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Immunology

Analysis of somatic hypermutation in mouse Peyer's patches using immunoglobulin κ light-chain transgenes

(antibody transgenes/germinal centers/mutation hotspots/gene conversion)

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ABSTRACT We have exploited mice transgenic for an immunoglobulin κ light chain in order to show that immunoglobulin genes in the B cells of Peyer's patches in unimmunized mice carry a high level of somatic mutations. Most of the mutations are found in the subpopulation of B cells which, based on peanut agglutinin binding, derive from the germinal centers. The number of mutations per clone and their distribution along the variable gene segment (indicative of untemplated point mutations) are very similar to those found in antigen-specific splenic B cells of normal mice after secondary immunization. The mutations accumulate mainly in complementarity-determining region 1, in particular in some specific codons (Ser-26, Ser-31, and Ser-77) which have been previously recognized as intrinsic hypermutational hotspots. These results suggest that, as in the spleen, somatic mutation occurs in B cells which have migrated to the germinal centers, probably as a consequence of stimulation by antigens present in the gut environment. Transgenic animals are increasingly being used to define the signals involved in hypermutation. However, their subsequent study is very time-consuming because it is based on immunization and analysis of hybridomas or antigen-selected cells. We propose that the use of Peyer's patches of unimmunized adult mice offers a reliable and simple approach to analyze hypermutation of transgenes.

It has been described that Peyer's patches (PPs), gut-associated lymph follicles along the small intestine, are areas that undergo chronic stimulation of lymphoid cells (1). Unlike those in the spleen, PP germinal centers are found in the absence of specific immunization, probably due to stimulation by naturally occurring antigens present in the gut environment (food, bacteria, etc) (1, 2). Germinal centers have been described to play a major role in the expansion and selection of antigen-specific B cells, in the hypermutation of immunoglobulin genes, and in the production of memory cells (3–5). As B lymphocytes from germinal centers bind high levels of peanut agglutinin (PNA), binding of this plant lectin is used as a marker of germinal center B cells (2, 6).

It has been shown that B lymphocytes from PPs of unimmunized adult sheep accumulate somatic point mutations, and this led to the suggestion that PPs perform a role as primary lymphoid organs (7). To test whether the same is true in mice, we decided to study germinal-center B cells from PPs of transgenic LK3 mice. The transgene contains a light-chain V_κOx1–J_κ5 variable–joining gene combination [predominantly found in the response to the hapten 2-phenyloxazolone (8, 9)], linked to a rat κ constant-region (C_κ) gene segment (10, 11). Previous analysis of hybridomas or specific B cells from immunized LK3 animals revealed that the transgene undergoes levels of somatic mutation similar to those of the endogenous κ chains, even when not forming part of the antibody (passenger transgenes) (10, 12). Here we use the transgenic animals to demonstrate hypermutation in the PPs of unimmunized animals. The mutations are found almost exclusively in the PNA^{high} B cells and have a similar distribution of hotspots and base substitution bias as found in spleen B cells from hyperimmunized animals (12).

Transgenic animals are used to define signals required for the hypermutation process (13–16). The analysis of somatic mutations of transgenes normally requires the immunization of animals (preferably hyperimmunized to maximize mutations), followed by isolation of hybridomas or PCR amplification of antigen-selected B cells. These studies are time-consuming, as adequate immunization is essential due to the very low levels of somatic mutations found in splenic B cells from unimmunized animals (17). Thus, there is a need for a more efficient and faster method to study hypermutation in transgenes. We suggest that the approach utilized in this paper, based on the use of germinal-center B cells from PPs, offers an excellent alternative to the analysis of the mutational efficiency of new transgenic constructs.

MATERIALS AND METHODS

Isolation of Cells. PPs from LK3 transgenic mice (10) were recovered by dissection from the small intestine. Single-cell suspensions were prepared by pressing PPs through a nylon mesh. The cell suspension was centrifuged and washed three times in cold Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Sigma). Clumps of dead cells were eliminated with a Pasteur pipette after each wash.

Immunofluorescence. Cells were incubated with biotinylated antibody RA3-3A1 (anti-B220) (18) at an appropriate dilution for 25 min at 4°C. After two washes with DMEM/5% fetal bovine serum, streptavidin-phycoerythrin conjugate (Dakopatts, Glostrup, Denmark) was added for 30 min at 4°C, and the cells were washed and suspended in phosphate-buffered saline with 5% fetal bovine serum. Fluorescein isothiocyanate-conjugated PNA (Sigma) was added to the labeled cells, which were then incubated on ice for 30 min, washed, and resuspended in medium. A FACStar^{Plus} (Becton Dickinson) was used to purify B220$^+$ PNA^{high} and B220$^+$ PNA^{low} cells.

Isolation of DNA. Cell pellets were resuspended in 10 μl of water, frozen immediately in liquid nitrogen, and kept at −70°C. Genomic DNA was isolated by treating the frozen pellets with proteinase K (100 μg/ml) in 20–50 μl of 10 mM Tris·HCl, pH 8.3/50 mM KCl/1.5 mM $MgCl_2$/0.5% Tween 20 at 56°C for 60 min and then at 95°C for 30 min.

DNA Amplification. Genomic DNA was amplified by PCR using the primers previously described (12, 19): VKOX-BACK (5′-CCGGGGAATTCTCAGCTTCCTGCTAATCA-3′, a back primer based on the sequence of the V_κOx1 leader region, containing an *Eco*RI site) and LKFOR (5′-CGCG-

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Abbreviations: PP, Peyer's patch; PNA, peanut agglutinin; CDR, complementarity-determining region.

GATCCCTTTCTATCCTGAAGTTCCT-3′, which primes in the $J_\kappa 5$–C_κ intron expressed in both endogenous mouse gene and transgene and which contains a *Bam*HI site). Amplification was carried out for 35 cycles (92°C, 2 min; 55°C, 1.5 min; 72°C, 2 min) in 10 mM Tris·HCl, pH 8.3/50 mM KCl/1.5 mM $MgCl_2$ containing 375 μM dNTPs and 3.5 units of *Taq* polymerase (Cetus) in a final volume of 50 μl. The PCR product was purified on a 1.2% agarose gel and the band was eluted by using a gene Clean II kit (Bio 101). Amplified DNA was digested with *Eco*RI and *Bam*HI in a volume of 100 μl and cloned into phage vector M13mp18.

Hybridization and Sequencing. Clones containing the transgenic light chain were positively and negatively selected by hybridization with the oligonucleotide AFR3 (5′-CCCACTTAAACGTCTAGAGGATCT-3′), which recognizes preferentially the transgene in the J–C intron region, and with the oligonucleotide p1400 (5′-ACGTCTAGAAGACCACGCTACCTGCAGTCAGACCC-3′), which recognizes a segment of the endogenous mouse J–C intron [removed during the construction of the transgene (10)]. DNA sequences were derived by standard procedures (20) using [α-[^{35}S]thio]dATP (Amersham) and Sequenase (United States Biochemical). The primer used for sequencing was JK5FOR (17).

RESULTS

Hypermutated Transgenes Are Found in PP Germinal-Center Cells of Unimmunized Animals. As previously reported for adult mice kept under ordinary laboratory conditions (2), PPs of transgenic mice contain a much higher proportion of germinal-center B cells than their spleens (Fig. 1). Indeed, the proportion of PNA^{high} cells among the B220-positive cells was very seldom as low as 5% and sometimes as high as 15%, values which very rarely are obtained in the spleens of immunized animals. Thus, double staining for PNA and B220 allowed clear separation of putative PP germinal-center B lymphocytes from the others. The transgene V_κOx1–J_κ5 present in LK3 mice (10) was then separately amplified from both B-cell populations, inserted into suitable vectors, and cloned. Positive clones were identified with specific probes, and the inserts were sequenced.

The results clearly showed that most of the sequences derived from the PNA^{high} population contained multiple point mutations, while those derived from the PNA^{low} cells only occasionally contained more than one mutation (4 clones of 33) (Fig. 2 and Table 1). The number of mutations in the transgene in the PNA^{high} population in PPs of unimmunized animals (Table 1) was even higher than in the same transgene in the spleen hybridomas from immunized animals (10). Indeed the distribution of mutations per clone observed in Fig. 2 is very similar to the one previously described for the

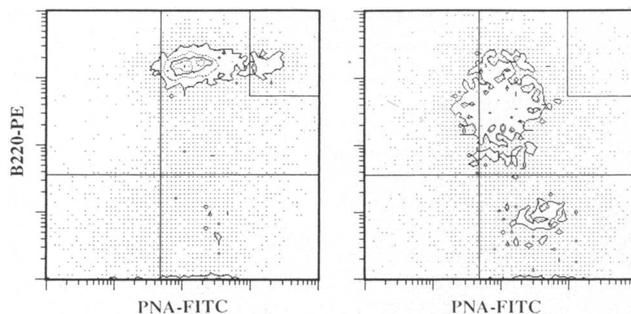

FIG. 1. Two-color FACS contour plots of LK3 PP (*Left*) or splenic (*Right*) lymphocytes stained with biotinylated anti-B220 followed by streptavidin-phycoerythrin (PE) conjugate (*y* axis) and PNA-fluorescein isothiocyanate conjugate (FITC) (*x* axis). Cells gated in the small square are B220$^+$ PNA^{high} lymphocytes (8.5% in PPs and 1% in spleen).

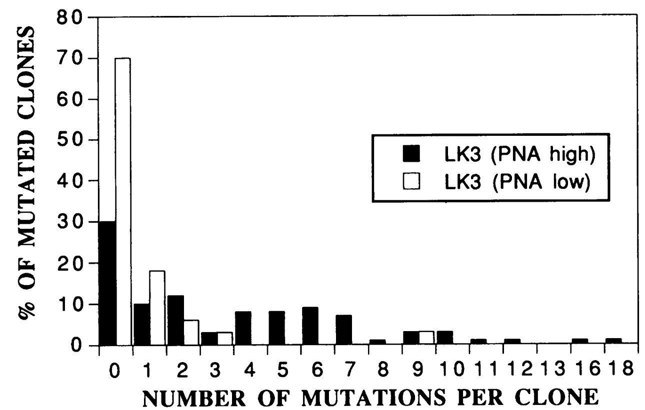

FIG. 2. Accumulation of somatic mutations in PNA^{high} (filled bars) and PNA^{low} (open bars) B220$^+$ cells from PPs.

same germ-line gene combination (V_κOx1), found in antigen-specific B cells at the end of a secondary response in the spleen of immunized BALB/c mice (17).

Fig. 3 shows the distribution of the mutations in sequences with two or more point mutations. Three sets of data are presented, from the PNA^{high} B220$^+$ population derived from several PPs from one mouse and a single PP from another, and the PNA^{low} B220$^+$ subpopulation from a pool of PPs. The single PP did not show a less diverse pattern than the mixed population. The only pair clonally related (clones 14 and 31) were from the PNA^{high} pool. Deleterious mutations were common: 9 out of 76 clones included stop codons. Two clones included deletions of 9 (clone 39 from the PNA^{high} single) and 21 (data not shown) nucleotides.

Similarity of Hypermutation in PPs and in Spleen Cells. The nature of the base substitutions is shown in Fig. 4. It includes the data of Fig. 3 for PNA^{high} and the values are corrected for the effect of PCR error preference [changes from T to C and A to G represent around 80% of the *Taq* polymerase errors (ref. 22 and unpublished observations)]. The pattern of substitutions in PPs was very close to the previously defined substitution preferences from pooled data from spleen sequences (12, 21). The predominant substitutions show the bias of transitions over transversions. The addition of these mutations to the previous compilation of data (21) generates a cumulative table that includes 1327 mutations in total (Fig. 4).

The distribution of mutations in the sequences from PPs (Fig. 5*A*) reveals the presence of dominant hotspots which are preferentially found around CDR1. The most prominent (second base of Ser-31, third of Ser-26, and second of Ser-77) have been previously identified as the most dominant intrinsic hotspots in a large data base of the same gene, in normal and transgenic mice immunized with different antigens (12). There are also other hotspots in Fig. 5*A*. The mutation of the third base of Val-30 is always silent and therefore cannot be

Table 1. Mutations in the V_κOx1–J_κ5 transgene of clones from PP B cells from LK3 mice

PP cells	No. of clones: Total	No. of clones: >1 Mut*	No. of mutations: Total	No. of mutations: >1 Mut*	Mutation frequency per base pair†
B220$^+$ PNA^{high}					
Pool (1 mouse)	39	21	124	119	1.1×10^{-2}
Single (1 mouse)	37	23	143	139	1.3×10^{-2}
B220$^+$ PNA^{low}					
Pool (1 mouse)	33	4	22	15	2.3×10^{-3}

*Data from clones with more than one mutation.
†PCR error is 0.8×10^{-3} mutation per base pair.

```
                                                                                    CDR1
                                                                      ---------------------------------
          1               5                   10                  15          20      * 25            30                  35              40              45
          Q   I   V   L   T   Q   S   P   A   I   M   S   A   S   P   G * K * T   M   T * A   S   S   S * V   S   Y   M   H   W   Y * Q   K   S   G   T   S * K
          CAA ATT GTT CTC ACC CAG TCT CCA GCA ATC ATG TCT GCA TCT CCA GGG AAG ACC ATG ACC GCC AGC TCA AGT GTA AGT TAC ATG CAC TGG TAC CAG AAG TCA GGC ACC TCC AAA
POOL(high)
1 (S)     --- --- --- --- --- --- --- --- C-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --A --- --- --- --- --- --- --- --- --- --- ---
3.        --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
4.        --- --- A-- --- --- --- --- --- --- --- --- --- --T --- --- --- --- --- C-- --- --- --- --- --- -CG --- --- --- --- --- --- --- --- --T --- --- --- ---
8.        --- --- -C- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --A --G -C- --- --- --- --- --- --- --- --- --- --- --- ---
9 (S)     --- --- --- --- --- T-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- G-- --A --- --- --- --- --- --- --- --- --- ---
10(S)     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --A --- --A --- --- --- --- --- ---
11.       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -T- --T --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
14. (+)   --- --- --- --- --- --- --- --- --G T-- C-- --- --- --- --C --- --- --- --- --- --- --T --- --- --- C-- --- --A --- --- --- --- --- --- --- --G --- ---
31 (+)    --- --- --- --- --- --A --- --- --G T-- C-- --- --- --- --C --- --- --- --- G-- --- --T --- --- --- C-- --- --A --- --- --- --- --- --- --- --G --- ---
15.       --G --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -T- --- --- --- --- --- --- --- --- G-- --- --A --- --- --- --- --- --- --- --- --- ---
16(S)     --- --- --- --- --- --- --- --G --- T-- --- --- A-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -C- --- --- --- --- --- --- --- --- ---
21.       --- --- --- --- --- --- --- --- --- --- --- C-- --- --- --- --- --- --- --- --- --- --T --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
24.       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -A- --- --- -C- --- --- --- --- --- --- --- --- --- --- ---
26.       --- --- -A- --- --- --- -T- --- -A- --- --- --- --- --- --- --- --A --- T-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
27.       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
28.       --- --- --- --- -G- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
29.       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- T-- -T- --- --- --- --- --- --- --- --- --- --- --- ---
34.       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- T-T -C- --- --- --- --- -G- --- --- --- --- --- --- ---
35.       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -A- --- T-- --- --- --- --- --- --- --- --- --- ---
37.       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- A-C --- --- --- --- --- --T --- --- --- --- --- --- ---
38.       --- --- --- --- --- --- --- --- -T- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- T-- --- --- --A --- --- --- --- --- --- --- --T --- ---
SINGLE(high)
11.       --- --- --- --- --- --- --- --- --- --- --- C-- --- --- --- --- --- --- --- --- --- --- --- --- --- -A- --- --- --T --- A-- --- --- --- --- --- --- ---
13(S)     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -T- --- --- --- --- --- --- C-- --- --- --- --A --- --- --- --- --- --- ---
17.       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- A-- -A- --- --- --- --- --- --- --- --- --- --- --- ---
19.       --- T-- --- --- --- --- --- --- --- --- --- --- --- --- --- A-- --- --- --- --- --- --T --- --- --- -A- --- --- --- --- --- --- --- --- --- --- --- ---
20.       --- --- -A- --- --- --- --- --- --- --- --- --- --- --- --- --- --- T-- --- --- --- --T --- --- --- CA- --- --A --- --- --- --- --- --- --- --- --- ---
21.       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --T --- --- --- --- --- --- --- --- ---
22.       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -A- --- -A- --- --A --- --- --- --- -C- --- --- --- --- ---
24.       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -T- --- --- --- --- --- --- --- --- --- --- --- ---
25.       --- --- --- --- --- --- --- --- -T- --- --- --- --- --- --- --- --- --- --- --- --- -A- --- --- --- --- --T --- --- --- --- --- --- --- --- --- --- ---
26.       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
28.       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
33.       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -G- --- G-- --- --- --- A-- --- --- ---
34.       --- --- --- --- --- --- --- --- A-- --- --- --- --- -T- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
36.       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- G-- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
38.       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -A- --- --- --- --- --- --- --- --- --- --- C-- ---
39(S)     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -T- --- --- --- --- --- -C- --- --- *** ****--- --- --- --- T-- --- TG-
5.        --- --- --- --- --- --- --- --- --- -C- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- TA- --- --- --- --- --- --- --- --- --- --- --- ---
6.        --- --- --- --- --- --- --- --- --- --- --- --- --C --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
8.        --- --- --- --- --- --- --- --- --- --- --- -T- --C --- --- --- --- --- --- --- --- --T --- --- --T --- --- --- --- --- --- --- --- --- --- --- --- ---
9 (S)     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --G --- --- --- --- --A --- --- --- --- --- --- --- ---
12(S)     --- -C- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --A --- T-- -C- --- -G- --- --- --- -G- T-- G-- --- --- --- A-- ---
23.       --- --C --- --- --- --- --- --- --- --- --- --- --- -T- -T- --- --- --- --- --- -T- --- -T- --- --- --- --- --- --- --- --- --- --- --- --T --- --- ---
37(S)     --- --- --- --- --- TC- --- --- C-- --- --- --- --- --- --T --- --- --- --- --- --- --- --- --- --T --- --T --- --- --A --- --- --- --- --- --T C-- ---
POOL(low)
5         --- --- --- --G --- --- --- --- --- --- --- --- A-G --- --- --- --- --- --- G-- --- -A- --- --- --- --- --- --- --- --- C-A --- --- --- --- --- --- ---
6 (S)     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
15        --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
33(S)                                                                 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
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```
                          CDR2                                                                                                 CDR3
                                                                                                       *
                      ------------------------                                                                                   ------------------------
                      50                  55 *        60          65    * 70                    75                  80    *85                   90                  95
          R   W   I   Y   D   T   S   K   L   A * V   P   A * F   S * S * S * S   Y   S   L   T   I   S   S * E   A   E * T   Y   Y   C * Q   W   S   S   N   P
          AGA TGG ATT TAT GAC ACA TCC AAA CTG GCT GTC CCT GCT TTC AGT AGT TCT TCT TAC TCT CTC ACA ATC AGC AGC GAG GCT GAA ACT TAT TAC TGC CAG TGG AGT AGT AAC CCA
POOL(high)
1 (S)     --- --- --- --- --- --- --- --- G-- --- --- --- --- --- --- --- --- --- --- G-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
3.        G-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -A- --- --- --- --- --- --- --- --- --- --- --- --- ---
4.        G-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
8.        --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -T- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
9 (S)     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- T-- -G- --- --- --- --- --- --- --- --- CT- ---
10(S)     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
11.       --- --- -G- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
14. (+)   C-- -T- --- --- --- --- --- -C- --- --- --- --C --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
31 (+)    C-- -T- --- --- --- --- --- -C- --- --- --- --C --- --- --- --- --- --- --T -T- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
15.       --- --- --- -T- --- --- --- -G- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
16(S)     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -T- --A --- --- --- --- --- -C- --- A-- --- --- --- --- --- --- ---
21.       --- --- --- --- --- --T --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -T- --- --- --- --- --- --- --- --- --- --- --- --- ---
24.       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- T-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- A-- --- --- ---
26.       --- --- --- --- --- --G --- --- --- --- --- --- --- --- --- --- --- --- --T --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
27.       --- --- G-- --- --- --- --- --- --- --- --- --- --- --- G-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
28.       --- --- --- --- --- --- --- --G --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -C- --- --- --- --- --- --- --- --- --C T-- -A- --- ---
29.       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
34.       --- --- --- --- --- --- --- --G --- --- --- --- --- --- --- --- --- --C --- --- --- --- --- --- --- --- --- --- --- --- -T- --- --- --- --- --- --- ---
35.       --- --- --- --- --- --- --- -C- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- C-- --- --- --- --- --- --- --- --- --- --- --- --- --- A--
37.       --- --- --- --- -G- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -A- --- --- --- --- --- --- --- --- --- --- --- --- ---
38.       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --T ---
SINGLE(high)
11.       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- T-- --- --- --- --- --- --- -C- --- --- --- --- --- --- --- --- --- ---
13(S)     --- --- --- --- --- --- --- --- --- --- --- --C --- --- --- --- G-- --- --- --- --- --- --- --- T-- --- --- --- --- --- --- --- --- --- --- -C- --- ---
17.       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
19.       --- --- --- -G- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --C --- --- --- ---
20.       --- --T --- --- --- --- --- --G --- --- --- --- T-- --- --- -T- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --A --- --- -
21.       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- C-- --- --- --- --- --- --T ---
22.       --- --- --- --- --- --- --- T-- --- --- --- --- A-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
24.       --- --- --- --- --- --- --- --- --- --- --- --- -T- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
25.       --- --- --- --- --- --- --- --- --- --- --- --C --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -T- --- --- --- ---
26.       --- --- --- --- --- --- --- --- --- --- --- --- --- C-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- C-- ---
28.       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --A --- --- --- --- -G- --- --- --- --- --- --- ---
33.       --- --- --- --- --- --- --- --- --- --- --- --- -T- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
34.       -A- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- C-- --- --- --- --- --- --- --- ---
36.       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -A- --- --- --- --- --- --- --- --- --- --- --- --- ---
38.       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --G --- --- --T --- --- --- --- G-- T-- ---
39(S)     --G --- --- --- --- --- --- --- --- --- --- --- --- C-- --- --- --- --- --- --- --- --- --- --- -A- --- --- --- --A --- --- --T --- --- --- --
5.        --- --- --- --- --- --T --- --- --- --- --- --- T-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- C-- ---
6.        --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -T- --- --- --- --- --- -G- --- -T- --- --- --- --- ---
8.        --- --- --- -T- --- --- --- --- --- --- --- --- --- --- --- --- --- -T- --T G-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -C- --- ---
9 (S)     --- --- --- --- --- --- --- --- --- A-- --- --- --- --- --- --- --- --- --- --- --- C-- -A- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
12(S)     --- --- --- --- --- -A- -A- -G- --- C-- -C- --- --- --- --- --- --- C-- --- --- --- -T- --- C-T --- --- --- ---
23.       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --T T-- --- -A- --- --- --- --- --- --- --- --- --- --- ---
37(S)     --- --- --- -G- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --
POOL(low)
5         -C- --- --- C-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
6 (S)     --- C-- --- --- --- --T --- --- --- --- --- --- --- --- --- --- --- --- --A --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
15        --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -C- -C- --- ---
33(S)     --- --- --- --- --- --- --- G-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- T-- --- --- --- --- ---
```

FIG. 3. (*Legend appears at the bottom of the opposite page.*)

FROM \ TO	T	C	A	G	TOTAL MUT.
T	----	0.57 / **0.53**	0.24 / **0.30**	0.18 / **0.17**	33 / **152**
C	0.79 / **0.75**	----	0.14 / **0.10**	0.07 / **0.15**	58 / **304**
A	0.35 / **0.30**	0.30 / **0.22**	----	0.35 / **0.48**	93 / **397**
G	0.20 / **0.11**	0.16 / **0.30**	0.63 / **0.59**	----	68 / **474**
					252 / **1327**

FIG. 4. Nature of the nucleotide substitutions in PPs. Data are expressed as percent total for each base. Numbers in bold represent the cumulative values that include the previous compilation of mutations in mutated heavy-chain and κ light-chain variable genes (V_H and V_κ) (21) and the mutations in PPs. PCR error preferences (substitutions from T to C and A to G) were subtracted at a rate of 0.64×10^{-3} mutation per base pair (80% of 0.8×10^{-3} mutation per base pair). MUT., mutations.

selected by antigen. It is not obvious whether changes in the third base of Met-33 [a minor hotspot not highlighted previously (12)] and some of the other minor hotspots involving replacement mutations are due to selection by antigen or otherwise.

The pattern and distribution of mutations is thus largely intrinsic to the mutation machinery. It is not as expected from gene conversion events, at least with closely related germ-line genes. This is illustrated by comparison with the diversity of V_κOx1 relative to the other 26 closely related members of the gene family (almost all of the relevant germ-line genes) (19, 23). Fig. 5*B* shows the number of germ-line genes which diverge from V_κOx1 at each position. The difference with Fig. 5*A* is revealing. For instance, segments of highest divergence were clustered in CDR2 rather than CDR1. Not only is the general pattern different but also many of the specific substitutions in PPs are rarely found or not present among other germ-line genes.

DISCUSSION

The preexisting germinal centers and the large number of germinal center B cells (PNA^high^) in PPs (at least 10-fold higher than in the spleen), together with the large number of mutations in transgenes, suggest that B lymphocytes in PPs are undergoing a high level of stimulation. Although in this paper we report only results from transgenic animals and in populations of B cells highly enriched for germinal-center cells, we have also found that mutated sequences are often found in the PNA^{high} subpopulation of B cells from PPs of BALB/c mice, even (albeit at much lower frequency) in unfractionated B lymphocytes (results not shown).

LK3 transgenes have been shown to mutate in spleen cells from immunized mice (10), and we have found an even larger number of mutations in germinal-center B cells from PPs. The hypermutation machinery in PPs generates the same intrinsic hotspots as those described in V_κOx1 passenger transgenes and in hybridomas from immunized spleen cells (either in normal or in transgenic LK3 mice) and specific B cells (12).

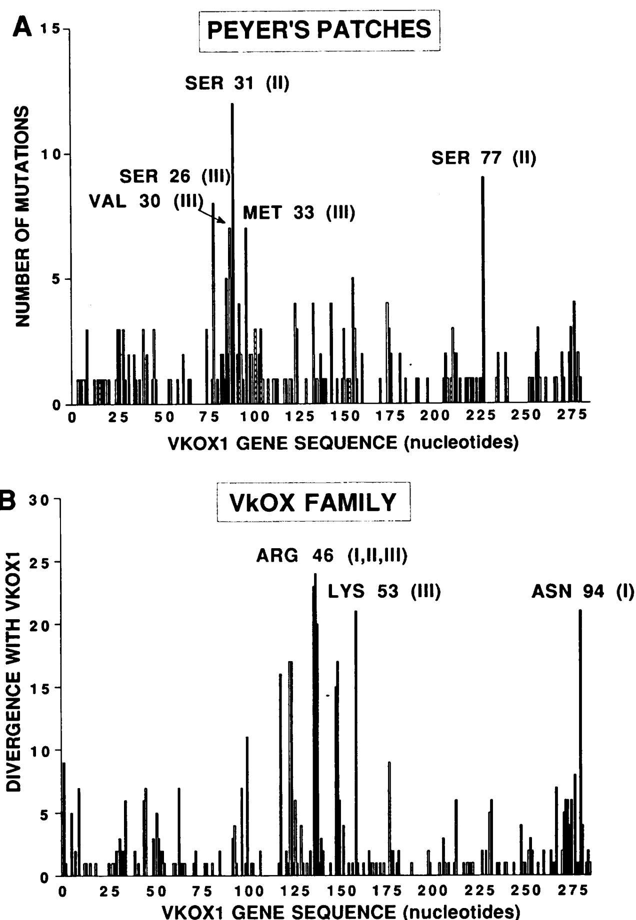

FIG. 5. (*A*) Distribution of mutations along the V_κOx1 transgene. Nucleotide substitutions in all the clones from PNA^{high} $B220^+$ cells were computed. Mutations that lead to amino acid substitution, silent mutations, and mutations that generate stop codons are shown as filled, open, and striped columns, respectively. The dominant individual hotspots are marked with the name and position of the amino acid, followed by the particular position in the codon (Roman numerals). (*B*) Divergences of other members of the V_κOx family with respect to the V_κOx1 transgene (data taken from refs. 19 and 23). The lack of correlation between the two sets of data indicates the untemplated nature of the mutations.

Overall, the pattern of mutations indicates that antigen selection does not play a major role in the generation of hotspots but that the majority of them are produced by the intrinsic bias of the machinery of hypermutation. However, the mutations in PPs were found in B cells which, although from unimmunized animals, were most likely stimulated by antigens, perhaps intestinal flora, food, or others. This apparent contradiction is explained if the transgene is not part of the receptor for such putative antigens. While this may be expressed in some B cells, it seems likely that in most cases it is behaving as a "passenger" (10, 12). This would explain the absence of predominant hotspots selected by antigen, as well as the very high proportion of stop codons, which seem to be selected to eliminate the expression of passenger transgenes (24).

Do the mutated sequences derive from cells activated only once or more frequently? We found highly mutated clones (in

FIG. 3 (*on opposite page*). Sequences of the V_κOx1 transgene obtained after PCR amplification. Clones derived from PNA^{high} B cells from a pool of PPs or from a single PP or derived from PNA^{low} B cells from a pool of PPs were compared with the V_κOx1 transgene (at the top). Unmutated codons are not shown (indicated by asterisks). (S) indicates clones which contain nucleotide substitutions that generate stop codons (in bold letters). One clone (Single high 39) with a deletion of 9 nucleotides is also shown. Two clones with very related sequences are indicated (+). CDR, complementarity-determining region.

some cases with 16 or 18 mutations per clone) which were, on average, more mutated than the same transgene from hybridomas after specific secondary immunization (10). While it is possible that the hypermutation rate in PPs is higher than in spleen, it seems more likely that they arise by repeated rounds of antigen stimulation. Indeed it seems probable that, as argued before (17), mutations arise not only in cells derived from primary stimulated B cells but also from the restimulated memory pool. The fact that mutations are found in the PNA^{high} (germinal-center B cells) and not in the PNA^{low} population reinforces the results obtained with studies in spleen identifying the germinal centers as the site of hypermutation (4, 5). It seems reasonable, therefore, to conclude that not only the nature of the hypermutation process, but also the compartmentalization in germinal centers of the process itself, is the same in spleen and PPs, and by implication, in all peripheral lymphoid organs. Although the approach we used is somewhat different from the one used by Reynaud *et al.* (7) in their analysis of sheep PPs, there are considerable similarities with our analysis in mice, but also interesting differences. The similarities refer to the extent and general nature of the hypermutation. The most interesting difference is that in the adult sheep, Reynaud *et al.* found that all the sequences analyzed were hypermutated, while in our cases the vast majority of the PNA^{low} cells (at least 80% of total PP cells) were not hypermutated. This is not an artifact of transgenic animals, since a similar situation was found with normal mice (results not shown). This discrepancy in the two species may reflect a fundamental difference in the role of PPs in each case: a primary lymphoid organ in the case of sheep and a peripheral lymphoid organ in mice.

We found no clear evidence of clonally related mutations in the single-PP analysis. This could be because each PP contains several follicles (2) and each cell contains three copies of the transgene (10). This may involve at least 100 independently generated hypermutated sequences, if we assume that there are >30 germinal centers in each PP and take into consideration that the three transgene copies mutate independently (10, 12, 16). In addition, PP germinal centers may be more heterogeneous than implied by the studies of primary immunized spleens (5, 25–27). Such heterogeneity could be due to repopulation with cells originating from the memory pool. If such cells migrate to germinal centers, they may start as PNA^{low}. Indeed there are a few PNA^{low}-derived clones which are mutated. They represent 10% of that population, but at this level it is difficult to exclude the possibility of cross contamination with PNA^{high} cells.

Leaving aside these considerations, it is clear that germinal center B cells from PPs represent a good source for the analysis of immunoglobulin transgenes. It allows measuring the rate of mutation of modified transgenes to provide information about the intrinsic machinery of hypermutation. To speed up the analysis it may be possible to analyze younger mice than those used here. While the proportion of T and B lymphocytes and cells that are PNA^{high} was similar in a 3-week-old mouse, the number of recovered cells from PPs increased with age up to about 6 to 8 weeks after birth (data not shown). It remains to be seen to what extent the frequency of somatic mutations depends on the age of the animals and on the environment in which the animals are housed. We have used animals of similar age (about 4 months old) kept under similar conditions and found no significant differences between them. The use of PPs for transgene analysis has advantages because (*i*) the number of germinal-center B cells is much larger than in the spleen, (*ii*) specific immunization of animals is not necessary, and (*iii*) it is easy to study a statistically significant number of clones.

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