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Mice Triallelic for the Ig Heavy Chain Locus: Implications for $V_{H}DJ_{H}$ Recombination

Vasco Barreto, Tommaso Meo, and Ana Cumano

$V_{H}DJ_{H}$ recombination has been extensively studied in mice carrying an Ig heavy chain rearranged transgene. In most models, inhibition of endogenous Ig rearrangement occurs, consistently with the feedback model of IgH recombination. Nonetheless, an incomplete IgH allelic exclusion is a recurrent observation in these animals. Furthermore, transgene expression in ontogeny is likely to start before somatic recombination, thus limiting the use of Ig-transgenic mice to access the dynamics of $V_{H}DJ_{H}$ recombination. As an alternative approach, we challenged the regulation of somatic recombination with the introduction of an extra IgH locus in germline configuration. This was achieved by reconstitution of RAG2$^{-/-}$ mice with fetal liver cells trisomic for chromosome 12 (Ts12). We found that all three alleles can recombine and that the ratio of Ig allotype-expressing B cells follows the allotypic ratio in trisomic cells. Although these cells are able to rearrange the three alleles, the levels of Ig phenotypic allelic exclusion are not altered when compared with euploid cells. Likewise, we find that most VDJ rearrangements of the silenced allele are unable to encode a functional $\mu$-chain, indicating that the majority of these cells are also genetically excluded. These results provide additional support for the feedback model of allelic exclusion.

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similar to control euploid hemopoietic cells (21, 22). In this study, we analyze chromosome 12 trisomic (Ts12) reconstitution chimeras with different IgH combinations for the ratio of allotype expression in B cells, the degree of IgH allelic exclusion, and the recombination status of the three alleles.

We found that all three alleles are available for recombination and that the allotypic ratio of Ig-expressing B cells reflects the allelic ratio. Although trisomic cells are able to rearrange all three alleles, the levels of IgH phenotypic allelic exclusion are not altered in these animals, as compared with euploid mice. Likewise, most V<sub>D</sub>1<sub>H</sub> rearrangements of the silenced allele are unproductive, indicating that the majority of these cells are also genetically excluded. These results provide further support for the feedback model of allelic exclusion. However, we also observed that the reading frame (RF) II usage in D<sub>H</sub> from the IgH Ts12 splenic B cells is higher than in controls, suggesting that some D-J<sub>H</sub> rearrangements might have occurred after productive V<sub>D</sub>1<sub>H</sub> rearrangement.

### Materials and Methods

#### Animals

Rh(4.12)/SbBarC57BL/6, Rh(6.12)/Sic/BALB/c, Rh(8.12)/SbBarC57BL/6, and Rh(8.12)/Sic/BALB/c wild-derived Robertsonian (Rh) mice were purchased from the Medizinische Universität zu Lübeck (Lübeck, Germany). A/J females were purchased from Charles River Breeding Laboratories (Clon, France); BALB/c and C57BL/6 females were purchased from Ifla Credo (L’Arbresle, France). RAG2<sup>−/−</sup>/129/Sn animals (23) and C57BL/6.6.5.1-congenic animals were purchased from the Centre de Développement des Techniques Avancées pour l’Expérimentation Animale-Centre National de la Recherche Scientifique (Orléans, France). Experiments were performed with animals bred in the Pasteur Institute animal facilities.

#### Reconstitution chimeras

Doubly heterozygous males Rh(4.12)/SbarRh(6.12)/Sic, Rh(4.12)/SbBarRh(8.12)/Sic, and Rh(8.12)/Sic/Rh(8.12)/Sic were obtained and mated with A/J, BALB/c, or C57BL/6 females. Pregnant females were killed by cervical dislocation between days 14 and 17 of gestation, the plug day considered to be day 1. Ts12 fetuses were identified by their exencephalic phenotype and by cytogentic tests. Ts12-nucleated fetal liver cells suspended in RPMI 1640 complete culture medium (10% FCS) were transplanted i.v. (>5 × 10<sup>6</sup> cells/animal) into 600 rad irradiated RAG2<sup>−/−</sup>/129/Sn mice (8–16 wk old). Ts12 cell hemopoietic reconstitution chimeras were identified on the basis of the presence of circulating IgGs 1 mo following injection. To generate mice with mixed Ts12 and euploid hemopoietic systems, Ts12 IgHab and euploid IgHab (BALB/c × C57BL/6L.6.1.5.1-<sup>−</sup>) bone marrows were donated to reconstitution chimeras (both in RAG2<sup>−/−</sup>/129/Sv) were mixed at different ratios and used as before to reconstitute RAG2<sup>−/−</sup>/129/Sn mice (8–16 wk old). Euploid and Ts12 cells were distinguished using a homemade anti-Ly-5.1, PE-labeled Ab. Throughout our study, chimeras were perpetuated by repeated transplantation of bone marrow cells into RAG2<sup>−/−</sup> recipients, for more than 1 year.

#### Cytogenetics

To obtain a karyotype of the trisomic embryos, a fraction (50,000–300,000 cells/animal; n = 3), which were put in a well with 6000 irradiated S17 cells and were stimulated with LPS, as described. After 4–5 days, 1.5 × 10<sup>5</sup> cells were harvested and DNA was prepared by standard phenol:chloroform extraction and precipitation. As control, DNA from S17 cells was also prepared. Detection of the IgH germline-specific amplicon was obtained in a PCR (35 cycles; 95°C, 30 s; 63°C, 30 s; 72°C, 1 min; final elongation at 72°C for 10 min) with the forward primer 5′-J<sub>H</sub>1 5′-CGCCGACCAGAG CAGG-3′ and the reverse primer 3′-J<sub>H</sub> 5′-GGCTTCTGGGGCCCA-3′ (25) under the following conditions: 2 mM dNTPs; 1.25 MgCl<sub>2</sub>; 12.5 pmol each oligo; 2.5 U Taq polymerase (Life Technologies); total volume, 25 μl. As a control for the amount of genomic template DNA, a RAG2 amplicon was amplified, under the same PCR conditions, with the forward primer 5′-TACGGAGAC GAGCAGGATGC-3′ and the reverse primer 5′-TGA CACGATATGAAAAGGAGG-3′. Serial dilutions covered a 2700-fold dilution, starting from 200 ng of NA.

#### PCR amplification of IgH rearrangements, cloning, and sequencing

(V<sub>D</sub>1<sub>H</sub>)<sub>αβ</sub> rearrangements were amplified from IgM-expressing B220<sup>−</sup>-sorted splenic B cells, which were further expanded in culture, as described. DNA was prepared by standard phenol:chloroform extraction, followed by precipitation. PCRs were performed starting with 5–200 ng of DNA, and the standard conditions were the following: 2 mM dNTPs, 2.5 mM MgCl<sub>2</sub>, 12.5 pmol of each oligonucleotide, and 1.25 U Taq polymerase (Life Technologies) in a final volume of 25 μl; final elongation at 72°C for 10 min.

To distinguish between BALB/c and C57BL/6 IgH alleles, we focused on (V<sub>D</sub>1<sub>H</sub>)<sub>αβ</sub> rearrangements due to the presence of an intrinsic single-base polymorphism between these two strains, which is very close to and 3′ of J<sub>H</sub>1 (26). For V<sub>D</sub>1<sub>H</sub> amplifications, a first reaction (30 cycles; 95°C, 30 s; 60°C, 30 s; 72°C, 1 min) was performed using the following V<sub>αβ</sub>-specific forward primers in separate reactions: V<sub>α</sub>A (JS58) 5′-GCCGA GTTTARGCCTGGGRCCTTCACTGAG-3′; V<sub>β</sub>B (Q52) 5′-GGCTTAC GCTCAGACAGGACGTCCTCACTGAG-3′; V<sub>αβ</sub>E (7183; DNA4) 5′- GCCGA GTCTGGTGAATCTGGGGGAGCTTA-3′, and the reverse primer 3′-TACGGAGAC GAGCAGGATGC-3′. Then, a second PCR (30 cycles; 95°C, 30 s; 58°C, 30 s; 72°C, 1 min) was performed on 1 μl of the first PCR, using the same V<sub>α</sub>-specific primers and the reverse primer 5′-AAAAA AGCCGACGTCCTCAGTA-3′. For DJ<sub>4</sub> amplifications, a PCR (35 cycles; 95°C, 30 s; 62°C, 30 s; 72°C, 30 s) was performed using a D5S-αGCTGACATTTGTT SAAGGGATCTACTAGT-3′ forward primer that recognizes all D elements except Q52 and the reverse primer 1A 5′-GGCTTAC GCCTAGCCGTCCTCAGGG-3′. For the amplification of DJ<sub>1</sub> rearrangements, a first PCR (35 cycles; 95°C, 30 s; 60°C, 30 s; 72°C, 1 min) was performed using D5S and J<sub>αβ</sub> as forward and reverse primers; 1 μl of the PCR was then used on a second PCR (30 cycles; 95°C, 30 s; 58°C, 30 s; 72°C, 1 min) with D5S and J<sub>αβ</sub> as forward and reverse primers, respectively.

(V<sub>D</sub>1<sub>H</sub>)<sub>αβ</sub> amplicons were cloned with the Topo TA cloning kit according to the instructions of the manufacturer (Invitrogen, San Diego, CA). The Dye Terminator Sequencing Kit (Perkin-Elmer, Foster City, CA) was used for sequencing.
used to determine the sequence of the cloned amplicons following the instructions of the manufacturers. V_{H}t-specific primers were used for the V_{H}D_{J_{H}} sequences; JA was used to sequence the DFS-JA-amplified D_{J_{H}}4 amplicons. Sequences were obtained in an ABI 370A DNA sequencer (Applied Biosystems, Foster City, CA). For the analysis of RF usage in DJ_{H} rearrangements, DJ_{H}1 and DJ_{H}4 rearrangements were pooled.

Results

Generation of Ig heavy chain three-allelic chimeras

For the generation of Ts12 B cells, we made use of male mice double heterozygous for two balanced Rb metacentric translocations carrying chromosome 12 as one of their arms: Rb(4.12;8.12) and Rb(4.12;6.12) mice. In these animals, the nonsegregation of the chromosome 12 pair is frequent, as each copy is fused to a different chromosome (Fig. 1A) (24). Upon fecundation, a third copy of the chromosome 12 is added, and these embryos, although with a limited life span, reach the 16 days postcoitum (dpc) stage. Additionally, they can be easily distinguished from euploid siblings by their exencephalic phenotype (24). Fetal liver cell Ts12 animals were used to reconstitute the hemopoietic system of irradiated RAG2^{-/-} mice. In a Ts12 hemopoietic system, not only is

FIGURE 1. Generation of Ts12 embryos. A, Breeding scheme for the generation of Ts12 embryos. Mice with the IgHa or the IgHb allotypes, homozygous for different Rb translocations, both involving chromosome 12 (for instance, Rb(8.12)/BALB/c and Rb(4.12)/C57BL/6), are crossed to generate double heterozygous animals for two balanced Rb translocation metacentrics carrying chromosome 12 as one of their arms. In these animals, during meiosis I, a quadrivalent figure is formed as the result from the pairing of homologous chromosomes. This figure can resolve in different ways, including the migration of both chromosome 12 (depicted as the thick chromosome) to the same pole and a normal segregation of the other chromosomes (thin arrow). Upon fecundation, a zygote trisomic for chromosome 12 and balanced for all others is formed. This gives rise to an embryo that at 12–17 dpc can be distinguished from euploid siblings due to its exencephalic phenotype. Chromosome 12 trisomy can be confirmed by cytogenetics analysis of fetal liver cells. Typically, trisomic fetuses have two metacentric-like Rb translocations (indicated by the arrows in the bottom photograph) and 37 acrocentric chromosomes, making a total of 41 chromosome arms, whereas in metaphases from BALB/c (C57BL/6 and A/J) euploid mice there are only 40 chromosomes and all are acrocentric. B, Ts12 allotypic combinations and respective parental origin.
the aneuploid karyotype stable over time, but the Ts12 hemopoietic cells (including lymphoid cells) are similar to the euploid ones as to morphology and function (21, 22). We initially tried to obtain embryos bearing three different allotypes for which specific Abs are available. Rb(4.12;8.12) and Rb(4.12; 6.12) double heterozygous males in a F1 (BALB/c × C57BL/6) background (IgHa and IgHb allotypes) were crossed with IgHe A/J females. The low breeding efficiency and the low frequency (<2%) of 13–17 dpc Ts12 embryos observed allowed the generation of one single Ts12 chimera (Fig. 1B) and prompted us to switch to other strains of females. C57BL/6 and BALB/c females were used instead of A/J. The frequency of trisomic embryos was then increased. This allowed us to establish RAG2−/− mice-reconstituted Ts12 fetal liver cells from five different embryos, from which the allotypic combinations are shown in Table I. These Ts12 hemopoietic systems were maintained by successive reconstitution of RAG2−/− mice with bone marrow cells from other RAG2−/− mice previously reconstituted with Ts12 fetal liver cells.

Ratio of IgH allotype-specific expressing B cells correlates with the genetic allotypic ratio

One month after reconstitution, Ts12 chimeras were analyzed for the presence of serum IgM and of B cells in the bone marrow and periphery. Levels of serum IgM and B cell numbers were similar to those of chimeras reconstituted with euploid fetal liver cells (data not shown). FACS analysis of bone marrow, spleen, and blood cells with allotypic-specific anti-IgM (IgD) Abs revealed that, both in the bone marrow and in the periphery, the ratio of IgMa (IgDa)- vs IgMb (IgDe)-expressing cells reflects the allotypic ratio at the level of the karyotype. In other words, ab trisomic chimeras have approximately one-third of the IgMa and two-thirds of IgMb B cells, whereas aab trisomic chimeras exhibit two-thirds of IgMa and one-third of IgMb+ B cells (Fig. 2). Similar results were obtained in chimeras with different allotypic combinations, since the ratio of IgDa vs IgDe-expressing cells also changes accordingly to the ratio of IgHa and IgHe alleles.

In an IgHaab chimera in which the IgHb and one IgHa are of paternal (Rb(4.12;6.12)/F1) origin and the other IgHa allele is maternal (BALB/c), the IgMa/IgMb ratio is similar to that of another IgHaab Ts12 chimera in which both IgHa alleles are paternal (Fig. 1B). This later chimera resulted from a crossing-over event between chromatids from chromosome 12 homologues in the Rb(4.12;8.12)/F1 male, leading to the cosegregation of both IgHa alleles during meiosis II. The observation that in these two IgHaab chimeras the IgMa/IgMb ratio is similar indicates that the rearrangement of the IgH alleles is not altered by paternal or maternal disomy in chromosome 12. Furthermore, reconstituted chimeras with euploid (Ly-5.1+) and Ts12 (Ly-5.1−) cells show a ratio of allotype-specific IgM-expressing cells, which reflects the allelic combination, suggesting that this ratio is solely determined by the genetic characteristics of the B cells (Fig. 2) and independent of external factors.

Levels of Ig heavy chain allelic exclusion in Ts12 mice are similar to those of normal mice

In euploid IgHab mice, one-half of B cells expresses IgMa and the other half expresses IgMb. By flow cytometric criteria, B cells expressing both allotypes are below 2%. These observations can be extended to IgHab Rb(4.12;8.12) and IgHab Rb(4.12; 6.12) mice, indicating that the fusion of chromosome 12 with another chromosome at the level of the centromere does not interfere with somatic rearrangement or with the expression of the Ig heavy chain (data not shown). Ts12 chimeras were compared with euploid F1 animals or chimeras reconstituted with euploid F1 fetal liver cells. The frequency of putative dual expressors in chromosome 12 chimeras is not different from that of euploid cells (Fig. 2 and Table I). The same comparison was also performed by intracytoplasmic staining of B cells stimulated ex vivo by LPS. As before, no significant difference was observed between Ts12 and euploid plasma cells (Table I), and the allotypic ratio follows what has been observed at the surface level. We recently developed an assay to estimate the frequency of B cells escaping allelic exclusion. Putative dual expressors are sorted, expanded in culture by LPS stimulation for 2–4 days, and reanalyzed by flow cytometry or by intracytoplasmic staining. We found that the frequency of dual expressors in euploid IgHa/b mice is in the order of 1:104 (27). The same assay performed on cells from three F1 animals and three Ts12-reconstituted chimeras revealed no significant differences in the numbers of dual expressor B cells (data not shown). We conclude that Ts12 reconstitution chimeras have levels of allelic inclusion comparable with the ones found in euploid mice.

Ts12 B cells can rearrange the three alleles

The ratio of Ig allotype-expressing cells in the Ts12 reconstitution chimeras does not provide any information concerning the rearrangement status of the three alleles in each cell. If within each cell one chromosome is randomly excluded from rearrangement, the allotypic ratio would be the same as if in all cells the three alleles are competent to rearrange. To distinguish between these two scenarios, we performed a semiquantitative PCR using a set of primers that amplifies a region between the most 3′DJH rearrangements on all three alleles.

Table I. Percentages of intracytoplasmic stainings of plasma cells obtained from sorted B cells (bone marrow or spleen) that differentiated in culture by LPS stimulation

<table>
<thead>
<tr>
<th></th>
<th>% IgM+</th>
<th>% IgM+</th>
<th>% Putative+</th>
<th>% IgM+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ts12[IgH[aab]]</td>
<td>236</td>
<td>68.5</td>
<td>31.3</td>
<td>0.2</td>
</tr>
<tr>
<td>Ts12[IgH[aab]]</td>
<td>1907</td>
<td>40.6</td>
<td>59.4</td>
<td>0.0</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Euploid (IgH[aab])</td>
<td>562</td>
<td>52.3</td>
<td>47.5</td>
<td>0.2</td>
</tr>
<tr>
<td>Ts12[IgH[aab]]</td>
<td>233</td>
<td>64.0</td>
<td>36.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* Number of cells observed.

** Of all of the cells analyzed, only one (from euploid ab) was clearly a dual expressor.

Having established that IgH (phenotypic) allelic exclusion is normal in Ts12 mice, we then focused on allelic exclusion at the
genetic level. By single cell PCR analysis of rearrangements in mature B cells, it was shown that euploid cells with two productive VH, D, J H rearrangements (genetically included) are present at low frequency (2.5%) (15). To evaluate whether the introduction of a third allele influenced the level of genetic allelic exclusion, we characterized VH, D, J H rearrangements of the BALB/c IgH allele in IgM^1 cells expressing the C57BL/6 allele (IgMb), from a T12 IgHabb chimera. DNA was prepared from sorted splenic IgMb ^1 cells (95% pure), which were expanded in culture by LPS stimulation. VH, D, J H rearrangements were amplified with primers specific for the J558, Q52, and 7183 V H families. The amplicons were then cloned and sequenced. To distinguish between BALB/c and C57BL/6 IgH alleles, we focused on VH, D, J H 1 rearrangements carrying a sequence polymorphism between these two strains (26).

BALB/c IgH alleles show a majority of nonproductive rearrangements (Table II). These results suggest that the proportion of cells with genetic allelic inclusion in T12 is a minority. These data are also consistent with the finding that a proportion of VH, D, J H C57BL/6 alleles in these cells had nonproductive rearrangements. Since there are two C57BL/6 alleles in these cells, one is likely to be silenced in most IgMb-expressing cells that rearranged both IgHb alleles V_H,D,J H.

**RFII usage in the D_J H-rearranged alleles of T12 B cells**

When a D_J H rearrangement is in a particular RF, RFII, D promoter elements drive the expression of the Dm protein, a truncated μ-chain that lacks the V region (28). It was proposed that this molecule signals the shut down of somatic recombination, thus...
arresting cell differentiation. This would account for the underrepresentation of D-JH elements in RFII in mature B cells (29). In support of this model, it has been shown that the presence of a DJα transgene leads to a partial block in B cell development (30). Moreover, a rearranged heavy chain transgene suppresses the RFII counterselection in D-JH rearrangements from the endogenous alleles, raising that frequency from 5% to 27% (31). We performed two independent experiments in which euploid control and Ts12 B cells were enriched by LPS stimulation for 5 days of splenic cells (Expt. 1) or purified by cell sorting (Expt. 2). Genomic D-JH1 and D-JH4 fragments were amplified, cloned, and sequenced. In the euploid control population, we reproduce most of what is known about RF usage (Table III), i.e., we found an overrepresentation of RFI, less of RFIII, and even less of RFII (8%). In the Ts12, we found a modest increase in the frequency of RF2 usage (15%). We performed two independent experiments in which euploid control and Ts12 B cells were enriched by LPS stimulation for 5 days of splenic cells (Expt. 1) or purified by cell sorting (Expt. 2). Genomic D-JH1 and D-JH4 fragments were amplified, cloned, and sequenced. In the euploid control population, we reproduce most of what is known about RF usage (Table III), i.e., we found an overrepresentation of RFI, less of RFIII, and even less of RFII (8%). In the Ts12, we found a modest increase in the frequency of RF2 usage (15%). We observe that in D-JH rearrangements from splenic B Ts12 cells, the frequency of RFII usage is clearly below one-third, in contrast with the data reported for an introduced rearranged heavy chain transgene (31). We conclude that most T12 B cell precursors rearranged all three alleles D to JH before starting VH to DJH. We propose that the modest increase in RFII reflects a situation in which a minority of T12 cells rearrange D to JH when at least one allele has or is simultaneously undergoing productive VH-DJH+ rearrangement, thus relieving the counterselection caused by DJα.

Discussion

In this study, we developed a new system to analyze allelic exclusion of the mouse Ig heavy chain. In our model, a third IgH allele in germline configuration and in its natural chromosomal location is introduced, by reconstituting immunodeficient mice with fetal liver cells from Ts12 embryos (21, 22). We used male mice double heterozygous for two different metacentric chromosomes that contain chromosome 12. In these mice, both chromosomes 12 frequently cosegregate during meiosis, allowing the formation of gametes that upon fecundation can give rise to trisomic embryos (Ts12) (24). This approach is complementary to the introduction of prerearranged transgenes in mice. In the later models, the strategy was the imposition of allelic exclusion by the transgene; in this study, we challenged both the regulated and the ordered models, by creating a scenario in which cells have three IgH alleles competent to rearrange. One appealing feature of the IgH triallelic system is that it is less evasive than all Ig transgenic models, in which the nondevelopmental regulation of the transgene expression and pressure for the diversification of the primary repertoire may bias the interpretation of what is occurring at the level of VH-DJH recombination. The use of a chromosome 12 trisomy ensures that the extra germline IgH locus is complete and in its natural position. An alternative strategy would have been the use of yeast artificial chromosome-derived transgenes that can be large enough to encompass most, if not all, of the IgH locus. Indeed, a transgenic mouse carrying a yeast artificial chromosome with a large fragment of the human heavy chain in germline configuration was recently obtained (32, 33). Unfortunately, <3% of B cells in this mouse expressed the human heavy chain.

We found that the levels of Ig heavy chain allelic exclusion in Ts12 mice are similar to those of normal mice. Similar observations were reported by Du Pasquier and Hsu (34) in a pioneering study using triploid and tetraploid Xenopus animals, heterozygous for the IgH. Although multiple IgH alleles were present, each cell only produced one Ab. At the time this study was performed, the stochastic model of allelic exclusion (35) was still debated. In its pure form, this model proposed that, given a low probability of rearranging one allele productively, allelic inclusion would have to be rare, as it would result from two infrequent events. Du Pasquier and Hsu (34) argued that a stochastic model of allelic exclusion could be compatible with their results only if the frequency of multiple successful rearrangements is very low, and that this is unlikely in frogs because it would lead to a great wastage of lymphocytes. Based on this rationale, they favored some sort of feedback mechanism. We know now that the actual frequency of productive rearrangement is not sufficiently low to explain allelic exclusion. For IgH, this frequency depends on the processing of the coding ends during somatic rearrangement, the proportion of VH pseudogenes, the proportion of aberrant (abortive) rearrangements, and also on the D element sequences that, if read in a particular RFIII, contain stop codons. As was already discussed

![FIGURE 3. Detection of IgH alleles in the germline configuration in mature B cells from Ts12 animals. Semiquantitative PCRs were performed on DNA from S17 cells (positive control) and from FACS-sorted B cells. The RAG2 amplicon is the PCR control for the amount of DNA template. The 3' D-5' J amplicon can only be obtained from alleles in the germline configuration. (See Materials and Methods for details.)](http://www.jimmunol.org/0000000)
likely that simultaneous VH recruitment should have an associated constraint on heavy chains similar to that of the surrogate light chain, the other being excluded from sur-
ficient as euploid cells exclude one allele from expression at the cell surface. At this level, allelic exclusion is a consequence of an active allelic exclusion mechanism of somatic rear-
and C57BL/6 alleles allowed us to analyze the rearrangement status of the silenced BALB/c allele (IgHa) from IgHb-expressing splenic B cells sorted from a IgHabb Ts12 chimera (26). Most VHDJH-recombined BALB/c alleles presented rearrangements that could not encode a full-length µ-chain, because sequences were not in frame at the level of the junctions. We concluded that there is no major alteration in the level of genetic allelic inclusion, as there is no selection for productively rearranged BALB/c alleles in the cells analyzed. Equivalent studies performed in euploid cells are somewhat contradictory. In the first evaluation of the rearrangement status of the silenced alleles in euploid cells, four of seven rearrangements had “no obvious impediments to expression” (37). More recent analyses based on single cell PCR indicate a much lower frequency of cells with genetic allelic inclusion (about 2.5%) (15). Although several cells were analyzed, the frequency of 2.5% comes from the detection of two cells, and PCR analyses do not detect rearrangements involving deletions or other alterations. Therefore, the actual frequency of allelically included cells has not been fully evaluated. Notwithstanding these cautionary notes, it seems clear that in the IgH Ts12

>15 years ago by Coleclough (36) and taking into account data accumulated thereafter, a strict stochastic model of allelic exclusion is untenable. The feedback model essentially solves this problem, but both are not incompatible; in fact, they are complementary. There is a stochastic component to allelic exclusion on the random choice of the allele that rearranges V to DJH, and it is likely that simultaneous VH recruitment should have an associated probability, as low as it may be. The difficulty in evaluating such probability is illustrated by the fact that the approaches with Ts12 animals would only change it by a factor of 2 or 3. Such minor change would be difficult to detect if the frequency of simultaneous rearrangements is, indeed, low.

The work of Hsu and Du Pasquier (34) was limited to serological and cell surface analysis. It should also be pointed out that in some of their hybrids, the possibility that different sets of Ig genes do not act as normal alleles could not be formally excluded. Furthermore, in one triploid combination, the Ig type from one haploid set was absent from all cells, which was interpreted not as a manifestation of Ig allelic exclusion, but rather as a phenomenon of genome haploid set suppression in certain triploid combinations. Our data in a mammalian model, however, are clearly the end result of an active allelic exclusion mechanism of somatic rearrangement. First, the fact that Ts12 embryos are exencephalic and die in utero suggests a gene-dosage effect due to the presence and expression of alleles from the extra chromosome 12. In addition, we found that the maternal or paternal disomy is not influencing the ratio of haplotype a- vs haplotype b-expressing cells, which argues against an imprinting phenomenon. Finally, in the characterization of the rearrangement status of the alleles, we demonstrate that, globally and within each cell, all three loci are capable of undergoing somatic rearrangement.

We show that Ts12 chimeras exclude phenotypically two of the three IgH alleles, at the level of individual cells, probably as efficiently as euploid cells exclude one allele from expression at the cell surface. At this level, allelic exclusion is a consequence of successive constraints for the expression of both alleles. The initial constraint is the limited number of cells carrying both alleles successfully rearranged (15, 37). Furthermore, even in these few cells, only one of the allelic forms was shown to be capable of pairing with the surrogate light chain, the other being excluded from surface expression (15). At the level of the BCR expression, it is likely that the conventional light chain will also introduce a pairing constraint on heavy chains similar to that of the surrogate light chains. Nevertheless, rare mature dual-expressing cells were shown to be present in the spleen of normal mice (27). Another factor contributing to the low frequency of dual-expressing cells is a higher probability of receptor editing and/or deletion of cells expressing autoreactive receptors, in the bone marrow (38). However, it is only at the level of V to DJH rearrangement that the introduction of another allele in the germline configuration could make a difference when compared with euploid cells. We, therefore, evaluated the level of genetic allelic inclusion in the Ts12 B cells. A particular sequence polymorphism in the J region between the BALB/c and C57BL/6 alleles allowed us to analyze the rearrangement status of the silenced BALB/c allele (IgHa) from IgHb-expressing splenic B cells sorted from a IgHabb Ts12 chimera (26). Most VHDJH-recombined BALB/c alleles presented rearrangements that could not encode a full-length µ-chain, because sequences were not in frame at the level of the junctions. We concluded that there is no major alteration in the level of genetic allelic inclusion, as there is no selection for productively rearranged BALB/c alleles in the cells analyzed. Equivalent studies performed in euploid cells are somewhat contradictory. In the first evaluation of the rearrangement status of the silenced alleles in euploid cells, four of seven rearrangements had “no obvious impediments to expression” (37). More recent analyses based on single cell PCR indicate a much lower frequency of cells with genetic allelic inclusion (about 2.5%) (15). Although several cells were analyzed, the frequency of 2.5% comes from the detection of two cells, and PCR analyses do not detect rearrangements involving deletions or other alterations. Therefore, the actual frequency of allelically included cells has not been fully evaluated. Notwithstanding these cautionary notes, it seems clear that in the IgH Ts12

Table II. BALB/c IgH alleles from IgMb B220 sorted cells from a Ts12 IgHabb chimera

<table>
<thead>
<tr>
<th>VH558</th>
<th>CDR3</th>
<th>In-Frame?</th>
<th>VH558 Pseudogene?</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGT GCA AGA GTA GTA TGA TGA CAA CAA CAA CAA TCG CAA TCG</td>
<td>No</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>TGT GCA AGA GTA GTA TGA TGA CAA CAA CAA CAA TCG CAA TCG</td>
<td>No</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>TGT GCA AGA GTA GTA TGA TGA CAA CAA CAA CAA TCG CAA TCG</td>
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<td></td>
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<td>ND</td>
<td></td>
</tr>
<tr>
<td>TGT GCA AGA GTA GTA TGA TGA CAA CAA CAA CAA TCG CAA TCG</td>
<td>No</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

The CD3 is defined as the sequence between the invariant cysteine codon (TGT) of the VH gene and the invariant tryptophan codon (TGG) from the J region. Stop codons are shown in bold italics. An in-frame sequence has the cysteine and the tryptophan codons in the same RF and lacks stop codons in between.

This sequence shows an in-frame stop codon (TAG) in the VH region. This VH gene has been sequenced previously (VH 7183.9; accession number AY008755), but it has an AAG codon (position 190) instead of the TAG codon. This could be explained by a PCR artifact which may have introduced an A/T transversion in our sequence. In support of this interpretation is the fact that an independent PCR product with the same (although poorly resolved) sequence lacks the stop codon. In some of their hybrids, the possibility that different sets of Ig genes do not act as normal alleles could not be formally excluded. Furthermore, in one triploid combination, the Ig type from one haploid set was absent from all cells, which was interpreted not as a manifestation of Ig allelic exclusion, but rather as a phenomenon of genome haploid set suppression in certain triploid combinations. Our data in a mammalian model, however, are clearly the end result of an active allelic exclusion mechanism of somatic rearrangement. First, the fact that Ts12 embryos are exencephalic and die in utero suggests a gene-dosage effect due to the presence and expression of alleles from the extra chromosome 12. In addition, we found that the paternal or maternal disomy is not influencing the ratio of haplotype a- vs haplotype b-expressing cells, which argues against an imprinting phenomenon. Finally, in the characterization of the rearrangement status of the alleles, we demonstrate that, globally and within each cell, all three loci are capable of undergoing somatic rearrangement.

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<table>
<thead>
<tr>
<th>Expt. 1</th>
<th>RFI</th>
<th>RFI*</th>
<th>RFIII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euploid ab B cells</td>
<td>17 (65%)</td>
<td>2 (8%)</td>
<td>7 (27%)</td>
</tr>
<tr>
<td>Ts12 aab B cells</td>
<td>23 (55%)</td>
<td>7 (17%)</td>
<td>12 (29%)</td>
</tr>
<tr>
<td>Expt. 2</td>
<td>RFI</td>
<td>RFI*</td>
<td>RFIII</td>
</tr>
<tr>
<td>Euploid ab IgMb cells</td>
<td>19 (58%)</td>
<td>3 (9%)</td>
<td>11 (33%)</td>
</tr>
<tr>
<td>Ts12 aab IgMb cells</td>
<td>19 (60%)</td>
<td>4 (12%)</td>
<td>9 (28%)</td>
</tr>
<tr>
<td>Total</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Euploid</td>
<td>36 (61%)</td>
<td>5 (8%)</td>
<td>18 (31%)</td>
</tr>
<tr>
<td>Ts12</td>
<td>42 (57%)</td>
<td>11 (15%)</td>
<td>21 (28%)</td>
</tr>
</tbody>
</table>

a RFI with stop codons in the junction were excluded from the analysis, and RF frequency biases due to JH usage are assumed to be negligible.

The observed difference between RFI total frequencies from euploid and Ts12 cells was found to be nonsignificant (χ² = 1.27 < χ² 0.05[1] (3.84)).
chimeras, most cells have nonproductive rearrangements in the silenced allels.

Curiously, we found a modest increase in the fraction of D-JH rearrangements carrying the D element in RFII. These are usually underrepresented in euploid cells since such rearrangements lead to the expression of a truncated μ protein capable of inhibiting further rearrangements in these cells and leading to their elimination (29, 30). The finding that Ts12 cells express an increased frequency of such rearrangements suggests that DIH and VH-DH rearrangements are not as orderly separated as in euploid cells, and that some D to JH might occur at the same time or even succeed VH to DIH recombination.

Note added in proof. The number of sequences from BALB/c IgH alleles was increased to a total of 22. Of these, 5 are + rearrangements (according to the family of the VH gene, the numbers of + to − rearrangements were 1:6 (4558), 2.5:7 (1783), 2.5:Q52), and 0.1 (MRL-DNA-4). The number of VH pseudogenes may be underestimated because sequence analysis of the amplicons does not detect mutations in VH regulatory regions or in coding sequences upstream of those amplified by PCR.

Acknowledgments

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References