V repertoire we have written a new bioinformatic program, which predicts potential primer annealing sites in any sequence. Using this program, we were able to determine a potential primer annealing site, which is present exclusively in the LTM α 15 V_H sequence. To this annealing site we have designed a 3' end primer for the RT-PCR or a minor groove binder (MGB) probe to perform the real-time PCR. To the best of our knowledge, this is the first report describing the de-

tection of a single natural immunoglobulin sequence in different donors. Our approach may therefore serve as a model for the detection and quantification of antibody sequences coding for natural antibodies.

2. Material and methods

2.1. DNA, RNA extraction, cDNA synthesis and plasmid DNA

Mononuclear cells of peripheral blood from 26 unselected healthy donors, 11 urticaria patients and of 25 cord blood samples were purified by Ficoll density gradient centrifugation (Lymphoprep, Pharmacia, Dübendorf, Switzerland). Total RNA was extracted using RNeasy Mini Kit (Qiagen AG, Basel, Switzerland). RNA was transcribed into cDNA using a primer specific for the first constant domain of the IgM gene (5'GCTCACACTAGTCTAGGCAATCACTGGAAG-AGG 3') and the Superscript TM II reverse transcriptase system (Invitrogen, Life technology, Basel, Switzerland). Genomic DNA was isolated using QIAamp DNA blood Maxi kit (Qiagen AG, Basel, Switzerland). Plasmid DNA containing the anti-Fc ϵ RI α antibody LTM α 15 heavy chain sequence was produced in our laboratory as described (Miescher et al., 2001).

2.2. Identification of specific primer annealing sites for semi-nested RT-PCR

Specific primers were designed in order to detect the LTM α 15 sequence in healthy donors and urticaria patients using PCR. The sequences of LTM α 15 V_H and V_L are available at EMBL GenBank under the numbers AJ276097 and AJ276098. We intended to design a forward primer annealing either in the complementary determining regions (CDR) 1 or CDR2 and a reverse primer hybridising to the CDR3 of LTM α 15 variable light (V_L) and variable heavy (V_H) chains. To identify specific primers, the CDR sequences of V_H and V_L of LTM α 15 were independently compared to 82,566 different immunoglobulin sequences (ImMunoGeneTics database, University of Montpellier, France and French National Centre for Scientific Research: CNRS, France (Ruiz et al., 2000)) applying a BLAST search (Basic Local Alignment Search Tool, version 2.2.8). BLAST was used with gap open and extension penalties of 5 and 2, respectively. The *E*-value threshold was set to 1000. For comparison, sequences of CDRs of LTM α 15 V_H and V_L , which were shorter than the desired primer length of 23 nucleotides, were extended to 23 nucleo-

tides by adding bases of the flanking frameworks. Immunoglobulin sequences containing similar CDRs as LTM α 15 were identified and were then checked for potential primer annealing sites. A potential primer annealing site was defined as a window of 20 or more nucleotides with at most two mismatches in total and no mismatch in the five nucleotides at the 3' end of the primer. The number of potential PCR products was then counted in the case that a forward and a reverse primer annealing site occurred simultaneously in sequences containing similar CDRs as LTM α 15. All scripts were written in Perl, using modules from Bioperl (Stein et al., 2002). After identification of primer annealing sites, primers specific for the LTM α 15 were designed using the PrimerExpress software (Applied Biosystems, Rotkreuz, Switzerland).

2.3. Semi-nested RT-PCR

The presence of LTM α 15V_H in 26 different healthy donors was analysed by semi-nested RT-PCR. Cord blood cDNA known to be positive for LTM α 15V_H was used as a control for testing primer specificity in RT-PCR. The first PCR was performed using 5 μ l of cDNA, CDR1V_H forward primer (5'CTGGATT-CACCTTTAGCAGCTATGC 3') that anneals to the CDR1 and C μ 1 reverse primer (5'GGAGTCGG-GAAGGAAGTCC 3') that matches the first constant domain of IgM. To perform the second PCR, the first PCR product was first gel purified from a 2% DNA agar gel (Axon Lab AG, Baden-Dättwil, Switzerland) using the MinElute gel extraction kit (Qiagen AG, Basel, Switzerland) and then amplified. The second PCR was carried out using 50 ng of purified first PCR product, CDR1V_H forward primer and CDR3V_H reverse primer (5' TCCATGTAGTAGGACGGTAGC 3'), which hybridises in the CDR3. In parallel and in order to investigate its quality, cDNA was directly amplified using two primers that hybridise to the first constant region of IgM gene C μ 1 for (5'GGAGTCGG-GAAGGAAGTCC 3') and C μ 1 rev (5'GCATCC-GCCCCAACCCCTT 3'). PCR amplifications were performed according to the manufacturer's instructions (Invitrogen, Life technology; Basel, Switzerland) in a total volume of 50 μ l using 2.5 U of Platinum Pfx DNA Polymerase. The first PCR was carried out at 94 °C for 5 min, followed by 30 cycles at 94 °C for 40 s, 58.9 °C for 30 s and 72 °C for 40 s. For final extension the samples were incubated for 10 min at 72 °C. A second PCR of 40 cycles was done as mentioned except for an annealing temperature of 57 °C. Products of the second PCR were purified and sequenced using primers of the second PCR.

2.4. Primers and TaqMan probes for real-time PCR

The primers and the TaqMan probes for real-time quantitative PCR were designed from appropriate sequences using the PrimerExpress software (Applied Biosystems). The primers and the TaqMan probes specific to the LTM α 15V_H and to the housekeeping gene porphobilinogen deaminase (PBGD) are: LTM α 15V_H for 5'CCGTATATTACTGTGCGAAAGGG 3'; LTM α 15V_H rev for 5'CATGGACGTCTGGGGCA 3'; LTM α 15V_H minor groove binder (MGB) TaqMan probe for 5'AAAGATGGCTACCGTCCTA 3'; PBGD for 5'TGACCCACAGTTGGTAGGCAT 3'; PBGD rev for 5'ATGCCCAAGTTCTGGGCA 3'; and PBGD TAMRA TaqMan probe for 5'CGTAACATTCCACGAGG-GCCCCA 3'. The primers were synthesised by Microsynth (Balgach, Switzerland) and the TaqMan probes by Applied Biosystems. LTM α 15V_H MGB TaqMan probe was labelled at the 5' end with 6-carboxy fluorescein (FAM) and at the 3' end with MGB/Non-Fluorescent Quencher (NFQ). PBGD TaqMan probe was labelled at the 5' end with FAM and the red fluorophore tetramethylrhodamine (TAMRA) at the 3' end was used as quencher.

2.5. Real-time PCR

Real-time PCR was performed using genomic DNA of 22 healthy donors (donors 5–26) and 11 urticaria patients (donors 27–37). All reactions were performed in triplicate (25 μ l each) using 12.5 μ l of TaqMan Universal PCR Master Mix (Applied Biosystems), 200 nM of TaqMan probe and 900 nM of each primer except for LTM α 15V_H rev primer which was used at a final concentration of 2 μ M. To check the amplification efficiency, PBGD and LTM α 15V_H were amplified using a serial dilution of DNA (2 μ g and 0.5 ng). A standard curve for PBGD and LTM α 15V_H was generated by plotting the different amounts of DNA on a log scale vs. threshold cycle number (C_T), which is the minimum number of cycles needed before the product can be detected (Applied Biosystems, 1997). Linearity of the amplified PBGD or LTM α 15V_H with respect to the amount of DNA could be observed ($R^2=0.9941$ and $R^2=0.9925$ for PBGD and LTM α 15V_H, respectively). This indicated that the use of these different genomic DNA concentrations would result in a linear progression of the amplification. 100 ng and 2 μ g of DNA was finally used to amplify PBGD and LTM α 15V_H, respectively. Real-time PCR was performed under the following conditions: 50 °C for 2 min; 95 °C for 10 min;

Table 1
Potential primer annealing sites in V_L and V_H genes

	V _L		V _H	
	Similar CDRs	Potential primer annealing sites	Similar CDRs	Potential primer annealing sites
CDR1	2182	146	2282	764
CDR2	327	119	10,673	4667
CDR3	450	65	926	3

CDR=complementarity determining region, V_L=variable light chain, V_H=variable heavy chain.

60 cycles at 95 °C for 15 s and 60 °C for 1 min on an ABI prism 7700 sequence detector equipped with a 96-well thermal cycler. Negative control without template (no genomic DNA) was included on each PCR plate. Data were collected and analysed with Sequence Detector software (Applied Biosystems).

2.6. Quantitative analysis and statistics

Quantitative analysis of gene copy number was done using the comparative C_T (ΔC_T) method according to the instructions of Applied Biosystems (Applied-Biosystems, 1997). To ensure that quantification of LTM α 15V_H was not affected by differences in the quality and quantity of the genomic DNA we used the housekeeping gene porphobilinogen deaminase (PBGD) as internal control and for normalisation. The average C_T values of the PBGD gene in the various genomic DNA samples differed less than 1.1 C_T values among each other, implying that the quality and quantity of genomic DNA per tube were comparable (data not shown). The arithmetic formula for the ΔC_T method is given by $2^{-\Delta C_T}$, where ΔC_T is the C_T value of LTM α 15V_H minus the C_T value of PBGD, which gives a normalised value. The normalised ΔC_T value of LTM α 15V_H is then subtracted from the normalised

ΔC_T value of a calibrator, which gives the $\Delta \Delta C_T$ value. To obtain positive values, the donor with the highest C_T value was chosen as calibrator. The formula $2^{-\Delta \Delta C_T}$ therefore gives a relative value when comparing the target to the calibrator. For each data point, the standard deviation of the difference was calculated from the standard deviations of the LTM α 15V_H and PBGD values as follows: S (S.D. of difference) = $\sqrt{S_1^2 + S_2^2}$ (AppliedBiosystems, 1997).

3. Results

3.1. Determination of primers and probes specific to LTM α 15

Previously, we have isolated identical anti-Fc ϵ RI α variable heavy (V_H) and variable light (V_L) chain genes from urticaria patients and healthy donors (Horn et al., 2001; Miescher et al., 2001; Pachlopnik et al., 2004). To investigate via PCR whether these V genes are differently distributed between urticaria patients and healthy donors, specific primers had to be designed. To identify specific primers, the complementarity determining region (CDR) sequences of V_H and V_L of LTM α 15 were compared to a human immunoglobulin database. Immunoglobulin sequences containing similar CDRs as LTM α 15V_L were identified (Table 1) and analysed for potential primer annealing sites. As shown in Table 1, a total of 146, 119 and 65 potential primer annealing sites in CDR1, CDR2 and CDR3 of V_L, respectively, were found, indicating an abundant usage of such CDRs in the V_L repertoire. We next analysed the number of potential PCR products. A PCR product would be expected if a single immunoglobulin sequence contained CDRs regions as in LTM α 15V_L where potential forward and reverse primer annealing sites had been previously identified. Fig. 1 shows that 61 potential

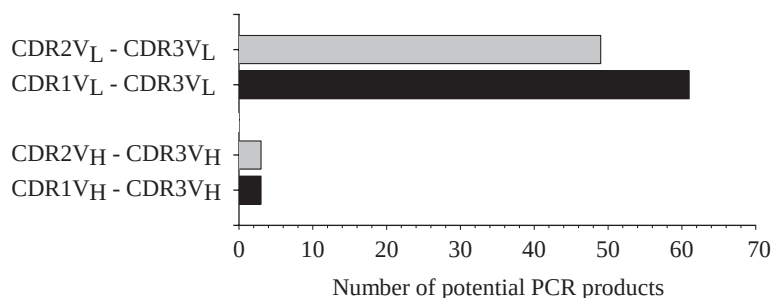


Fig. 1. Number of potential PCR products. LTM α 15 CDR regions of V_H and V_L were compared to all other known immunoglobulin sequences contained in the IMGT database and analysed for potential primer annealing sites. The results are shown as numbers of possible PCR products to be expected if a potential forward primer annealing site in the CDR1 (black bars) or in CDR2 (grey bars) is combined with a potential reverse primer site in the CDR3 of V_L and V_H. CDR=complementarity determining regions; V_H=variable heavy chain; V_L=variable light chain.

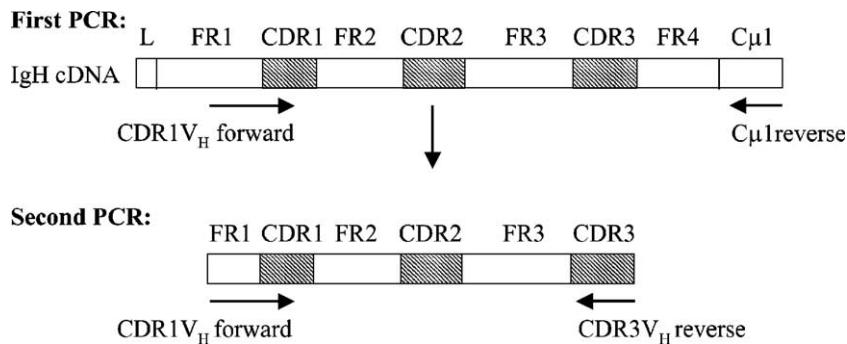


Fig. 2. Semi-nested RT-PCR strategy to detect LTMα15V_H sequence. cDNA is amplified by a first PCR using a 5' primer located in the CDR1V_H and a 3' primer annealing in the first constant domain of IgM (Cμ1). The second PCR is performed with purified product of the first PCR, the same CDR1V_H forward primer as for the first PCR together with specific CDR3V_H reverse primer. CDR=complementarity determining regions; FR=framework; V_H=variable heavy chain.

PCR products were obtained if a forward primer in CDR1V_L and a reverse primer in CDR3V_L were designed and 49 if a forward primer annealed in CDR2V_L and a reverse primer in CDR3V_L. This high number of different PCR products prevented the design of V_L primers for the detection of LTMα15V_L. The same approach was used to identify sequences containing similar CDRs as LTMα15V_H (Table 1). Analysis of potential forward primer annealing sites in these sequences revealed 764 and 4667 sites in the CDR1 and CDR2 of V_H indicating an abundant usage of such CDRs in the V_H repertoire (Table 1). In contrast, only 3 out of 926 similar CDR3 of V_H contained potential reverse primer annealing sites (Table 1). Detailed analysis revealed that these sites were contained in the three anti-FcεRIα antibodies LTMα15, UMα16 and UGα8, which carry identical CDR3 of V_H. As shown in Fig. 1, a limited number of three potential PCR products were found if forward primers were designed to anneal either in CDR1V_H or in CDR2V_H and were combined with a reverse primer in CDR3V_H. This low number of PCR products allowed us to design V_H primers for the detection of LTMα15V_H. As there were less potential forward primer annealing sites in the CDR1 than in the CDR2 of

V_H (764 vs. 4667), a forward primer was designed which anneals to CDR1 of V_H. Detailed analysis of the bioinformatic results also showed that four nucleotides in the CDR3 of LTMα15V_H are exclusively present in this CDR3 and in no other immunoglobulin sequence using the same germline family as LTMα15. To provide specific amplification of the LTMα15V_H sequence, we designed the reverse primer such that two of these four nucleotides are located at the 3' end of the primer sequence. Forward and reverse primer annealing sites for the semi-nested RT-PCR are shown in Fig. 2. To perform the real-time PCR, the minor groove binder (MGB) TaqMan probe was designed such that these four nucleotides exclusively present in the CDR3 of the LTMα15V_H sequence are located in the middle of the MGB probe as shown in Fig. 3.

3.2. Semi-nested RT-PCR for detection of the LTMα15V_H sequence in healthy donors

The presence of the LTMα15V_H sequence in the human genome was monitored by RT-PCR. To increase sensitivity a semi-nested RT-PCR was done as shown in Fig. 2. The semi-nested RT-PCR was performed using

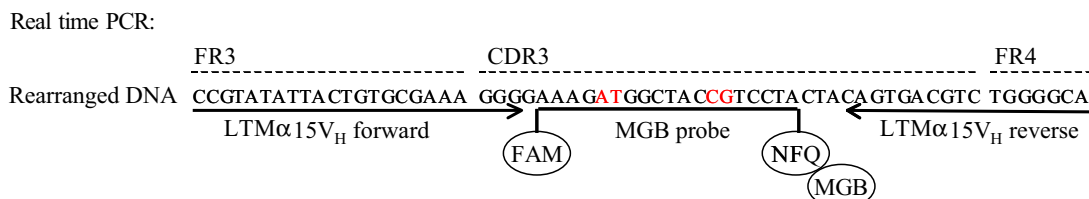


Fig. 3. Real-time PCR strategy for detection of LTMα15V_H CDR3 sequence. Real-time PCR was performed using primers annealing to the 3' and 5' end of the CDR3 region and a TaqMan MGB probe covering 19 bases in the CDR3 region. The four nucleotides identified as unique for the CDR3 of LTMα15V_H are highlighted in red. FAM=6-carboxy fluoroscein; MGB=minor groove binder; NFQ=non-fluorescent quencher; CDR=complementarity determining regions; FR=framework; V_H=variable heavy chain.

cDNA of 26 randomly selected healthy donors. Cord blood cDNA known to be positive for the LTM α 15V_H sequence was taken as positive control. For the first PCR a pair of primers was designed which anneal to the CDR1 of LTM α 15V_H and the first constant region of the IgM gene, respectively. The first PCR yielded for all tested donors as well as for the cord blood sample a single product band of expected size (400 bp, data not shown). The second PCR was carried out with purified first PCR products, the same forward primer used for the first PCR and a reverse primer annealing with its 3' end to two nucleotides unique for the CDR3 of LTM α 15V_H. As shown in Fig. 4, all product samples of the second PCR contained a major band of the same size (254 bp) as the one obtained with the control plasmid DNA containing LTM α 15V_H. Sequencing revealed that in cord blood cDNA as well as in cDNA of donors 3, 10, 11, 12, 14 and 15 the same V_H was found as the one present in LTM α 15. In all other donor samples, except donor 2, the V_H germline family, which is present in LTM α 15V_H (V_H3–23), was amplified. However, in these donor samples, different CDR3V_H sequences were amplified, indicating that the two nucleotides at the 3' end of the reverse primer sequence did not provide enough specificity to amplify exclusively the LTM α 15V_H sequence. We have repeated the semi-nested RT–PCR using a non-proofreading polymerase to investigate whether this increases the specificity of the reverse primer (Huang et al., 1992; Nasis et al., 2004). Our results demonstrated that the use of a non-proofreading polymerase did not increase the specificity of the reverse primer (data not shown). These data are in contrast to other studies showing that a mismatch of one nucleotide at the 3' terminus of a primer sequence resulted in no amplification (Ma et al., 2003). To avoid time-consuming optimisation of the semi-nested RT–PCR, we chose the real-time PCR approach, which

should be more specific because of the use of an additional probe.

3.3. Quantitative analysis of the LTM α 15V_H sequence in healthy donors and urticaria patients

To compare the frequency of LTM α 15V_H in healthy donors and urticaria patients, genomic DNA samples of 22 healthy donors (donors 5–26) and 11 urticaria patients (donors 27–37) were tested for the presence of LTM α 15V_H sequence. DNA of donors 1–4 was unavailable. Rearranged genomic DNA instead of RNA was chosen as template because of the reproducibility and sensitivity problems linked to RT–PCR. The real-time PCR was carried out using a forward and a reverse primer hybridising to the framework 3 and the CDR3V_H, respectively, and a probe, which anneals to the CDR3V_H region containing the four nucleotides unique for the CDR3V_H of LTM α 15V_H. To increase specificity of real-time PCR, a 3' minor groove binder (MGB) TaqMan probe was used instead of the commonly used TaqMan probe. The MGB molecule allows TaqMan probes to be shorter compared to conventional ones, hence increasing the stability and specificity of the probe as a single-base mismatch prevents hybridisation (Kutyavin et al., 2000).

As internal control of genomic DNA quality and quantity, the housekeeping gene porphobilinogen deaminase (PBGD) was chosen. Quantification analysis was performed using the comparative C_T method. This was possible because the efficiencies of amplification of the LTM α 15V_H and the PBGD gene were equal ((AppliedBiosystems, 1997) data not shown).

For quantification, the LTM α 15V_H rearranged gene copy number of each donor was normalised against PBGD and compared to a normalised calibrator. Donor 21, who showed the highest C_T value, was

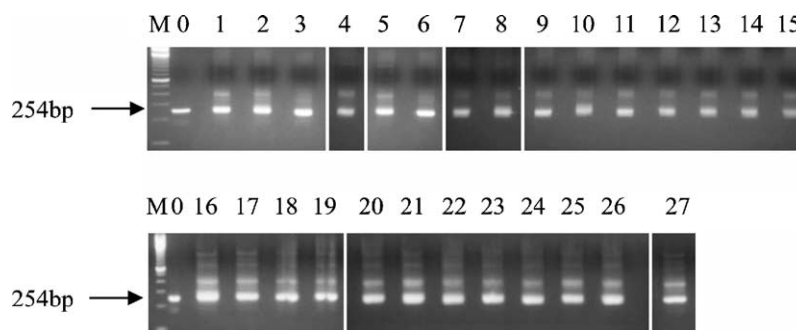


Fig. 4. The products of the second reaction of the semi-nested RT–PCR were analysed on a 2% agar gel. Lanes 1–26 are products from cDNA of 26 healthy individuals, lane 0 from plasmid DNA containing LTM α 15V_H gene, lane 27 from cord blood cDNA. M=100 bp DNA molecular weight marker.

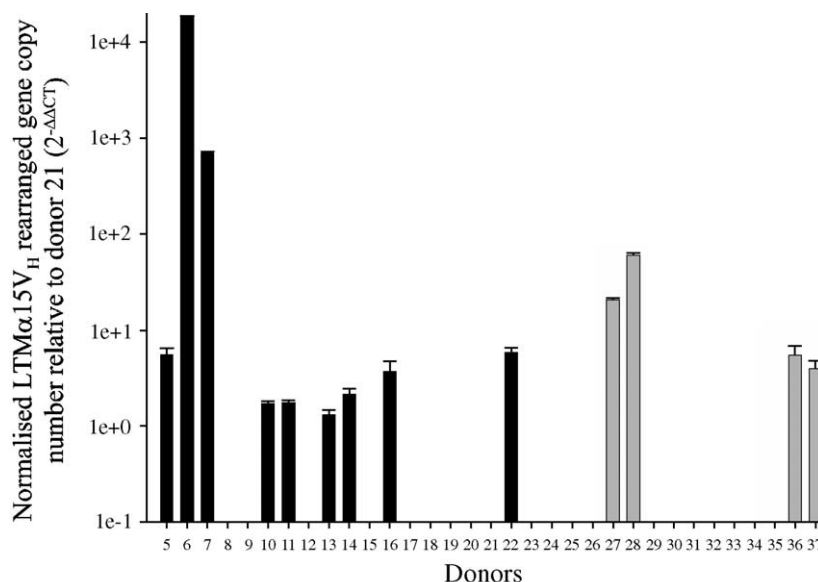


Fig. 5. Relative quantification of LTMα15V_H rearranged gene copy number using the comparative C_T method. DNA from peripheral blood mononuclear cells of 22 healthy donors and 11 urticaria patients were used as templates for real-time PCR containing either primer and probe for LTMα15V_H or for PBGD. Relative rearranged gene copy number of LTMα15V_H normalised to PBGD and relative to the normalised calibrator (donor 21) was given by $2^{-\Delta\Delta C_T}$. Black and grey bars represent mean values of triplicates for healthy donors and urticaria patients, respectively.

chosen as calibrator. The relative quantity of the LTMα15V_H was given by $2^{-\Delta\Delta C_T}$. As shown in Fig. 5, the LTMα15V_H sequence was detected in 9 out of 22 healthy donors and in 4 out of 11 urticaria patients demonstrating that the presence of this V_H is not restricted to urticaria patients. The results also show

that the relative quantity of LTMα15V_H rearranged gene copy number differed considerably between the different donors, over a range of more than four orders of magnitude and there was no correlation with the source of the sample originating either from healthy donors or urticaria patients.

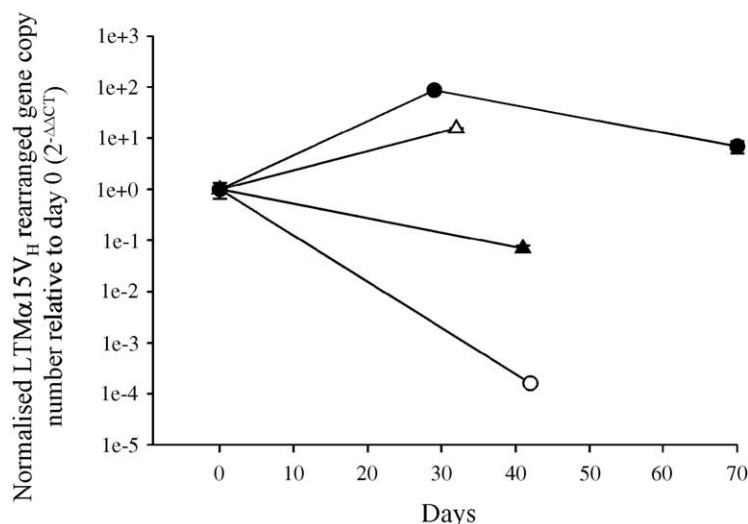


Fig. 6. Time-related relative quantification of LTMα15V_H rearranged gene copy number using the comparative C_T method. DNA from peripheral blood mononuclear cells of 4 healthy donors were isolated at different time points. The relative rearranged gene copy number of LTMα15V_H normalised to PBGD and relative to the normalised calibrator (time point 0; first blood donation) was given by $2^{-\Delta\Delta C_T}$. The data are mean values of triplicates of donor 6 (○), donor 14 (●), donor 16 (△) and donor 20 (▲).

3.4. Time-related analysis of LTM α 15V_H sequence in healthy donors

So far our data have demonstrated that the distribution and quantity of LTM α 15V_H rearranged gene copy number differs from donor to donor. We next asked the question whether in a single healthy donor LTM α 15V_H rearranged gene copy number is stable over a certain time period. Thus blood samples were collected from four healthy donors (6, 14, 16, and 20) at different time points. The first blood donation of each donor was defined as day 0 and used as calibrator. The normalised amount of LTM α 15V_H rearranged gene copy number was calculated as described above and given by $2^{-\Delta\Delta C_T}$. The healthy donors showed gene copy numbers over time which ranged up to four orders of magnitude difference for a given donor suggesting a time-related fluctuation of circulating B cells carrying the LTM α 15V_H rearranged gene (Fig. 6).

4. Discussion

Autoantibodies against the α -chain of the high affinity IgE receptor were claimed to be a selective marker for autoimmune urticaria (Fiebiger et al., 1995). However, we have recently shown that natural anti-Fc ϵ RI α autoantibodies are also present in the sera of healthy donors which could give false positive results in serological diagnostics for autoimmune urticaria (Pachlopnik et al., 2004). The distribution of such natural autoantibodies was further analysed at a monoclonal level in healthy donors and urticaria patients. To determine the distribution of rare antibodies, polyclonal B cell activation with limiting dilution assays and detection of specific antibodies are usually employed (Vischer et al., 1988; Crotty et al., 2003). As this approach is time consuming, a simplified screening test would be desirable. Detection of *Mycobacterium tuberculosis* or group B *Streptococcus*, using RT-PCR and real-time PCR, respectively, have become standard procedures and are approved by the US Food and Drug Administration (FDA) (Yang and Rothman, 2004). In recent years, these approaches have also become important to predict the clinical outcome of minimal residual disease in leukaemia. Junctional regions of immunoglobulin and T cell receptor gene rearrangements can be regarded as leukaemia-specific DNA fingerprints. Using PCR analysis of these junctional regions, it was possible to detect one leukaemic cell in a background of approximately 10^4 to 10^6 normal cells (Voena et al., 1997; Pongers-Willemse et al., 1998; Verhagen et al., 2000; Togel et al., 2002). These studies

indicated the feasibility of the detection of one single immunoglobulin sequence in a high background of other immunoglobulin sequences using PCR approaches. In this paper we report the detection of a single immunoglobulin heavy chain sequence of the natural anti-Fc ϵ RI α antibody LTM α 15 using semi-nested RT-PCR and real-time PCR approaches.

Specific primers for the semi-nested RT-PCR were identified by comparing the CDR regions of LTM α 15 V_H and V_L to other immunoglobulin sequences. Immunoglobulin sequences containing LTM α 15 like CDRs were analysed for potential primer annealing sites using a new bioinformatic program. Our results demonstrated that CDRs similar to LTM α 15V_L are abundantly used in the V_L repertoire, thereby preventing the specific detection of LTM α 15V_L using semi-nested RT-PCR. Interestingly, LTM α 15V_L belongs to the V λ 6a gene family, which normally represents 1.9%–4% of a healthy antibody repertoire (Ignatovich et al., 1999; Perfetti et al., 2002). Therefore, such an abundant usage of LTM α 15 like CDRV_L was not expected. Detailed analysis of our bioinformatic results demonstrated that immunoglobulin sequences containing LTM α 15 like CDRV_L were mostly isolated from SLE and primary systemic amyloidosis patients. As it was shown that the expression of the V λ 6a gene family is tenfold increased in such patients (Dorner et al., 1999; Comenzo et al., 2001; Perfetti et al., 2002), the observed abundant usage of LTM α 15 like CDRV_L in the V_L repertoire most probably represents a bias in the published immunoglobulin sequences. Using the same bioinformatic program a number of immunoglobulin sequences were identified containing CDR1 and CDR2 similar to those of LTM α 15V_H. This was expected as LTM α 15V_H is in germline configuration of the V_H3–23 family which is abundantly used in the human antibody repertoire (Brezinschek et al., 1997). Our bioinformatic analysis also revealed that no other immunoglobulin sequence contained a 100% identical CDR3 as that used in LTM α 15V_H. The CDR3 of LTM α 15V_H may therefore account for its antigen specificity. This is supported by other studies showing that the CDR3 of V_H is the key determinant of specificity in antigen recognition and is sufficient to obtain highly specific IgM antibodies to protein antigens (Xu and Davis, 2000; Mageed et al., 2001). However, the CDR3 of LTM α 15V_H consists of diversity (D) and joining (J) segments containing only three mutations and three randomly inserted nucleotides compared to germline. Parts of the CDR3 sequence of LTM α 15V_H are therefore also present in CDR3V_H of other immunoglobulin sequences using the same D and J segments. Analysis

of our bioinformatic results showed that only four nucleotides are exclusively present in the CDR3 of LTM α 15V_H. As it was shown that one mismatch at the 3' end of a primer sequence results in no amplification (Ma et al., 2003), we designed a reverse primer annealing with its 3' terminus to two of these four nucleotides of the CDR3 of LTM α 15V_H. This reverse primer was used together with a germline forward primer annealing in the CDR1, which was expected to amplify LTM α 15V_H, but not exclusively. Our semi-nested RT-PCR results demonstrated that the V_H germline family, which is present in the LTM α 15V_H (V_H3–23), was detected. However, in 16 out of 26 tested donors, different CDR3V_H than the LTM α 15V_H CDR3 were amplified indicating that the reverse primer was not specific enough to exclusively amplify the LTM α 15V_H sequence. Additionally, the successful detection of LTM α 15V_H sequence was not reproducible in all donors (data not shown). This may be explained by the kinetics of a PCR. Based on the assumption that in early cycles of the PCR a mispriming of the reverse primer occurs, templates containing the V_H germline family of LTM α 15V_H but also other CDR3V_H than LTM α 15V_H are exponentially amplified and can interfere with an efficient PCR of the LTM α 15V_H sequence. To increase specificity, we have performed a real-time quantitative PCR that provides a reliable method for rapid quantification of specific gene copies. This method has been successfully used to detect minimal residual disease in leukaemia (Pongers-Willems et al., 1998; Verhagen et al., 2000; Togel et al., 2002). In these studies, CDR3 regions of rearranged immunoglobulin, which contained several randomly inserted and mutated nucleotides compared to germline, were analysed. The design of either primers or conventional *TaqMan* probe to these randomly inserted and mutated nucleotides assured specificity. In contrast, the CDR3 region of LTM α 15V_H as mentioned above was only slightly mutated compared to the germline sequence. Thus, we used a novel fluorescent probe annealing to the four nucleotides of the CDR3V_H previously identified as unique for LTM α 15V_H by our bioinformatic program. This probe contains a minor groove binder (MGB) at its 3' end, which stabilises the complex DNA-probe by binding to the minor groove of DNA. Compared to ordinary *TaqMan* probes, this probe has an increased specificity and it has been demonstrated that, using such a probe, one single mismatch between the DNA template and the probe results in no amplification signal (Kutyavin et al., 2000). Such MGB *TaqMan* probes have been successfully used for single nucleotide polymorphism (SNP) analysis (de Kok et al., 2002), gene

expression analysis (Yoshitomi et al., 2003) and viral load determination (Lin et al., 2004). Moreover, in a parallel study we have demonstrated the use of an MBP probe for the detection of a particular anti-IgE sequence in 21 different donors (Vogel et al., 2004).

Our real-time PCR analysis revealed the presence of the LTM α 15V_H sequence in both healthy donors and urticaria patients, confirming our previous studies where this V_H sequence could be isolated from healthy donors and urticaria patients using phage display technology (Horn et al., 2001; Miescher et al., 2001; Pachlopnik et al., 2004). Moreover, our results showed that the rearranged gene copy number of LTM α 15V_H does not correlate with health status. The rearranged gene copy number can be even higher in healthy donors than in urticaria patients and can therefore not be used as a biomarker for urticaria. Furthermore, our kinetic experiments revealed that the gene copy number ranged up to four orders of magnitude difference in one single healthy donor over a period of more than 70 days. This finding is consistent with previous studies reporting that V_H family distribution varied with time and isotype in one individual, thus implying that repertoire fluctuation might occur during the lifetime of a healthy donor (Huang and Stollar, 1993; Wang and Stollar, 1999; Van Dijk-Hard and Lundkvist, 2002).

In summary, we have been able to detect one single natural autoantibody sequence in healthy donors and urticaria patients. Using a new bioinformatic program, we were able to determine a region in the CDR3V_H that is unique for our natural antibody sequence. An MGB *TaqMan* probe, which hybridises to this region was designed and allowed us to detect one single natural autoantibody sequence by real-time PCR. To the best of our knowledge, this is the first report describing the detection of a natural antibody sequence, which is in germline configuration. Therefore, our approach may be a model to monitor natural autoantibody sequences that are in germline configuration and to compare the gene copy number of such sequences in the human antibody repertoire at a monoclonal level.

References

- AppliedBiosystems, 1997. User Bulletin#2 ABI PRISM 7700 Sequence Detection System, User Bulletin#2 ABI PRISM 7700 Sequence Detection System.
- Brezinschek, H.P., Foster, S.J., Brezinschek, R.I., Dorner, T., Domiati-Saad, R., Lipsky, P.E., 1997. Analysis of the human V_H gene repertoire. Differential effects of selection and somatic hypermutation on human peripheral CD5(+)/IgM+ and CD5(-)/IgM+ B cells. *J. Clin. Invest.* 99, 2488.

- Comenzo, R.L., Zhang, Y., Martinez, C., Osman, K., Herrera, G.A., 2001. The tropism of organ involvement in primary systemic amyloidosis: contributions of Ig V(L) germ line gene use and clonal plasma cell burden. *Blood* 98, 714.
- Crotty, S., Felgner, P., Davies, H., Glidewell, J., Villarreal, L., Ahmed, R., 2003. Cutting edge: long-term B cell memory in humans after smallpox vaccination. *J. Immunol.* 171, 4969.
- de Kok, J.B., Wiegerinck, E.T., Giesendorf, B.A., Swinkels, D.W., 2002. Rapid genotyping of single nucleotide polymorphisms using novel minor groove binding DNA oligonucleotides (MGB probes). *Human Mutat.* 19, 554.
- Dorner, T., Farmer, N.L., Lipsky, P.E., 1999. Ig lambda and heavy chain gene usage in early untreated systemic lupus erythematosus suggests intensive B cell stimulation. *J. Immunol.* 163, 1027.
- Fiebiger, E., Maurer, D., Holub, H., Reininger, B., Hartmann, G., Woisetschlager, M., Kinet, J.P., Stingl, G., 1995. Serum IgG autoantibodies directed against the alpha chain of Fc epsilon RI: a selective marker and pathogenetic factor for a distinct subset of chronic urticaria patients? *J. Clin. Invest.* 96, 2606.
- Hide, M., Francis, D.M., Grattan, C.E., Hakimi, J., Kochan, J.P., Greaves, M.W., 1993. Autoantibodies against the high-affinity IgE receptor as a cause of histamine release in chronic urticaria. *N. Engl. J. Med.* 328, 1599.
- Hide, M., Francis, D.M., Grattan, C.E., Barr, R.M., Winkelmann, R.K., Greaves, M.W., 1994. The pathogenesis of chronic idiopathic urticaria: new evidence suggests an auto-immune basis and implications for treatment. *Clin. Exp. Allergy* 24, 624.
- Horn, M.P., Gerster, T., Ochsenberger, B., Derer, T., Kricek, F., Jouvin, M.H., Kinet, J.P., Tschernig, T., Vogel, M., Stadler, B.M., Miescher, S.M., 1999. Human anti-Fc epsilon RIalpha autoantibodies isolated from healthy donors cross-react with tetanus toxoid. *Eur. J. Immunol.* 29, 1139.
- Horn, M.P., Pachlopnik, J.M., Vogel, M., Dahinden, M., Wurm, F., Stadler, B.M., Miescher, S.M., 2001. Conditional autoimmunity mediated by human natural anti-Fc(epsilon)RIalpha autoantibodies? *FASEB J.* 15, 2268.
- Huang, C., Stollar, B.D., 1993. A majority of Ig H chain cDNA of normal human adult blood lymphocytes resembles cDNA for fetal Ig and natural autoantibodies. *J. Immunol.* 151, 5290.
- Huang, M.M., Arnheim, N., Goodman, M.F., 1992. Extension of base mispairs by *Taq* DNA polymerase: implications for single nucleotide discrimination in PCR. *Nucleic Acids Res.* 20, 4567.
- Ignatovich, O., Tomlinson, I.M., Popov, A.V., Bruggemann, M., Winter, G., 1999. Dominance of intrinsic genetic factors in shaping the human immunoglobulin Vlambda repertoire. *J. Mol. Biol.* 294, 457.
- Kutyavin, I.V., Afonina, I.A., Mills, A., Gorn, V.V., Lukhtanov, E.A., Belousov, E.S., Singer, M.J., Walburger, D.K., Lokhov, S.G., Gall, A.A., Dempcy, R., Reed, M.W., Meyer, R.B., Hedgpeth, J., 2000. 3'-Minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. *Nucleic Acids Res.* 28, 655.
- Lin, H.H., Wang, S.J., Liu, Y.C., Lee, S.S., Hwang, C.K., Chen, Y.S., Wann, S.R., Shih, Y.L., 2004. Quantitation of severe acute respiratory syndrome coronavirus genome by real-time polymerase chain reaction assay using minor groove binder DNA probe technology. *J. Microbiol. Immunol. Infect.* 37, 258.
- Ma, C.L., Fang, D.X., Chen, H.B., Li, F.Q., Jin, H.Y., Li, S.Q., Tan, W.G., 2003. A mutation specific polymerase chain reaction for detecting hepatitis B virus genome mutations at nt551. *World J. Gastroenterol.* 9, 509.
- Mageed, R.A., Harmer, I.J., Wynn, S.L., Moyes, S.P., Maziak, B.B., Bruggemann, M., MacKworth-Young, C.G., 2001. Rearrangement of the human heavy chain variable region gene V3–23 in transgenic mice generates antibodies reactive with a range of antigens on the basis of VHCDR3 and residues intrinsic to the heavy chain variable region. *Clin. Exp. Immunol.* 123, 1.
- Miescher, S.M., Horn, M.P., Pachlopnik, J.M., Baldi, L., Vogel, M., Stadler, B.M., 2001. Natural anti-Fc epsilon RIalpha autoantibodies isolated from healthy donors and chronic idiopathic urticaria patients reveal a restricted repertoire and autoreactivity on human basophils. *Hum. Antib.* 10, 119.
- Nasis, O., Thompson, S., Hong, T., Sherwood, M., Radcliffe, S., Jackson, L., Otevrel, T., 2004. Improvement in sensitivity of allele-specific PCR facilitates reliable noninvasive prenatal detection of cystic fibrosis. *Clin. Chem.* 50, 694.
- Pacheco, N., Mago, V., Gomez, I., Gueneau, P., Guelrud, M., Reyes, N., Pericchi, L.R., Dominguez-Bello, M.G., 2001. Comparison of PCR and common clinical tests for the diagnosis of *H. pylori* in dyspeptic patients. *Diagn. Microbiol. Infect. Dis.* 39, 207.
- Pachlopnik, J.M., Horn, M.P., Fux, M., Dahinden, M., Mandallaz, M., Schneeberger, D., Baldi, L., Vogel, M., Stadler, B.M., Miescher, S.M., 2004. Natural anti-Fc epsilon RIalpha autoantibodies may interfere with diagnostic tests for autoimmune urticaria. *J. Autoimmun.* 22, 43.
- Perfetti, V., Casarini, S., Palladini, G., Vignarelli, M.C., Klersy, C., Diegoli, M., Ascari, E., Merlini, G., 2002. Analysis of V(lambda)-J(lambda) expression in plasma cells from primary (AL) amyloidosis and normal bone marrow identifies 3r (lambdaIII) as a new amyloid-associated germline gene segment. *Blood* 100, 948.
- Pongers-Willems, M.J., Verhagen, O.J., Tibbe, G.J., Wijkhuijs, A.J., de Haas, V., Roovers, E., van der Schoot, C.E., van Dongen, J.J., 1998. Real-time quantitative PCR for the detection of minimal residual disease in acute lymphoblastic leukemia using junctional region specific *TaqMan* probes. *Leukemia* 12, 2006.
- Ruiz, M., Giudicelli, V., Ginestoux, C., Stoeck, P., Robinson, J., Bodmer, J., Marsh, S.G., Bontrop, R., Lemaître, M., Lefranc, G., Chaume, D., Lefranc, M.P., 2000. IMGT, the International Immunogenetics Database. *Nucleic Acids Res.* 28, 219.
- Sabroe, R.A., Fiebiger, E., Francis, D.M., Maurer, D., Seed, P.T., Grattan, C.E., Black, A.K., Stingl, G., Greaves, M.W., Barr, R.M., 2002. Classification of anti-Fc epsilon RI and anti-IgE autoantibodies in chronic idiopathic urticaria and correlation with disease severity. *J. Allergy Clin. Immunol.* 110, 492.
- Stein, L.D., Mungall, C., Shu, S., Caudy, M., Mangone, M., Day, A., Nickerson, E., Stajich, J.E., Harris, T.W., Arva, A., Lewis, S., 2002. The generic genome browser: a building block for a model organism system database. *Genome Res.* 12, 1599.
- Togel, F., Kroger, N., Koriath, F., Fehse, B., Zander, A.R., 2002. Molecular methods for detection and quantification of myeloma cells after bone marrow transplantation: comparison between real-time quantitative and nested PCR. *J. Hematother. Stem Cell Res.* 11, 971.
- Tomlinson, I.M., Walter, G., Marks, J.D., Llewellyn, M.B., Winter, G., 1992. The repertoire of human germline V_H sequences reveals about fifty groups of V_H segments with different hypervariable loops. *J. Mol. Biol.* 227, 776.
- Tomlinson, I.M., Cox, J.P., Gherardi, E., Lesk, A.M., Chothia, C., 1995. The structural repertoire of the human V kappa domain. *EMBO J.* 14, 4628.
- Van Dijk-Hard, I., Lundkvist, I., 2002. Long-term kinetics of adult human antibody repertoires. *Immunology* 107, 136.

- Verhagen, O.J., Willemse, M.J., Breunis, W.B., Wijkhuijs, A.J., Jacobs, D.C., Joosten, S.A., van Wering, E.R., van Dongen, J.J., van der Schoot, C.E., 2000. Application of germline IGH probes in real-time quantitative PCR for the detection of minimal residual disease in acute lymphoblastic leukemia. *Leukemia* 14, 1426.
- Vischer, T.L., Werner-Favre, C.F., Wen, L., Zubler, R.H., 1988. Quantitative analysis of precursors frequency of rheumatoid factor (RF) producing human B cells. *Scand. J. Rheumatol., Suppl.* 75, 123.
- Voena, C., Ladetto, M., Astolfi, M., Provan, D., Gribben, J.G., Boccadoro, M., Pileri, A., Corradini, P., 1997. A novel nested-PCR strategy for the detection of rearranged immunoglobulin heavy-chain genes in B cell tumors. *Leukemia* 11, 1793.
- Vogel, M., Tschopp, C., Bobrzynski, T., Fux, M., Stadler, M.B., Miescher, S.M., Stadler, B.M., 2004. A highly conserved interspecies V(H) in the human genome. *J. Mol. Biol.* 341, 477.
- Wang, X., Stollar, B.D., 1999. Immunoglobulin V_H gene expression in human aging. *Clin. Immunol.* 93, 132.
- Williams, S.C., Fripiat, J.P., Tomlinson, I.M., Ignatovich, O., Lefranc, M.P., Winter, G., 1996. Sequence and evolution of the human germline V lambda repertoire. *J. Mol. Biol.* 264, 220.
- Xu, J.L., Davis, M.M., 2000. Diversity in the CDR3 region of V(H) is sufficient for most antibody specificities. *Immunity* 13, 37.
- Yang, S., Rothman, R.E., 2004. PCR-based diagnostics for infectious diseases: uses, limitations, and future applications in acute-care settings. *Lancet, Infect. Dis.* 4, 337.
- Yoshitomi, K.J., Jinneman, K.C., Weagant, S.D., 2003. Optimization of a 3'-minor groove binder-DNA probe targeting the *uidA* gene for rapid identification of *Escherichia coli* O157:H7 using real-time PCR. *Mol. Cell. Probes* 17, 275.