Cutting Edge: Absence of Expression of RAG1 in Peritoneal B-1 Cells Detected by Knocking into RAG1 Locus with Green Fluorescent Protein Gene

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It has been proposed that Ig gene rearrangement in the peritoneal cavity (Pc) B-1 cells might be involved in autoantibody generation. To study possible secondary B cell maturation, we prepared mice carrying a target integration of gfp gene into a rag1 locus (rag1/gfp mice). The GFP* cells express rag1 mRNA and are undergoing Ig gene rearrangement. RAG1 expression was studied in Pc B-1 cells to detect cells during the stage of Ig gene rearrangement. In contrast to previous reports, Pc B-1 cells did not show RAG1 expression in adolescent or elderly mice. RAG1 expression was not induced in Pc B-1 cells in vivo after stimulation by oral or i.p. administration of LPS. Our results suggest that RAG1 expression in Pc B-1 cells is inhibited for a long period under normal condition and that this suppression is an essential state which maintains allelic exclusion of Ig genes. The Journal of Immunology, 1999, 163: 6355–6359.

B cells are generated from hematopoietic precursors by a specific molecular mechanism regulating rearrangements of Ig genes (1, 2). Expression of recombinase components RAG1 and RAG2 is also regulated for the repertoire formation of a set of B cell clones generated in primary lymphoid organs. Reconstitution studies of various progenitor sources showed functionally distinct B cell populations as B-1 cells, derived from progenitors that are present in fetal omentum and fetal liver but are largely absent from adult bone marrow (BM) (3, 4). In contrast to conventional B cells (term B-2 cells), which are replenished throughout life by differentiation of unrearranged progenitors based in the BM, B-1 cells maintain their numbers in adult animals by self-replenishment (4). The B-1 repertoire is fixed early in development and becomes progressively restricted as animals age, because new entrants to the B-1 pool are prevented (due to the feedback mechanism), and clonal populations expand to occupy a progressively greater proportion of the pool. Many studies suggested that autoantibody-producing cells are generated from B-1 cells in autoimmune-prone mice and in autoantibody-producing transgenic (TG) mice (5, 6).

A recent paper demonstrated an increase of rag mRNAs in Pc B-1 cells from normal and the autoimmune New Zealand Black (NZB) mice (7). The results did not agree with the previous observation that B-1 cells possess a rather restricted range of Ab repertoire, that had been created before the recruitment from fetal liver or omentum of the Pc. The finding of the increased rag mRNAs in B-1 cells suggested a mechanism for secondary repertoire formation in the B-1 pool. The possibility of continuous Ig rearrangement could potentially be a major factor for leading to generation of autoantibody-producing B-1 cells. Therefore, we designed experiments to study in vivo mechanisms of induction of RAG1 reexpression in Pc B-1 cells.

Materials and Methods
Targeting vector
A 9.0-kb rag1 fragment was subcloned into pBluescript II KS(−). EcorI/StuI fragment of rabbit β-globin poly(A) gene from pCXN2 (8) was ligated to EcoRI-StuI site of pEGFP (GFP). We prepared an EpsI-CA-NcoI sequence into BamHI-EcoRI of vector, to which a NcoI-HindIII of pEGFP poly(A) was ligated. A 1.3-kb EpsI-Apal GFP was ligated with endogenous rag1 EpsI and the Apal of pBluescript II. The sequence (CAAC) from EpsI to the ATG codon of rag1 was replaced with the sequence (CACC), giving a 1-bp difference in the 5′-flanking sequence.

Establishment of GFP knock-in mice
TT2 were used for rag1/gfp knock-in embryonic stem (ES) cell (8). Genomic NcoI/BamHI-digested DNAs were hybridized with AM or 3U (see Fig. 1A) from rag1 cDNA (1933–3115 bp and 3489–4706 bp). Mice were screened by PCR using RAG1-5-2; 5′-AGGTAGCTTAGCCAA-3′, primer R6 and GFP3 (9).

Flow cytometry
The mAbs are: PE-anti-mouse (m) B220 (RA3-6B2; PharMingen, San Diego, CA), biotin-anti-mCD43 (S7), biotin-anti-mIgM (R6-60.2; PharMingen), and biotin-anti-mCD5 (53-7-3; PharMingen). Streptavidin-RED670 (Life Technologies, Rockville, MD) was used.

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3Abbreviations used in this paper: BM, bone marrow; GFP, green fluorescent protein; Pc, peritoneal cavity; TG, transgenic; LM-PCR, ligation-mediated PCR.
RNA and RT-PCR

B220<sup>+</sup> cells by MACS beads were sorted into GFP<sup>+</sup> or GFP<sup>−</sup> cells on FACSVantage. RNAs from sorted cells were analyzed by RT-PCR. The cDNAs were amplified for rag1 with F3 and R6 (9), for gfp with F3 and GFP3; 5'-GCTCAGGTAGTGGTTGTCGG-3', for hprt with HPRT5; and 5'-GCTGGTGAAAAGGACCTC-3'.

Ligation-mediated PCR (LM-PCR)

Genomic DNA was ligated to BW linker at 20 pmols (10) and PCR (11) was carried out using the following primers: BW-1H and MuO 2 for the first PCR; BW-1H and MuO for the nested PCR. The blot was hybridized with the probe between primers. Control was with CD14 (10).

Results and Discussion

Establishment of mice with the GFP knock-in to rag1 gene

The gfp knock-in to the coding region of one endogenous allele of the rag1 gene (Fig. 1A) will potentially represent results in expression of both GFP and RAG1 in the identical tissue-specific and cell stage-dependent patterns in lymphoid lineage cells. Two lines screened by PCR were confirmed by Southern blot analysis (Fig. 1B). The 3UT and AM showed the adequate integration into rag1 gene as seen with the 6.1-kb band by Ncol and BamHI, and the hybridization was with AM or 3UT. The probes show bands at 8.2 kb of the wild-type and 6.1 kb of the targeted allele. C, PCR of mouse tail DNA. Genomic DNA was amplified with primers of RAG1-5-2, R6, and GFP3. Normal and the targeting alleles were shown with either pair of RAG1-5-2 and R6 (515 bp) or RAG1-5-2 and GFP3 (626 bp). D, RT-PCR with BM RNAs were transcribed into cDNAs and amplified with primers for rag1, gfp, and hprt.

FIGURE 1. Generation of rag1/gfp knock-in mice. A, A targeting vector is compared with the rag1 locus and the homologously recombined allele. DTA is a diphtheria toxin A gene for selection, A+T is the mRNA destabilizing sequence of G-CSF, and pau indicates a pausing signal of minute virus of mice (MVM) (8). The hatched box is the RAG1 coding region. PCR was conducted with neo1, 5'-TCGTGCTTTACGG TATCGCCGCTCCCGATT-3'; and control 2, 5'-CTGGCCTCACAAGGCTTCAG GCAATCTC-3'. B, Southern blot analysis of genomic DNA. DNAs were digested with NcoI and BamHI, and the hybridization was with AM or 3UT. The probes show bands at 8.2 kb of the wild-type and 6.1 kb of the targeted allele. C, PCR of mouse tail DNA. Genomic DNA was amplified with primers of RAG1-5-2, R6, and GFP3. Normal and the targeting alleles were shown with either pair of RAG1-5-2 and R6 (515 bp) or RAG1-5-2 and GFP3 (626 bp). D, RT-PCR with BM RNAs were transcribed into cDNAs and amplified with primers for rag1, gfp, and hprt.
65.63% (G2), 89.68% (G3), and 55.25% (G4) (Fig. 2A). We next examined the capability of GFP cells to mediate rearrangement of Ig genes. B220+ cells of heterozygous rag1+/gfp+ mice were separated by GFP as BM+ and BM−. The endogenous rag1 mRNA was detected abundantly in GFP+ cells (Fig. 2B). Recombinase activity was measured by LM-PCR of GFP+ and GFP− cells (Fig. 2C). PCR products indicating the occurrence of DJ rearrangement were detected in GFP+ cells at various sizes. BM− cells and GFP− B220+ spleen cells did not show any signal. BM cells from wild-type littermate showed a single band of DJ rearrangement but the homologously replaced (gfp+/gfp+) mice showed no rearrangements. These results indicate that the GFP expression is regulated under the endogenous rag1 promoter, suggesting that GFP+ cells are undergoing Ig gene rearrangement.

**Pc B-1 cells do not express RAG1 signal in normal mice**

We studied the expression of RAG1 in B-1 cells. Pc B cells are composed of two types: B220lowIgM^{+}CD5+ (B-1a or B-1) cells and B220hiIgM^{+}CD5− (B-2) cells. RAG1/GFP is detected in B-2 cells (Fig. 3A). The B-2 cells are presumably continuously supplied with newly rearranged B cell immigrants from the BM as that are detected before the GFP protein decays below detectable levels. These freshly repopulating cells make up ~15–45% of the B-2 pool in the Pc. The GFP+ cells in the B-2 population did not express transcripts of rag1 or gfp gene (data not shown), indicating that the signal of RAG1/GFP is more sensitive to chase newly generated B cells than the measurement by RT-PCR. There are no RAG1/GFP+ cells (<1.5%) in the B-1 cells. This is consistent with the idea that the B-1 pool was formed presumably when the mice were neonates earlier stage and has been maintained for the longer period without rag1 gene expression in the Pc for a length of time sufficient for GFP protein to decay below the limits of detection. The result that very few B-1 cells showed RAG1/GFP expression is surprising because a recent report conversely demonstrated an increase of rag1 mRNA in Id negative B-1 cells of mice carrying replacement of IgVH and IgVL (7). We used the same mAbs to gate the B-1 cells and the cells also showed CD5, indicating that the Pc B-1 cells from 6-wk-old mice showed a very low level of RAG1 under special pathogen free conditions (Fig. 3A). The B-1 cell is maintained its pool by self-replenishment with the reduced recombination activity in the Pc.

It is possible that B-1 cells from older mice may show the induction of RAG1 during aging of the B-1 pool either by the re-population of newly generated B-1 cells or by activation of secondary Ig gene rearrangement as observed in the germinal center (GC) region (9, 12). Therefore, we evaluated the effect of aging on
the regulation of RAG1/GFP expression in B-1 cells. The Pc B-1 cells from 6-mo-old mice did not show any induction of RAG1/GFP (Fig. 3B). While RAG1/GFP expression in the B-2 pool is detected as 33.15% in the adolescent mice, the RAG1/GFP expression is absent in Pc B-1 cells of both adolescent and elderly mice. RT-PCR detected neither \( \text{rag1} \) nor \( \text{rag2} \) transcripts in the B-1 cells (data not shown). Administration of LPS activates Pc B-1 cells and causes the induction of autoantibody production or exacerbation of autoimmune symptoms in the TG mouse model (13). Oral administration of LPS caused an increase of Pc cells in B-1 population; 56% of the control mice (from 3.20 to 4.98); however, we did not detect any increase of RAG1/GFP expression in B-1 cells ( Table I ). We further examined the effect of LPS on RAG1/GFP expression by direct administration into the Pc. Stimulation with LPS did not induce reexpression of RAG1/GFP in Pc B-1 cells in vivo, although we detected a slight increase of GFP\(^+\) cells in B-2 population (from 17.48 to 20.04% by oral administration of LPS and from 10.85 to 12.16% by i.p. injection in vivo). These results suggest that the Pc B-1 cell pool that is maintained by self-replenishment is presumably stable in the Ab specificity because Ig gene recombination is suppressed in normal mice.

A similar chase study of RAG2 expression in mature B cells using \( gfp \)-TG and knock-in mice was reported recently (14–16). The mice showed that RAG2 was observed at the lower level in immature B cells of the spleen but an antigenic challenge would not easily induce reexpression of RAG2. We also examined RAG1/GFP expression after stimulating B-1 cells in vitro with various B cell activators, but none of the attempt was successful for induction of RAG1 at the detectable level (data not shown). These results might not agree with recent observations of frequent expression of RAG and the induction of secondary Ig gene rearrangements in the germinal center by stimulation with T-dependent Ag (9, 12).

The \( \text{rag1}/\text{gfp} \) knock-in mice showed that a single copy \( \text{rag1} \) promoter mediated expression of GFP that is sufficient to mark the RAG1 expressing B-1 cells in vivo. Our results provide important

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**FIGURE 3.** GFP expression in B-1 or B-2 cells in the Pc. A, Pc cells were isolated from adolescent mice (4 – 8 wk) and stained with mAbs against B220, IgM, and CD5. B-1 and B-2 cells were gated with expression of IgM\(^{\text{high}}\)B220\(^{\text{low}}\) or CD5\(^{\text{high}}\)B220\(^{\text{low}}\) as B-1 cells, and IgM\(^{\text{low}}\)B220\(^{\text{high}}\) or CD5\(^{\text{low}}\)B220\(^{\text{high}}\) as B-2 cells. GFP of gated cells is shown and the number indicates the percentage of GFP\(^+\) cells. B, GFP\(^+\) cells in Pc B-1 and B-2 cells from adolescent or elderly mice are shown.
information that is in contrast with previous reports of rag1 gene up-regulation in Pc B-1 cells (7). The postulated association of RAG reexpression with the tendency of autoreactivity of the B-1 repertoire have now need to be re-evaluated at least in the Pc B-1 cells.

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References


Table I. Effect of administration of LPS in vivo upon expression of RAG1/GFP in peritoneal B-1 and B-2 cells

<table>
<thead>
<tr>
<th>Route of Administration</th>
<th>No. of Cells</th>
<th>GFP Positive Cells (%) in</th>
<th>No. of Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral</td>
<td>PBS</td>
<td>3.20 ± 0.62</td>
<td>1.78 ± 1.29</td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>4.98 ± 1.03</td>
<td>1.42 ± 1.07</td>
</tr>
<tr>
<td>i.p.</td>
<td>PBS</td>
<td>2.00</td>
<td>2.28</td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>4.00</td>
<td>1.00 ± 0.48</td>
</tr>
</tbody>
</table>

* Oral and i.p. administration of LPS was carried out as described (13), and the effect was confirmed in each mouse by the increase of Pc in comparison to the control of PBS administration. The results are shown as the mean with or without 1 SE.

The percentage of RAG1/GFP positive cells is shown after gating the peritoneal B-1 and B-2 cells as described in Materials and Methods.

a Number of cells recovered from peritoneal cavity × 10^6.