

A Mouse with a Monoclonal Primary Immunoglobulin Repertoire not Further Diversified by V-Gene Replacement

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(Revised 01 December, 1998; In final form 09 December, 1998)

We have generated a monoclonal B-cell mouse by introducing homozygous, nonfunctional RAG-2 alleles and a $\lambda 1$ light-chain transgene into the quasi-monoclonal (QM) mouse, which contains a "knocked-in" V_HDJ_H rearrangement. Thus, this mouse, which we call MonoB, is devoid of T cells and contains preformed heavy- and light-chain genes encoding immunoglobulin with an anti-NP specificity. The MonoB mouse allows us to examine immunoglobulin diversity in the absence of processes mediated by V(D)J recombination and T cells. Here we report that not only is the MonoB's primary immunoglobulin repertoire monoclonal, but also that its secondary repertoire is not further diversified by V-gene replacement or gene conversion. Among 99 heavy-chain and 41 λ light-chain genes from peripheral B cells of the MonoB mouse, there were no V-gene replacements. When compared to the QM mouse, which has RAG activity, and for which V-gene replacement is the major diversifying mechanism, these data suggest that V-gene replacement is mediated by V(D)J recombination and not by other recombination systems.

Keywords: MonoB mouse, QM mouse, RAG, transgenic, knockout, knockin, flow cytometry

INTRODUCTION

V_H replacement, as originally described *in vitro* (Kleinfield et al., 1986; Reth et al., 1986; Beck-Engeser et al., 1987; Kleinfield and Weigert, 1989; Shirasawa et al., 1992) and in acute lymphoblastic leukemia (Steenbergen et al., 1993), can generate a heavy (H) chain with a different specificity (Kleinfield et al., 1986; Reth et al., 1986; Cascalho et al.,

1996) or destroy a productive gene (Taki et al., 1995). V_H replacement is thought to be mediated by a heptamer recombination signal sequence (RSS) embedded near the 3' end of many V_H 's (Covey et al., 1990; Usuda et al., 1992; Chen et al., 1995; Fanning et al., 1998) and by a putative nonamer sequence found ca. 12 bp upstream in the transgene (Chen et al., 1995). Because V-gene replacement shares the same RSS as V(D)J recombination, it has been proposed that the two share the same mechanism (Covey et al., 1990;

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Usuda *et al.*, 1992; Chen *et al.*, 1995; Fanning *et al.*, 1998).

In order to determine whether V-gene replacement is dependent on V(D)J recombinase activity, we bred a mouse with preformed IgH and IgL genes onto a RAG-knockout background. For the heavy- (H-) chain locus, we used the quasi-monoclonal (QM) mouse, which is heterozygous for two gene-targeted alleles: On one homologue is a targeted insertion in which the stretch of DNA containing the J_H segments has been replaced by a rearranged V_HDJ_H exon encoding an H-chain V region bearing the 17.2.25 idiotope (id) (Cascalho *et al.*, 1996); on the other homologue is a nonfunctional "knockout" in which all J_H gene segments have been deleted (Chen *et al.*, 1993a). The J_K stretch has been knocked out on both homologues (Chen *et al.*, 1993b), and, as a consequence, QM mice produce only λ -type light (L) chains. We bred the QM mouse to the RAG-2-deficient strain (Shinkai *et al.*, 1992) to generate a mouse homozygous for a disrupted RAG-2 allele and containing the inserted 17.2.25 V_HDJ_H exon. As the absence of RAG expression also prevents V to J rearrangement at the λ L-chain locus, we introduced a $\lambda 1$ transgene (Young *et al.*, 1994). The absence of RAG-2 expression also means that the resulting mouse lacks T cells. This mouse, which we call MonoB, has a nominally monoclonal primary B-cell repertoire with a B-cell receptor (BCR) consisting of an H chain encoded by an inserted V_HDJ_H (with a constant region) and the $\lambda 1$ transgene; this BCR is specific for the hapten NP [(4-hydroxy-3-nitrophenyl) acetyl] (Cascalho *et al.*, 1996).

We report here experiments to investigate whether the secondary repertoire of the MonoB mouse becomes diversified by V-gene replacement, gene conversion, or any other process involving transfer of donor sequences.

RESULTS

Flow Cytometric Analysis

Figure 1 shows flow-cytometric profiles of peripheral blood lymphocytes (PBL) from MonoB, QM, and RAG-2^{-/-} mice stained with an anti-idiotypic antibody

(Cascalho *et al.*, 1996) specific for the H chain encoded by the inserted 17.2.25 V_HDJ_H and either anti-B220 (Figs. 1A-1C) or anti- μ^a (Figs. 1D-1F), Figure 2 shows flow-cytometric profiles of peripheral blood lymphocytes from MonoB, QM, and RAG-2^{-/-} mice stained with an anti- λ chain antibody (Cascalho *et al.*, 1996) and either anti-B220 (Figs. 2A and 2B) or anti-CD19 (Figs. 2C to 2E).

As can also be seen in Fig. 1, there are no apparent μ^a -negative, id-positive (switched) cells in the MonoB mouse (Fig. 1D), although as expected, the QM mouse has switched cells (Fig. 1E). Analysis of serum Ig also failed to detect H-chain isotypes other than IgM (data not shown).

As shown in Fig. 1 and 2, well over 20% of the peripheral B cells in MonoB mice do not have the idiotypic BCR. About 30% of the B220-positive (Fig. 1A) as well as the μ^a -positive (Fig. 1D) cells are id-negative; about 25% of the B220-positive (Fig. 2A) and CD19-positive (Fig. 2C) cells are λ -negative. In QM mice, about 15% of the B220-positive (Fig. 1B) and μ^a -positive (Fig. 1E) cells are id-negative, and only a few (<3%) of the B220-positive (Fig. 2B) or CD19-positive (Fig. 2D) are λ -negative. As the number of B220-positive cells in the periphery of MonoB is about one-third that of QM mice, the frequencies given in Figs. 1 and 2 mean that there are more $\lambda 1$ -negative but about the same or fewer id-negative cells in the PBL of MonoB than in those of QM mice.

As shown in Fig. 3, 7.6% of MonoB and 1.4% of QM PBL were B220-positive, CD43-positive. These cells are immature B-lineage cells, and they stain only dimly for B220 in both strains; as they mature, they will lose CD43 and increase the amount of B220 on their surface. But the id-negative and λ -negative populations of Figs. 1 and 2 stain brightly with anti-B220, so they are not likely to be immature B lymphocytes that have not yet expressed their BCR.

Immunoglobulin RNA and DNA Sequence Analysis of Id-Negative and λ -Negative Cells

Table I gives a summary of Ig cDNA and genomic DNA sequences cloned from sorted id-negative and

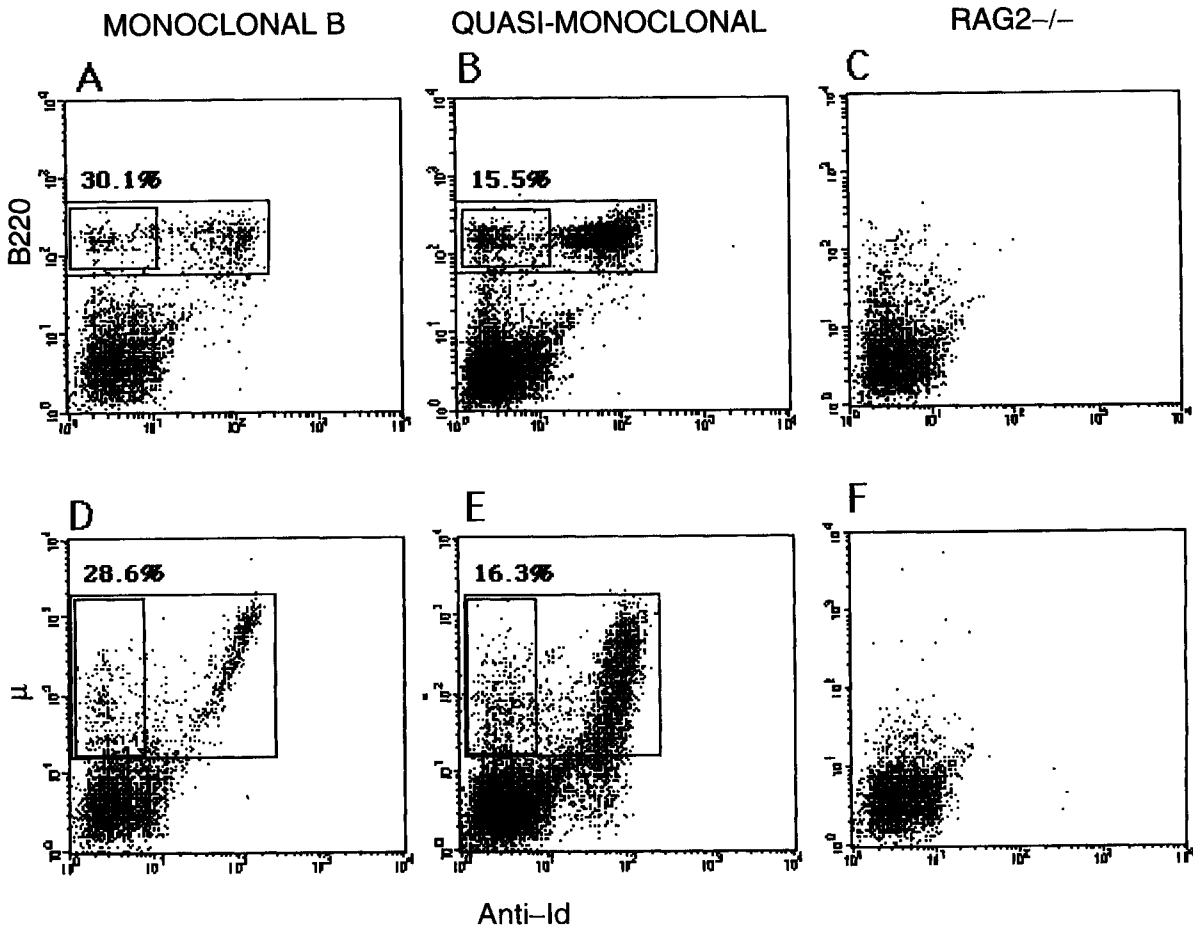


FIGURE 1 Flow-cytometric analysis of peripheral blood cells from MonoB (A, D), QM (B, E), and RAG-2^{-/-} mice (C, F). y axis; PE-conjugated anti-B220 monoclonal antibody (A-C) or anti-μ^a (D-F) x-axis: FITC-conjugated anti-idiotype 17.2.25 (anti-id) monoclonal antibody

from λ-negative cells as well as Ig cDNA sequences cloned from LPS-stimulated cells. All 140 sequences were clearly the 17.2.25 V_HDJ_H or λ1, the knocked-in H-chain and transgenic L-chain genes present in the germline of MonoB mice, respectively. That is, there were no V-gene replacements. For the expressed λ-chain transgene, this is not surprising, for there are no likely donors for its replacement. But the frequency of V-gene replacements in the id-negative fraction of the QM mouse was previously found to be almost 100% (Cascalho et al., 1996).

There was also no evidence of gene conversion among the 140 variable-region gene segments sequenced. Moreover, in the same cDNA preparation and using the same reagents (except for the primers), there were more mutations in the V_HDJ_H segment than in a similarly sized C_μ1-2 segment – 41 nucleotide changes in 6496 bases of the V_HDJ_H segment vs. 5 nucleotide changes in 7935 bases of the C_μ1-2 segment. Nevertheless, because of the large number of PCR cycles used, an accurate assessment of the mutant frequency would require more extensive methodological controls.

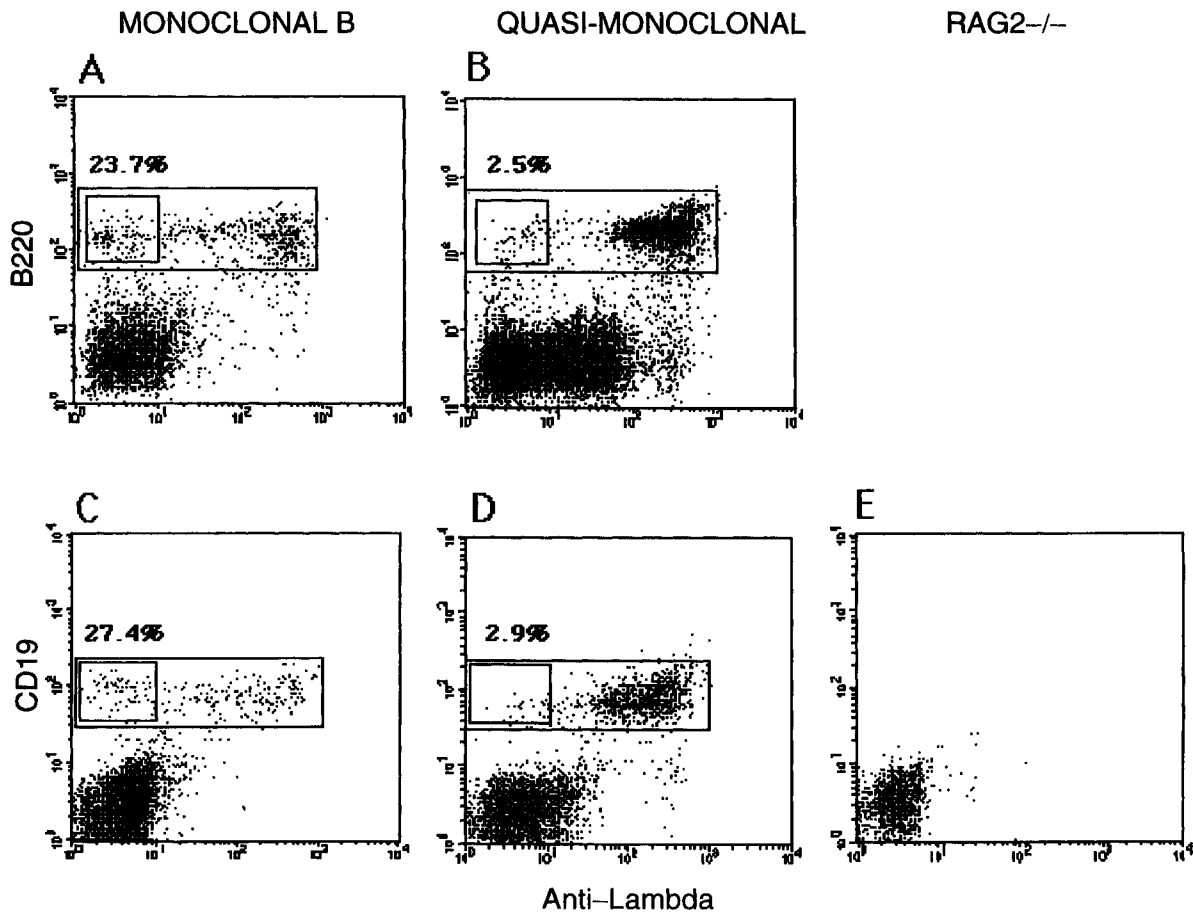


FIGURE 2 Flow-cytometric analysis of peripheral blood cells from MonoB (A, C), QM (B, D), and RAG-2^{-/-} mice (E). y axis; PE-conjugated anti-B220 monoclonal antibody (A-B); or anti-CD19 (C-E). x-axis; FITC-conjugated anti- λ monoclonal antibody, which reacts with λ 1 and λ 2 L chains

DISCUSSION

What Are the Idiotype-Negative B Lymphocytes in MonoB Mice?

The high frequency of B-lineage cells lacking the idiotypic BCR of MonoB mice remains a puzzle. We have experimentally excluded that their lack of idiotype results from its loss by V-gene replacement, gene conversion, or any other process involving transfer of donor sequences. Moreover, we have experimentally excluded that these cells are immature B cells that

have not yet expressed their BCR. Although we have not formally excluded the possibility that the lack of reaction with anti-idiotypic and anti- λ antibodies is due to somatic hypermutation, the mutation frequency is not high enough to make this a likely possibility unless the loss of idiotype per se confers a selective advantage.

It is possible that the relevant epitopes in these cells are masked by cross-reactive antigen(s), perhaps endogenous, perhaps environmental. Under normal circumstances, B cells with bound antigen home to the secondary lymphoid organs and, thus, are taken

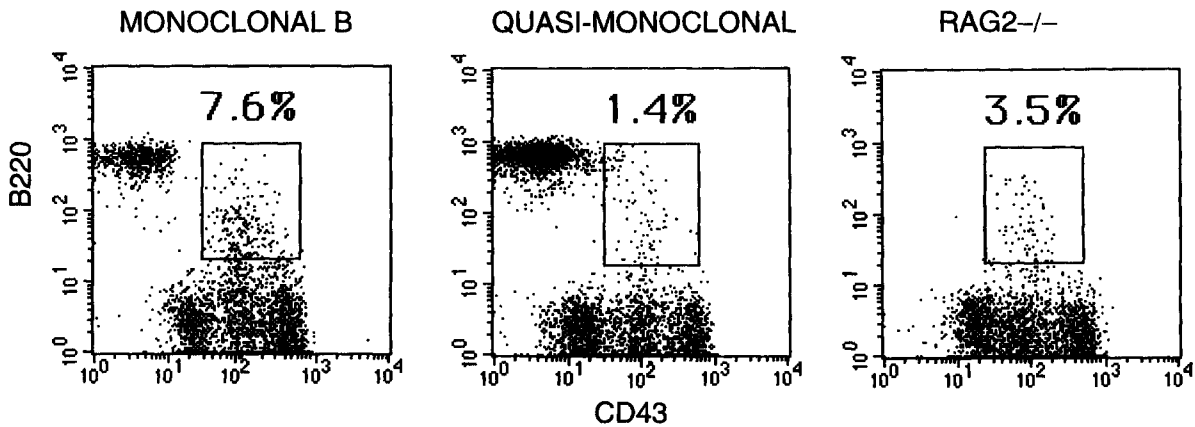


FIGURE 3 Flow-cytometric analysis of peripheral blood cells from MonoB, QM, and RAG 2^{-/-} mice. y-axis; PE-conjugated anti-B220 monoclonal antibody. x axis: FITC-conjugated anti-CD43

out of the peripheral blood. Because T cells are important in promoting the germinal center reaction (Jacob and Kelsoe, 1992; MacLennan, 1994), the absence of T cells in MonoB mice might compromise the homing of lymphocytes that have bound antigen, thus leaving many of them circulating in the peripheral blood with masked λ and id epitopes. Because reacting MonoB PBL with the cognate hapten bound to a carrier abrogated the binding of the anti-id antibody (M. Madan, unpublished), the masked epitope notion is our working hypothesis.

Consistent with this hypothesis is the lack of cells with switched isotypes and the lack of circulating antibody other than IgM in MonoB mice, because switching also takes place in germinal centers. Interestingly, there are switched isotypes in a similar strain with another inserted V_HDJ_H, B1-8, and the same λ 1 transgene on a RAG-2-deficient background (Lansford et al., 1998). As the only known significant difference between the two strains is the sequence of the inserted V_HDJ_H gene segment and, as a consequence, the fine specificity of the BCR (White-Scharf and Imanishi-Kari, 1982; David et al., 1992), it is credible that the difference in switching behavior is due to antigen binding.

Why Is There No Diversification by Transfer of Donor Sequences in MonoB Mice ?

In the QM mouse and other gene-targeted mice (Chen et al., 1995; Taki et al., 1995; Cascalho et al., 1996; 1997), V-gene replacements are strongly selected and most new BCRs are generated by this mechanism. The selective forces must be at least as great in the MonoB mouse, which has an even smaller primary repertoire. Here we have shown that the primary repertoire of MonoB mice is not further diversified – by V-gene replacement, gene conversion, or any other process involving transfer of donor sequences – at a frequency detectable by current methods, even in a system with strong selective pressures. The interpretation of this result is complicated by the fact that the absence of RAG-2 expression in MonoB mice affects B cells in two ways – directly through the absence of V(D)J rearrangement and indirectly through the absence of T cells.

Whatever the nature of the cells lacking the idiotypic BCR of MonoB mice, any cells with V-gene replacement at the H-chain locus would be found in the id-negative fraction (Cascalho et al., 1997). Among 83 V_HDJ_H segments sequenced from the id-negative population, there were no replacements. As the id-negative population used was 25% to 30%

of all B cells, we have effectively sampled about 300 cells, which means that at the 95% confidence level, there are less than 1% replacements. That is, the frequency of V-gene replacement is at least 100-fold lower than in the QM mouse. As it is not likely that the absence of T cells could result in such a large depression of the replacement frequency, the absence of V-gene replacement must be, at least in part, a direct consequence of the absence of V(D)J recombination.

Gene conversion, another diversifying mechanism that is common in chickens and rabbits (Reynaud *et al.*, 1987; Knight and Becker, 1990; David *et al.*, 1992; Takeda *et al.*, 1992), was also not observed. If it occurs at all in mouse germinal-center B cells, it is very rare (Ford *et al.*, 1994). From our results, it is not clear whether its absence is due to dependence on RAG-2, T cells, or its rarity.

MATERIALS AND METHODS

Mice Strains and Breeding

The MonoB mouse was generated by breeding three strains: the QM mouse (Cascalho *et al.*, 1996), a $\lambda 1$ transgenic (Young *et al.*, 1994), and a RAG-2^{-/-} (Shinkai *et al.*, 1992). After appropriate breeding, mice were screened by flow cytometry for the absence of T cells in peripheral blood lymphocytes (PBL) with an anti-CD3 antibody, a kind gift of Phillipe Poussier (Toronto), and for the presence of idiotype 17.2.25 (id) positive B cells with antibody R2.438.8, a kind gift of Thereza Imanishi-Kari (Tufts), who produced it; a detailed characterization of R2.438.8 has not yet been published. Expression of λ in PBL was also verified by immunofluorescence on cytospin slides or in flow cytometry with a goat anti-mouse λ polyclonal antibody (Pharmingen 01274D). In some breedings, the presence of the $\lambda 1$ transgene and the 17.2.25

H-chain gene was detected by PCR analysis of tail DNA. For $\lambda 1$, the primers and conditions are described in what follows in the cloning strategy; for H chain, see reference Cascalho *et al.* (1997). The genotype of each MonoB mouse was verified by staining peripheral blood lymphocytes (PBL) and PCR-based sequencing of the $\lambda 1$ transgene.

Cell Sorting and Flow Cytometry

Cells were analyzed with a Becton Dickinson (BD) FACScan or FACSCalibur and sorted with a FACStar or FACStarplus; data were analyzed with the BD Cell Quest program. PBL were prepared from total blood by hemolyzing erythrocytes with lysis buffer (8.2 g NH₄Cl, 1 g KHCO₃, 200 μ l 0.5 M EDTA, pH 8.0, in 1 liter), washed, and stained by incubating them with antibodies for 30 min at 4°C before analysis and/or sorting. Dead cells were excluded by propidium iodide staining or with Via-Probe (Pharmingen 34321X 7-AAD). The following fluorescent antibodies were used for staining cells: phycoerythrin (PE) conjugated rat anti-B220 (Caltag RM2604), PE-conjugated mouse anti-B220 (mAb14.8, Pharmingen 01975B), PE-conjugated rat monoclonal anti-CD19 (mAb ID3, Pharmingen 09655B), PE-conjugated mouse monoclonal anti- μ^a (Pharmingen 05095B), monoclonal fluorescein isothiocyanate (FITC) coupled anti-17.2.25 V_HDJ_H anti-idiotypic mAb R2.438.8, FITC-conjugated anti- λ (Pharmingen 01274D), which reacts with $\lambda 1$ and $\lambda 2$.

LPS-Stimulated Spleen-Cell Cultures

Spleen-cell suspensions, prepared by standard methods, were placed in 24-well plates at 70,000 cell/well in RPMI, 10% FCS, and 10 μ g/ml LPS. Six days later, wells were harvested, and clones were generated for sequencing as described in what follows for V_HDJ_H and C _{μ} 1–2 cDNA.

TABLE I Summary of MonoB H and L-Chain Gene Segments Sequenced

Cells ^a	Ig chain	Genomic or cDNA	Clones sequenced	Total bp sequenced	V-gene replacement
Sorted B220 ⁺ id ⁻ or μ^+ id ⁻ PBL	H	cDNA	45	12,720	0
Sorted μ^+ id ⁻ PBL (LPS-activated spleen)	H(H)	Genomic (cDNA)	38 (16)	13,300 (4,165)	0 (0)
Totals	H		99	30,185	0
Sorted μ^+ id ⁻ PBL or B220 ⁺ id ⁻ PBL	λ 1	cDNA	21	7,670	0
Sorted B220 ⁺ λ PBL	λ 1	cDNA	20	7,551	0
Totals	λ 1		41	15,221	0

id⁻: cells not stained by anti-idiotype 17.2.25 monoclonal antibody (see Fig. 1). PBL: peripheral blood lymphocytes.

Preparation of cDNA and DNA and amplification by PCR

An Oligotex direct mRNA kit (Quiagen 72012) was used to prepare mRNA, which was reverse transcribed with Superscript (Gibco-BRL 180-014) to obtain cDNA. The synthesis was primed with oligo d(T)₁₂₋₁₈ (Gibco-BRL 18418-012). For DNA, a published direct-cell lysate procedure was used (Pennycook et al., 1997). For both DNA and cDNA, a partial V_HDJ_H was amplified by PCR with 5 pmol V_H 5' primer AGGTC/ga/cAa/gCTGCAGc/gAGTCa/tGG (Orlandi et al., 1989) and J_H4 3' primer GAG-GAGACGGTGACTGAGGTTCCCTTG ; λ 1 cDNA was amplified with V _{λ 1} 5 primer GGAATTCCTGCACTCACCATCACCTGG and C _{λ 1} 3' primer GTGGATCCTACCTTCCAGTCCACTGTCCACC; C _{μ} cDNA was amplified with C _{μ 1} 5' primer GAGTCAGTCCTTCCCAAATG and C _{μ 2} 3' primer CTCGATGGTCACCGGATCTG. Reactions were done in 50 μ l total volume with 2.5 U Taq polymerase (BMB 1146173), 200 μ M of dNTPs 1X PCR buffer, with 1.8 mM MgCl₂ for the V_HDJ_H segment, and 1.5 mM MgCl₂ for the λ 1 gene and C _{μ} gene segment. For the V_HDJ_H and C _{μ} gene segments, the program ran 30 cycles, each 30 sec, 94°C; 1 min, 60°C; 2 min, 72°C; and for λ 40 cycles, each 1 min, 94°C; 1 min, 59°C; and 2 min, 72°C. The last cycle included a prolonged extension step (10 min). The PCR fragments were cloned into pCR 2.1 (Invitrogen K2000-01).

Sequencing

Double-stranded DNA was prepared and clones were screened for the presence of the insert by digestion with EcoRI. Positive clones were sequenced with the Sequenase kit (USB 70770) and the T7 Sequencing Kit (Pharmacia Biotech 27-1682-01) and universal or reverse primers.

Acknowledgements

We are indebted to Claude Cantin for flow cytometry and cell sorting and to Charley Steinberg for help with the manuscript. This work was supported by NIH grant R01 AI41570, by the National Cancer Institute of Canada Terry Fox Marathon of Hope, by the Medical Research Council of Canada (MRC), by an Engalitcheff Award of the Arthritis Foundation, by the Leukemia Research Fund of Canada, by funds from the Markey Trust, by a Howard Hughes Transgenic Mouse Grant, and by a grant from the Junta Nacional de Investigaç o Cient fica e Tecnol gica – Praxis XXI-BD 3763/94 to MC. DM is the recipient of a MRC studentship; GEW is an MRC Scientist.

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