Reexpression of RAG-1 and RAG-2 Genes in Activated Mature Mouse B cells

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Recombination activating genes (RAG-1 and RAG-2), involved in V(D)J rearrangement of immunoglobulin genes, have been thought to be expressed only in immature stages of B-cell development. However, RAG-1 and RAG-2 transcripts were found to be reexpressed in mature mouse B cells after culture with interleukin-4 in association with several different co-stimuli. Reexpression was also detected in draining lymph nodes from immunized mice. RAG-1 and RAG-2 proteins could be detected by immunofluorescence microscopy in the nuclei of B cells cultured in vitro and in the germinal centers of draining lymph nodes. These findings suggest that RAG gene products play a heretofore unsuspected role in mature B cells.

The rearrangement of immunoglobulin (Ig) and T cell receptor (TCR) genes is a crucial step in the maturation process of B cells and T cells. Recombination of Ig and TCR genes is catalyzed by the products of the two recombination activating genes, RAG-1 and RAG-2 (*1–3*). A defect in either RAG-1 or RAG-2 results in the retention of Ig and TCR loci in germline configuration and blocks the development of mature lymphocytes (*4*). RAG-1 and RAG-2 have been considered to be expressed exclusively in immature lymphocytes in bone marrow and thymus, being readily down-regulated in mature lymphocyte populations, such as IgD^+ B cells and TCR^+ T cells (*2, 5, 6*). RAG expression in early B cells occurs in two waves, the first being responsible for V(D)J (V, variable; D, diversity; J, joining) rearrangement of Ig heavy-chain genes in proB and preB-I cells and the second for VJ recombination of Ig light-chain genes in small preB-II cells (*5*). We describe a third wave of RAG-1 and RAG-2 expression, induced in activated mature B cells in vitro and in vivo.

Spleen B cells from C3H/HeN mice cultured for 2 days with lipopolysaccharide (LPS) plus interleukin-4 (IL-4) expressed RAG-1 and RAG-2, as assessed by the combination of reverse transcriptase–polymerase chain reaction (RT-PCR) and Southern (DNA) blotting (Fig. 1A). The presence of bands of correct size was strictly dependent on RT, indicating that PCR-amplified materials were not derived from contaminated genomic DNA. RAG-1 and RAG-2 expression was not detected in the cells before the culture, in unstimulated B cells, and in cells stimulated with either LPS or IL-4 alone. LPS combined with other cytokines—including IL-2, IL-3, or IL-5—was ineffective (*7*). To confirm that surface IgD^+ ($sIgD^+$) mature B cells respond to LPS plus IL-4, we undertook similar experiments in B cells purified by panning on plates coated with monoclonal antibody (mAb) to IgD (Fig. 1B). These enriched B-cell preparations also expressed RAG genes in response to LPS plus IL-4.

Various stimuli other than LPS were examined for their capacity to elicit the expression of RAG genes in the presence of IL-4 (Fig. 1C). Monoclonal antibody to CD40 was as effective as LPS, and antibody to μ heavy chain plus 8-mercaptoguanosine, a potent B-cell activator (*8*), was also effective; both these stimuli were ineffective in the absence of IL-4. The same stimuli—IL-4 with LPS, with mAb to CD40 and with antibody to μ heavy chain plus 8-mercaptoguanosine—also cause B cells to switch the isotype of secreted Igs from IgM to IgG1 and IgE (*9*). Thus, it is likely that Ig class switching and RAG expression require a similar B-cell activation status.

Can in vitro RAG expression be reproduced in vivo? Mice were immunized with the antigen trinitrophenyl–keyhole limpet hemocyanin (TNP-KLH) in the hind footpads, and the draining lymph nodes (LN) were examined for the expression of RAG-1 and RAG-2. Inguinal and popliteal LN cells expressed these gene transcripts on day 6 and day 8 postimmunization (Fig. 2).

To further confirm RAG gene expression, we performed immunofluorescence microscopy, using mAbs to RAG-1 and RAG-2 (Fig. 3). These mAbs detected RAG-1 and RAG-2 proteins in mouse thymocytes used as a positive control (*5, 10*). More than 80% of the B cells that were stimulated in vitro with LPS plus IL-4 stained with both mAbs; unstimulated B cells and isotype-matched control mAb showed no positive immunofluorescence. RAG-1– and RAG-2–positive cells were not detected before culture. About 70% of the B cells used were recovered as viable cells at the end of the culture period. Taken together, these observations suggest that the development of RAG-expressing cells was not due to the expansion of a small number of immature B cells possibly present in the initial B cell preparation.

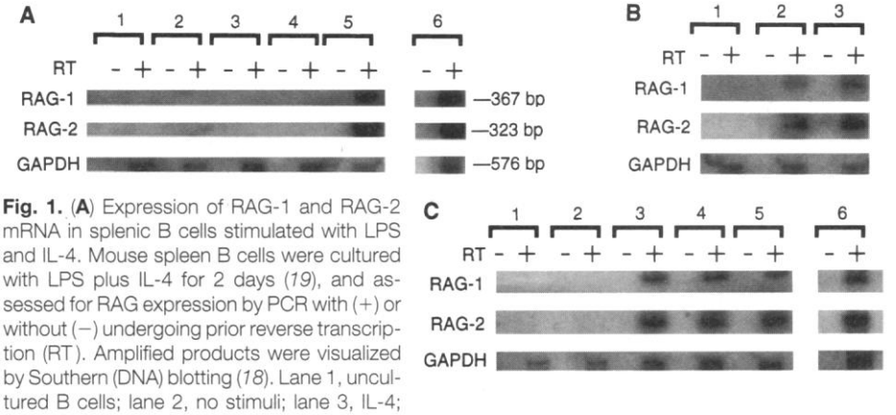

Fig. 1. (**A**) Expression of RAG-1 and RAG-2 mRNA in splenic B cells stimulated with LPS and IL-4. Mouse spleen B cells were cultured with LPS plus IL-4 for 2 days (*19*), and assessed for RAG expression by PCR with (+) or without (−) undergoing prior reverse transcription (RT). Amplified products were visualized by Southern (DNA) blotting (*18*). Lane 1, uncultured B cells; lane 2, no stimuli; lane 3, IL-4; lane 4, LPS; lane 5, LPS plus IL-4; and lane 6, positive control (thymocytes). IL-4-dependent RAG expression was observed reproducibly in at least 10 separate experiments, and a representative result is shown. (**B**) Purified $sIgD^+$B cells express RAG-1 and RAG-2 mRNA when stimulated with LPS and IL-4. $sIgD^+$B cells were purified by panning on culture plates coated with mAb to IgD (*20*) and stimulated with (lane 2) or without (lane 1) LPS plus IL-4 as described above. Lane 3 shows the positive control (thymocytes). (**C**) Effects of various B-cell stimuli on the induction of RAG-1 and RAG-2 expression. Mouse spleen B cells were cultured for 2 days with the following stimuli (*19*): Lane 1, no stimuli; lane 2, IL-4; lane 3, LPS plus IL-4; lane 4, mAb to CD40 plus IL-4; lane 5, antibody to μ heavy chain, 8-mercaptoguanosine, and IL-4; and lane 6, positive control (thymocytes).

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Cryosections of draining LN from mice immunized with TNP-KLH were stained with mAb to RAG-1 (Fig. 3B). RAG-1–positive cells were detected in inguinal and popliteal LN sections. Germinal center (GC) B cells were clearly visualized by staining with fluorescein isothiocyanate–conjugated peanut agglutinin (FITC-PNA) (*11*). RAG-1 products appeared to be expressed almost exclusively within the FITC-PNA–binding GC populations and were not present in the marginal zone around GC. PNA- and RAG-1–positive cells were not detected in the unimmunized LN sections. Further, an observation has been made which suggests that RAG genes are expressed in tingible body cells in GC (*12*).

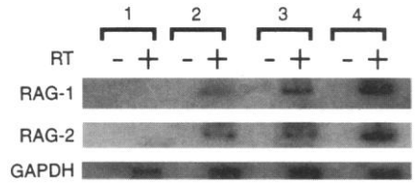

Fig. 2. Expression of RAG-1 and RAG-2 in draining LN of TNP-KLH–immunized mice. Mice were immunized with TNP-KLH in the hind footpads (*19*). Inguinal LN cells were assessed for RAG expression on day 0, 6, and 8 postimmunization, as indicated in Fig. 1 (*18*). Lane 1, day 0; lane 2, day 6; lane 3, day 8; and lane 4, positive control (thymocytes).

It has been reported that RAG genes are expressed in some sIg-positive B cell lines with some mature phenotypes (*13*), and a trace amount of RAG transcripts was detected in normal mouse lymphoid tissues (*14*). The latter may have been because of a small number of immature B cells. However, we have now shown that significant RAG-1 and RAG-2 gene expression occurs, both in primary culture of mature B cells and in the peripheral lymphoid tissues of immunized animals.

In mice transgenic for genes encoding autoantibodies to DNA (*15*) or H-2K (*16*), B cells that escaped deletion after encountering the self antigen bear altered sIg receptors. These receptor modifications (termed receptor editing) were shown to accompany RAG expression in bone marrow cells, and are believed to contribute to the removal of autoreactive B cells during earlier developmental stages (*16*).

It is interesting to note that RAG expression was induced by an antigen in GC where Ig class switching, somatic hypermutations, and the selection process for affinity maturation take place (*17*). Although further investigations are necessary to prove that RAG gene products induced under these experimental conditions are functional, it is possible that RAG-dependent revision of Ig genes can occur in mature B cells.

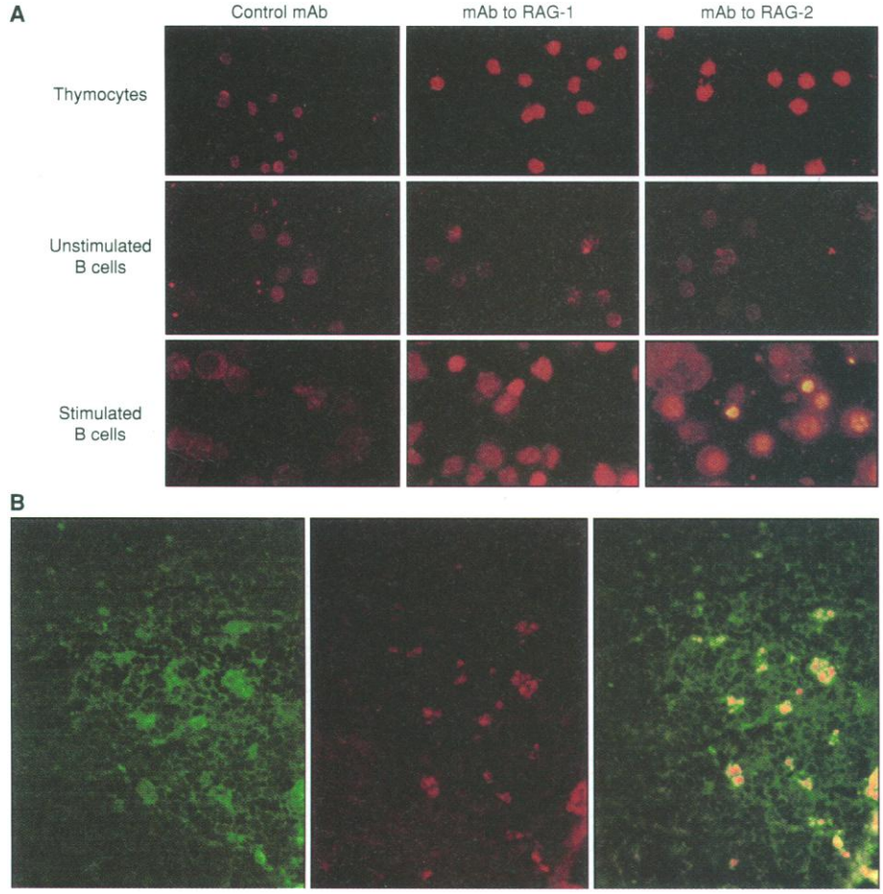

Fig. 3. Immunofluorescence analysis of RAG-1 and RAG-2 expression in cultured B cells and in draining LN. (**A**) Isolated thymocytes, B cells cultured without stimuli, and B cells stimulated with LPS plus IL-4 for 2 days (*19*) were reacted with biotinylated irrelevant IgG2b, mAb to RAG-1, or mAb to RAG-2, followed by staining with rhodaminated avidin (*21*). (**B**) Cryosections of inguinal LN from mice immunized with TNP-KLH and alum (*19*) were prepared on day 8 postimmunization and were reacted with biotinylated mAb to RAG-1, followed by double-staining with rhodaminated avidin and FITC-PNA (*21*). Irrelevant IgG2b gave much weaker rhodamine fluorescence. (Left), FITC-PNA; (middle), mAb to RAG-1; (right), superimposed image of (left) and (middle). Magnification ×200.

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18. Total RNA was extracted from 1 × 10^6 cultured B cells by the RNA Zol B method as described [P. Chomczynski and N. Sacchi, *Anal. Biochem.* **162**, 156 (1987)]. The extracted RNA preparations were reverse transcribed, and the resultant cDNA was amplified by PCR with the following sense and antisense primers: 5′-ATGGCTGCCTCCTTGCCGTCT-3′ and 5′-GTATCTCCGGCTGTGCCCGTC-3′ for RAG-1, 5′-ATGTCCCTGCAGATGGTAACA-3′ and 5′-TAAATCTTATCGGAAAGCTCA-3′ for RAG-2, and 5′-CCATCACCATCTTCCAGGAG-3′ and 5′-CCTGCTTCACCACCTTCTTG-3′ for GAPDH. PCR conditions were 27 cycles of 1 min at 95°C, 2 min at 60°C, and 2 min at 72°C for RAG-1 and RAG-2; and 20 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C for glyceraldehyde phosphate dehydrogenase

(GAPDH). These PCR cycles were confirmed to be in the exponential phase of the amplification. PCR products were electrophoresed on 7.5% polyacrylamide gel and visualized by Southern (DNA) blotting using ^{32}P-labeled probes, the Dde I–Dde I 163–base pair (bp) internal fragment of RAG-1 cDNA, the Pst I–Hinf I 124-bp internal fragment of RAG-2 cDNA, and the entire coding region of GAPDH cDNA. Hybridized filters were exposed to Fuji imaging plate (Fuji Film) for 2 days and visualized by a Bioimaging Analyzer, BAS 1000 (Fuji Film). RAG-1 and RAG-2 cDNAs were given by D. G. Schatz (Yale University, New Haven, CT).

19. Mouse B cells were prepared by treating spleen cells from male C3H/HeN mice (8 to 10 weeks of age, Japan Charles River) with 1/1000-diluted mAb to Thy 1.2 mAb (SeroTec), followed by incubation with low-toxic rabbit complement (Cederlane) as described [K. Haruna *et al.*, *Cell. Immunol.* **151**, 52 (1993)]. The B cells (3×10^6 cells per milliliter) were cultured with LPS (20 μg/ml) from *Escherichia coli* 055 B5 (Sigma) and mouse recombinant IL-4 (500 U/ml; PeproTech) in 1 ml of RPMI-1640 medium containing 10% fetal bovine serum, 10 μM 2-mercaptoethanol, penicillin G (100 U/ml), and streptomycin (50 μg/ml). In some cases, mAb to mouse CD40 (1 μg/ml; rat mAb LB429 presented by N. Sakaguchi, Kumamoto University, Japan) or F(ab')$_2$ fragment of goat antibody to mouse μ heavy chain (10 μg/ml; Cosmo Bio) plus 1 mM 8-mercaptoguanosine (Sigma) was used as a stimulus. Because it was confirmed that RAG expression peaked on day 2 of the culture and declined thereafter, all cultures were carried out for 2 days. In in vivo experiments, mice were immunized with 20 μg of TNP-KLH and 0.45 mg of alum in each hind footpad. Inguinal or popliteal LN cells from three mice were pooled on day 0, 6, and 8 postimmunization and assessed for the expression of RAG-1 and RAG-2 mRNA (*18*).
20. Culture plates of 100 mm diameter were coated with 100 μg/ml of mAb to mouse IgD (Biosys). Sixty million mouse spleen cells that had been depleted of T cells and erythrocytes were placed in the plate and incubated for 1 hour at room temperature. Then the plate was gently washed with phosphate-buffered saline four times to remove nonadherent cells. It was confirmed by flow cytometric analysis (FACScan) that adherent cells recovered from the plate were more than 99% positive for sIgD and B220.
21. Monoclonal antibodies to mouse RAG-1 (G109-256.2, mouse IgG2b) and RAG-2 (G110-461, mouse IgG2b) were obtained from Pharmingen. A myeloma-derived murine IgG2b (MOPC 195) was used as an irrelevant negative control (ICN Biomedicals). These mAbs were biotinylated under the same conditions, using a biotinylation kit (American Qualex). Staining of RAG proteins in thymocytes or cultured B cells was carried out as described (*10*), with some modifications. Briefly, the cultured cells were fixed on glass slides in methanol-acetone (1:1) for 5 min, rehydrated in phosphate-buffered saline, and preblocked for 1 hour with TBST [10 mM tris (pH 8.0), 150 mM NaCl, and 0.05% Tween 20] containing 1% bovine serum albumin (BSA) and MOPC 195 (50 μg/ml). The slides were then incubated in TBST containing 1% BSA and one of each biotinylated mAb (5 μg/ml) for 1 hour at room temperature. Slides were then washed three times in TBST and reacted for 1 hour with rhodaminated avidin (2 μg/ml; Sigma) in TBST containing 1% BSA. For the immunofluorescent staining of LN sections, 6-μm-thick cryosections mounted on slides were allowed to air dry for 15 min and were fixed in ice-cold acetone for 10 min. After rehydration and preblocking as described above, the sections were treated with biotinylated anti–RAG-1 (5 μg/ml) for 1 hour, followed by double-staining with rhodaminated avidin (2 μg/ml) and FITC-PNA (4 μg/ml; Seikagaku Kogyo) for 40 min. All reagents were diluted in TBST containing 1% BSA. After washing with TBST, samples were finally mounted with low-fluorescent glycerol and cover slip protection, and were observed with a Zeis fluorescence microscope.
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Neoteny in Lymphocytes: *Rag1* and *Rag2* Expression in Germinal Center B Cells

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The products of the *Rag1* and *Rag2* genes drive genomic V(D)J rearrangements that assemble functional immunoglobulin and T cell antigen receptor genes. Expression of the *Rag* genes has been thought to be limited to developmentally immature lymphocyte populations that in normal adult animals are primarily restricted to the bone marrow and thymus. Abundant RAG1 and RAG2 protein and messenger RNA was detected in the activated B cells that populate murine splenic and Peyer's patch germinal centers. Germinal center B cells thus share fundamental characteristics of immature lymphocytes, raising the possibility that antigen-dependent secondary V(D)J rearrangements modify the peripheral antibody repertoire.

Periodic expression of the recombination-activating genes *Rag1* and *Rag2* controls the assembly of immunoglobulin (Ig) genes and defines the principal stages of B lymphopoiesis in the bone marrow (*1*). Transcription of the *Rag* genes ends with the expression of competent Ig on the surface of immature B cells, precluding further V(D)J recombination in the mature lymphocyte pool (*2*). However, we and others have found that lymphocytes in germinal centers (GCs) exhibit features of immature T and B cells, including the expression of membrane markers typically present on developing lymphocytes (*3*) and exquisite sensitivity to activation-induced apoptosis that is independent of the Fas molecule (*4*). Perhaps most remarkable is the similar spectrum of nucleotide exchanges introduced during antigen-driven V(D)J hypermutation in murine GCs and by the developmentally regulated generation of point mutations in the Ig genes of B cells in ileal Peyer's patches (PP) of fetal lambs (*5*).

Germinal centers are sites of antigen- and T cell–dependent cellular reactions that develop in secondary lymphoid tissues. Germinal centers are necessary for immunological memory in the B cell compartment (*6*, *7*) and are the site of V(D)J hypermutation and selection that is required for affinity maturation of antibody responses (*8*). Two populations of B lymphocytes, the mitotically active Ig$^-$ centroblasts and the nondividing Ig$^+$ centrocytes, make up the majority of GC cells; centrocytes arise from centroblasts, and in turn, some centrocytes reenter the proliferating pool (*3*, *9*). Evidence suggests that the centrocyte population is subject to selective apoptosis (*4*). Splenic GCs first appear 4 to 5 days after primary immunization and may be identified by their distinctive ability to bind peanut agglutinin (PNA$^+$) and the monoclonal antibody GL-7 (GL-7$^+$) (*3*). The GC reaction is transient, peaking by day 12 of the response and waning after 3 weeks (*9*). In contrast, GCs are constitutively present in murine PPs, being chronically stimulated by food antigens and the gut flora (*10*).

To determine if the immature character of GC B cells extended to the level of *Rag* expression, we used affinity-purified antibodies specific for active RAG1 and RAG2 proteins (*11*) to label histologic sections of spleen and PP from immunized and normal mice (*12*). Mature GCs, those present in spleen 16 days after immunization (Fig. 1) or in the PPs of unimmunized mice (Fig. 2), contain PNA$^+$, GL-7$^+$ B cells that express substantial amounts of immunoreactive RAG1 and RAG2 protein. The distribution of labeled cells coincided with the distribution of B7-2 expression, suggesting that RAG proteins are predominantly expressed in the centrocytes of the GC light zone (*7*). Virtually identical staining patterns for immunoreactive RAG1 were achieved with rabbit IgG specific for the NH_2-terminal residues of RAG1 and a murine monoclonal antibody that binds to the COOH-terminal region of RAG1 (*13*). Histologic demonstration of RAG2 was more difficult, even in sections of thymus, a site of active V(D)J recombination and high RAG expression (*1*). Two rabbit antibodies were used to localize RAG2 protein; one, made against amino acids 70 through 516 of murine RAG2, gave equivocal labeling, whereas the other (antibody 435), specific for a 20–amino acid stretch of RAG2 (*13*), adequately labeled both GCs (Figs. 1C and 2B) and cortical thymocytes.

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