V-Region and Class Specific RT-PCR Amplification of Human Immunoglobulin Heavy and Light Chain Genes from B-Cell Lines

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We have designed and tested primers that amplify complete human kappa and lambda light chain genes, and human Fd fragments from gamma, mu and alpha heavy chain genes. These primers were tested for efficiency and specificity on monoclonal sources of human immunoglobulin RNA, obtained from human B-cell lines of known immunoglobulin gene expression. Analysis of the sequences derived from these B-cells confirms the specificity of the PCR primers and the extent of somatic mutation seen in different B-cell malignancies supports existing concepts for differing aetiologies in the tumours concerned.

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INTRODUCTION

The production of combinatorial antibody libraries in bacteria is based on the efficient cloning of active immunoglobulin genes into bacterial expression vectors. This is usually achieved by using the polymerase chain reaction (PCR) to amplify complementary DNA (cDNA) of the active immunoglobulin genes with primers containing restriction sites which enables directional cloning into bacterial expression vectors [1–5]. The complexity and representation of the library achieved depends largely on the ability of the primers used to amplify a broad spectrum of immunoglobulin variable region genes. Increased representation within a library can also be achieved by increasing the number of classes of immunoglobulin genes included. In designing primers for repertoire cloning, we decided to examine the specificity of PCR primers used for the amplification of human active human immunoglobulin V-region genes, and to extend the number of immunoglobulin classes included. In order to be compatible with existing Fab phage display vectors [2, 3], primers were designed to amplify the major V_{κ} and V_{λ} gene families as intact light chains, and to amplify V_H gene families as Fd fragments from IgG, IgM and IgA. To test these primers we amplified and cloned several light and heavy chain immunoglobulin genes from clonal populations of B-cells. As a source of clonal B-cell populations to test our primers, we selected B-cell lines from a Burkitt's lymphoma

(Daudi) [6], two multiple myelomas (RPMI 8226 [7] and IM-9 [8]), an undifferentiated B-cell lymphoma (MC116) [9] and *in vitro* Epstein–Barr Virus (EBV) transformed B-cells (Dakiki) [10].

MATERIALS AND METHODS

Cell culture. Cell lines RPMI 8226 (CCL 155), IM-9 (CCL 159), Daudi (CCL 213), Dakiki (TIB 206) and MC116 (CRL 1649) were obtained from ATCC (Rockville, MD, USA) and grown in RPMI 1640, supplemented with 25 μ g/ml Gentamicin, 2 mM L-Glutamine and 20% FBS. Each cell line was grown until the number of cells exceeded 10^8 , at which point they were harvested, washed once in PBS, and then used for the preparation of RNA.

RNA extraction and cDNA synthesis. Total cellular RNA was extracted using standard techniques [11]. Briefly, 10^8 – 10^9 cells from each cell line were dissolved by homogenization in 10 ml 4M guanidine thiocyanate supplemented with 0.1% sarcosine and 80 μ l beta-mercaptoethanol. DNA was then sheared by 10 passes through a 21 gauge needle, followed by 10 passes through a 25 gauge needle. This cellular homogenate was then loaded onto a cesium chloride cushion (density = 1.62 g/ml), and centrifuged at 300 000 g for 16 h/20°C in a Beckman Ti50 rotor. Total RNA was recovered as a pellet, washed with ethanol, resuspended in 500 μ l DEPC-treated water, precipitated with 50 μ l of 3M sodium acetate and 1 ml of ethanol, resuspended in a final volume of 300 μ l and quantified by absorbance at 260 nm. Typical yields were 1–4 μ g/ μ l for a total of 300 μ g to 1.2 mg from 10^8 cells. 5–25 μ g of total RNA was denatured by incubation at room temperature (23°C) for 5 min with 1 mM methyl

Table 1. Primers used for PCR amplification of human immunoglobulin genes

Chain primer	Sequence	Specificity
Light	1a AGA TGT <u>GAGCTC</u> CAG ATG ACC CAG TCT CC	V _{κ1} &V _{κ4}
	1b CAG TGG <u>GAGCTC</u> GTG ATG ACT CAG TCT CC	V _{κ2} &V _{κ6}
	1c ACC GGA <u>GAGCTC</u> GTG TTG ACG CAG TCT CC	V _{κ3} &V _{κ5}
	2 GCG CCG <u>TCTAGA</u> ACT AAC ACT CTC CCC TGT TGA AGC TCT TTG TGA CGG GCG AAC TCA G	C _κ
	3a GCG ATC <u>GAGCTC</u> TCT GTG CTG ACT CAG CC	V _{λ1}
	3b TCC TGG <u>GAGCTC</u> TCT GCC CTG ACT CAG CC	V _{λ2}
	3c TCT GTG <u>GAGCTC</u> TAT GTG CTG ACT CAG CC	V _{λ3}
	3d TCT GTG <u>GAGCTC</u> TCT GAG CTG ACT CAG GA	V _{λ4}
	3e TCC AAT <u>GAGCTC</u> ACT GTG GTG ACT CAG GA	V _{λ7}
	4 GCG CCG <u>TCTAGA</u> CTA AGA ACA TTC TGC AGG GGC CA	C _λ
	5a G GTC CTG <u>CTCGAG</u> GTG CAG CTG GTG CAG TCT GG	V _{H1} &V _{H5}
	5b G GTC CTG <u>CTCGAG</u> GTG CAG CTG CAG GAG TCG GG	V _{H4}
	5c GTC CTG <u>CTCGAG</u> GTG CAG CTG GTG GAG TCT GG	V _{H3}
	5d GTC CTG <u>CTCGAG</u> GTC ACC TTG AAG GAG TCT GG	V _{H2}
Heavy	5e G GTC CTG <u>CTCGAG</u> GTG CAG CTA CAG CAG TGG GG	V _{H4-21}
	5f T GTC CTG <u>CTCGAG</u> GTA CAG CTG CAG CAG TCA GG	V _{H6}
	6a CAG AGT <u>ACTAGT</u> CTT GTC CAC CTT GGT GTT GCT	IgG
	6b GTG AGT <u>ACTAGT</u> ACA AGA TTT GGG CTC AAC T	IgG ₁
	6c CTC AGC <u>ACTAGT</u> TGG TAG AGG CAC GTT CTT TT	IgM
	6d CAG AGT <u>ACTAGT</u> TGG GCA GGG CAC AGT CAC AT	IgA

Restriction sites inserted for cloning are underlined.

mercury hydroxide. The RNA was then put on ice, mixed with the remaining reagents, then incubated for 1 h at 42°C. The reaction conditions were: 35 mM beta-mercaptoethanol; 1 mM each dNTP; 0.1 µg/µl random hexamers; 10 mM Tris HCl pH 8.8; 50 mM KCl; 6.5 mM MgCl₂; 0.01% Gelatin; 500 units/mL RNasin; 2 units/µl AMV RT. cDNA was used directly for PCR.

Polymerase chain reaction. Reaction volumes were 100 or 200 µl using 5 or 10 µl of a cDNA reaction for each PCR reaction. Conditions for PCR were 10 mM Tris-HCl pH 8.8; 50 mM KCl; 1.5 mM MgCl₂; 0.01% gelatin, 160 µM each dNTP, 2.5 µM each primer. Reaction parameters were 94°C/1 min; 45°C/2 min; 72°C/1 min, 40 cycles. The final cycle was followed by a 7 min/72°C primer extension phase.

The primers used for the PCR amplification of human immunoglobulin genes are shown in Table 1. Primers 1a (V_{κ1} and V_{κ4}), 1b (V_{κ2} and V_{κ6}) or 1c (V_{κ3} and V_{κ5}) were used with primer 2 in separate reactions for the amplification of full length kappa chains. Primer 2 was designed to incorporate a C to T mutation to eliminate a naturally occurring Sac-I site in human C_κ gene. Primers 3a(V_{λ1}), 3b (V_{λ2}), 3c (V_{λ3}), 3d (V_{λ4}) and 3e (V_{λ7}) were used with primer 4 to amplify full-length lambda immunoglobulin light chains.

Amplification of heavy chain Fd fragments was achieved using primers 5a through 5f with primers 6a (γ₁₋₄), 6b (γ₁), 6c (μ) or 6d (α₁ and α₂). Primer 5a was designed to recognize group I heavy chain variable (V_{H1}) domains, but due to the homology between groups V_{H1} and V_{H5}, should also recognize V_{H5} genes. Primer 5b was designed to amplify V_{H2} genes and primer 5c to recognize V_{H3} genes. Primers 5d and 5e were designed to amplify the V_{H4} family of genes, primer 5e being designed to recognize the V_{H4-21} group of germline sequences. Primer 5f was designed to recognize sequences derived from the single V_{H6} germline sequence.

Cloning. PCR products were separated by electrophoresis through a 1.5% agarose gel. Bands at approximately 650 bp were isolated using with DEAE paper [11], then digested with the appropriate restriction enzymes and cloned into pBluescript-II KS- (Stratagene, La Jolla, CA, USA) using standard techniques [11]. Clones containing inserts were sequenced using standard dideoxy techniques [11]. The heavy chain Fd fragment derived from cell line Dakiki was initially refractory to cloning. Post restriction digestion analysis of the PCR product revealed bands of approximately 500bp and 150bp. This Fd fragment was then cloned in two parts, as an Xho-I/Spe-I fragment of 140bp, and as an Spe-I/Spe-I fragment of 510bp, demonstrating that the additional Spe-I site was in the V_H region, which was confirmed by sequencing.

Database homology searches. The sequences described in this report were compared with the December 1994 update of genbank and EMBL databases. Genbank has assigned the following accession to the sequences described in this report: Cell line IM-9; IgG Fd: U07985; κ chain: U07989; Cell line RPMI 8226; λ Chain: U07992. Cell line Dakiki, IgA Fd: U07986. Cell line Daudi; IgM Fd: U07987, κ chain: U07990. Cell line MC116; IgM Fd: U07988, λ chain: U07991.

RESULTS

Immunoglobulin light chains

We were able to amplify four active light chain genes from the 5 B-cell lines tested, the sequences of which are shown in Table 2. Kappa light chains were amplified from cell lines Daudi and IM-9 with the V_{κ1} primer alone, and sequencing

confirmed that both V-region genes were from the $V_{\kappa 1}$ family. PCR amplification of lambda light chains using cDNA from MC116 RNA as a template gave positive reactions for $V_{\lambda 1}$ and $V_{\lambda 3}$, with the reaction for $V_{\lambda 1}$ being considerably stronger than that for $V_{\lambda 3}$. Using cDNA from RPMI 8226 RNA as a template, positive reactions were obtained with primers for $V_{\lambda 1}$ and $V_{\lambda 2}$, with the reaction for $V_{\lambda 1}$ being stronger than that for $V_{\lambda 2}$. Sequence analysis of both sequences revealed that they belong to the $V_{\lambda 2}$ family. The greater amplification using a $V_{\lambda 1}$ primer was therefore surprising and we failed to amplify immunoglobulin light chain from cDNA derived from Dakiki RNA. A positive PCR signal was obtained with primers designed to amplify only the C1 region and a positive signal corresponding to a full length lambda transcript was obtained on a northern blot probed with a C_{λ} probe. Additional primers were synthesized corresponding to variants of $V_{\lambda 1}$ and $V_{\lambda 4}$, but these also failed to amplify the full length lambda transcript. We conclude from these observations that the lambda light chain expressed in cell line Dakiki is significantly different at the site of our 5' V_{λ} PCR primers than any of the primers tested thus far.

The Daudi kappa chain contains a $J_{\kappa 4}$ sequence and the IM-9 light chain uses a $J_{\kappa 1}$ J-region. Cell lines MC-116 and RPMI8226 both utilize $J_{\lambda 2}$ derived J-regions and $C_{\lambda 2}$ constant regions.

Comparison of the nucleotide sequences showed that the sequence for the Daudi V_{κ} region differed from the previously reported sequence [12] at three nucleotides, one of which resulted in an amino acid change (a V to D change at a.a. 56). We have sequenced three clones of the Daudi V_{κ} from three separate cDNA syntheses; all three clones have the same sequence, indicating that the observed divergence from the previously reported sequence was real. It is not known whether these differences reflect sequencing error in the original report, or on-going somatic mutation in the cell line.

The Daudi $V_{\kappa 1}$ gene shows the greatest homology to germline gene L11 [13], with which it was only 84.6% identical. Comparison with rearranged $V_{\kappa 1}$ genes indicated that the Daudi $V_{\kappa 1}$ region shared less than 90% with all sequences on the database. The IM-9 $V_{\kappa 1}$ gene was 96.3% identical to germline $V_{\kappa 1}$ gene L12 [14], and 94.9% identical to germline gene $H_{\kappa 102}$ [15]. The MC116 V_{λ} region was highly homologous (89–96%) to many rearranged $V_{\lambda 2}$ genes, but was most closely related to one germline $V_{\lambda 2}$ sequence (DPL13) [16], with which it shared 96% homology.

The RPMI8226 $V_{\lambda 2}$ gene showed 91.4% identity to the same germline $V_{\lambda 2}$ gene to which the $V_{\lambda 2}$ gene cloned from cell line MC116 (DPL13) [16] was most closely related. Given the limited homology of the RPMI8226 $V_{\lambda 2}$ gene to DPL13, it is possible that this gene is related to a previously undescribed germline $V_{\lambda 2}$ gene. The RPMI8226 $V_{\lambda 2}$ gene was, however, completely identical to a rearranged $V_{\lambda 2}$ gene ($V_{\lambda 001}$) [17]. It is remarkable that two rearranged genes from separate B-cell lines should have identical $V_{\lambda 2}$ -J $_{\lambda 2}$ sequences, particularly when the V_{λ} sequence concerned has diverged from the

nearest known germline gene by almost 9% (25 altered nucleotides), possibly indicating cross-contamination of the two cell lines.

Immunoglobulin heavy chains

Four immunoglobulin heavy chain Fd fragments were successfully amplified and cloned, the sequences of which are shown in Table 3. An IgG₁ Fd fragment was cloned from IM-9, an IgA₁ Fd fragment was cloned from Dakiki, and IgM Fd fragments were cloned from cell lines Daudi and MC116. No attempt was made to amplify Fd fragments from cell line RPMI 8226 on the basis of previous reports of light chain expression only [7]. The active VH domains from cell lines Daudi, Dakiki and IM-9 are from the V_{H3} family, while that from cell line MC116 is from the V_{H1} family. Under the conditions employed, there was little cross-recognition of V_H regions by primers designed to amplify different V_H regions. Using cDNA derived from Dakiki, we obtained specific amplification with primers 5c and 6d (V_{H3} /IgA), using cDNA from IM-9, amplification was only observed with primers 5c and 6a (V_{H3} /IgG), and using cDNA derived from Daudi amplification was only seen with primers 5c and 6c (V_{H3} /IgM). Only in the case of MC116 was some reactivity seen with primers 5c and 6d on a V_{H1} template, in addition to amplification with primers 5a and 6d. Primers 6a and 6b both amplified an IgG₁ Fd fragment from IM-9 derived cDNA with primer 5c. Both PCR products were cloned and sequenced, and both were identical, with the exception of the 3' end where the clone obtained using primer 6b contained the N-terminal part of the IgG₁ hinge as expected. Analysis of J-gene usage showed that three of the four heavy chains cloned (Daudi, Dakiki and MC116) contained J_{H4} related J-regions, while the IM-9 heavy chain contained a J_{H3} related sequence.

The V_{H3} gene from cell line Daudi was most closely related to human germline gene DP53 [18]/H11 [19], showing 84% homology, but was almost as related (80–82% homologous) to germline genes DP87 [20], DP58 [18], HHG19 [21] and DP54 [18], as well as numerous rearranged genes. The V_{H3} gene cloned from IM-9 was 94.6% identical to germline gene DP31 [18], and not closely related to any other germline V_H gene. The Dakiki V_{H3} gene was 93% homologous to three germline genes DP47 [18], V-B19.7 [22] and VH26 [23], and was more than 90% homologous to many rearranged V_H gene sequences. The V_{H1} gene cloned from MC116 was closely related to several germline genes: it showed 97.3% and 96.9% identity to germline genes DP75 and DP8, respectively [18], and more than 90% identical to two other germline genes, as well as five rearranged genes.

DISCUSSION

We have designed oligonucleotide PCR primers which allow amplification of human kappa and lambda light chains, and

alpha, gamma and mu heavy chain Fd fragments. The kappa light chain primers were efficient in amplifying $V_{\kappa 1}$ containing kappa chain genes, and are similar to previously described primers [5, 24]. We were able to amplify two of the three lambda light chains we attempted, but not necessarily with the primers predicted, indicating that further work may be required to optimize the site and/or design of V_{λ} primers. Amplification of heavy chain Fd fragments was specific, both for class of heavy chain and for family of V_H region. We had tried without success to amplify the active heavy chain Fd fragments with previously described V_H region primers [5], and arrived at the present V_H region sequences by trial and error. The strategy used in the placement of the Xho-I site, and the overall V_H region primer sequences are similar, although not identical, to recently published V_H region primers [25].

The primers we described have been designed to be compatible with previously described vectors for the display of Fab fragments on the surface of filamentous phage. While we have improved the primers available to work with these vectors and expanded the number available to include both IgM and IgA, there are some additional questions that have arisen. Specifically, the discovery of an internal Spe-I restriction site in one of four heavy chain variable region genes we cloned clearly indicates that any Spe-I site containing genes would clearly not be represented in an antibody library constructed using Spe-I. This, in turn, suggests that the use of Spe-I for antibody library construction is less than ideal. An additional question exists regarding the feasibility of cloning of IgM Fd genes and their expression as Fab fragments, due to the lack of a simple hinge in IgM. While it has been demonstrated that such a molecule can be expressed using a flexible linker to join the heavy and light chains [26], it may also be possible to modify the IgM reverse primer (6c in Table 1) to encode the sequence PCP (as with the IgA primer 6d) in place of PLP, thereby allowing the Fd fragment to covalently link to the light chain. We have tested such a primer and found it to amplify IgM Fd fragments with the same efficiency as primer 6c.

From the immunoglobulin sequences that we obtained from these B-cell lines, there are clearly differences in the extent to which the different genes have varied from known germline genes. We were also able to compare the extent of variation of V_H and V_L genes from the same B-cell line for three of the cell lines we used. The genes from MC116 (derived from an undifferentiated lymphoma) were closest to germline sequences. The V_H gene was closely related (97.3% and 96.9% identical) to two V_{H1} germline genes, and the V_{λ} gene was 96% homologous to a germline V_{λ} gene. Similarly, the V-region genes from multiple myeloma-derived cell-line IM-9 were 94.6% (V_{H3}) and 96.3% ($V_{\kappa 1}$) homologous to the nearest germline genes. In contrast, the V region gene sequences from Burkitt's lymphoma derived cell line Daudi were highly divergent from both germline and rearranged gene sequences. Both V_{H3} and $V_{\kappa 1}$ genes were

approximately 84% homologous to the nearest germline sequences, and almost equally distant from the most homologous rearranged genes. This lack of homology could indicate extensive somatic mutation, which would be unusual for a cell that had not undergone the class switching from IgM to IgG (or IgA) normally thought to be associated with somatic mutation of active immunoglobulin genes. A recent report has demonstrated ongoing intraclonal variation within a follicular lymphoma expressing an IgM immunoglobulin [27], thought to be due to ongoing somatic mutation driven by antigen. Although no such observation has been made in Burkitt's lymphoma, it is clearly possible for extensive somatic mutation to occur in the absence of class switching. A possible alternative explanation is that the germline genes from which the Daudi sequences were derived have not yet been characterized.

Overall, the sequences derived from these B-cell malignancies support previous observations suggesting that Burkitt's lymphomas are derived through malaria-parasite-antigen driven mechanisms [28] and that VDJ regions from multiple myelomas contain somatic mutations [29]. Our observations on the active immunoglobulin genes of MC116 suggest that undifferentiated B-cell lymphomas have limited somatic mutation of their immunoglobulin genes, which would argue against an antigen driven mechanism of transformation in these tumors. The extent to which both V_H and V_L genes show the same degree of divergence from the nearest germline V-region genes suggests that the mechanisms of somatic mutation that give rise to these changes act in parallel on both heavy and light chain variable region genes.

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