Brief Definitive Report

Rearrangement of \( \lambda \) Light Chain Genes in Mature B Cells In Vitro and In Vivo. Function of Reexpressed Recombination-activating Gene (RAG) Products

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Summary

V(D)J (V, variable; D, diversity; J, joining) combination of immunoglobulin (Ig) genes established in premature B cells has been thought to be conserved throughout differentiation at mature stages. However, germinal center (GC) B cells have been shown to reexpress recombination-activating gene (RAG)-1 and RAG-2 proteins in immunized mice. Here, we present several lines of evidence indicating that RAG proteins thus induced are functional as the V(D)J recombinase. DNA excision product reflecting \( \lambda_1 \) to \( J_1 \) rearrangement was generated in parallel with the expression of RAG genes in mature mouse B cells that were activated in vitro with LPS and IL-4. Similar \( \lambda \) chain gene rearrangement was observed in the draining lymph node of immunized mice. Further, B cells that underwent \( \lambda \) gene rearrangement were shown by in situ PCR to be localized within GCs. Thus, secondary rearrangement of Ig genes (receptor editing) can occur in mature B cells.

Materials and Methods

Preparation and Culture of Mouse B Cells. Spleen cells from male C3H/HeN mice (7–9 wk of age; Japan Charles River, Kanagawa, Japan) were treated with 1/1,000-diluted anti-Thy 1.2 mAb (SeroTec, Kidlington, UK) followed by incubation with 20 \( \mu \)g/ml LPS from Escherichia coli 055 on May 1, 2019 jem.rupress.org Downloaded from http://doi.org/10.1084/jem.187.5.795 Published Online: 2 March, 1998 | Supp Info:
Analysis of RAG-2 Transcripts. Total RNA was extracted from 10^6 cells and reverse transcribed as described previously (16). The resultant cDNA was amplified by PCR using following sense and antisense primers (11, 13): RAG-2, 5'-CACATCCACAGGAGATATAC-3' and 5'-GGTTCCAGGACATCTCCTACTAAG-3'; and GAPDH, 5'-CCATCACCACCTTCCAGGAG-3' and 5'-CCTGCTTACACCCACCTTCTTG-3'. Both primer pairs span an intron to avoid the amplification of contaminating genomic DNA. To heighten the specificity of the amplification, the cycling conditions were 20 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C.

Flow Cytometric Analysis. Cultured B cells were incubated with 10 μg/ml biotinylated anti-κ or λ chain mAb at 4°C for 30 min followed by staining with streptavidin-PerCP (Becton Dickinson, San Jose, CA). The anti-λ mAb was reactive with λ1 and λ2 chains. Additionally, the cells were stained with phycoerythrin-labeled anti-B220 mAb (PharMingen, San Diego, CA) and FITC-labeled mAb to mouse IgG1 or IgM (PharMingen), respectively. Three-color analyses were carried out using AmphiTag Gold (Perkin-Elmer Corp., Foster City, CA) as the polymerase according to the manufacturer's protocol. PCR was carried out as follows for RAG-2, at 94°C for 12 min followed by 40 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min; for GAPDH, 26 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The amplified products were electrophoresed on a 7.5% polyacrylamide gel and visualized by SYBER green I (FMC BioProducts, Rockland, ME) staining. RAG-1 and RAG-2 expression in LPS/IL-4-stimulated mature mouse B cells has been confirmed by reverse transcriptase-dependent PCR (RT-PCR) followed by Southern blotting as described previously (11).

Proliferative Response of B Cells. Unseparated, κ− or λ− B cells were purified from mouse spleen B cells using immunomagnetic beads. In brief, 10^8 spleen B cells were incubated with 5 μg/ml biotinylated anti-κ or λ chain mAb to mouse κ or λ chain in 1 ml of phosphate-buffered saline at 4°C for 30 min. After washing, the cells were mixed with 1 mg/ml streptavidin-coated magnetic beads (Dynal, Oslo, Norway) and incubated at 4°C for 30 min. Rosette cells were separated by placing the tubes in magnetic particle concentrator (Dynal) for 2 min. This magnetic separation was repeated twice. Purity of each cell preparation was >99% as assessed by flow cytometry.

Enhanced Expression of λ L C chains on B Cells Stimulated with LPS Plus IL-4 In Vitro. As reported previously (11), stimulation of mature mouse B cells with LPS/IL-4, but not with LPS alone resulted in the expression of RAG-2 in vitro (Fig. 1A). It was then investigated whether LPS/IL-4-induced RAG gene products can mediate receptor editing in mature mouse B cells. One of the criteria for the occurrence of receptor editing is the induction of λ chains in κ chain−bearing B cells since this process involves de novo rearrangement of λ genes (5, 17). Thus, mouse spleen B cells were stimulated in vitro with LPS/IL-4 and examined for the induction of λ chains after the culture. Before the culture, >95% of B220− B cells were IgM+ and λ−/B220− cells were <1% of total cells (Fig. 1B). IgG1+ (1+) cells were 2.3% before culture, but increased to 15.6% after culture with LPS/IL-4 due to IL-4-induced Ig class switching (16). Interestingly, LPS/IL-4 stimulation resulted in an increase of λ+/B220+ cells from 0.94 to 4.67%, a majority of which belonged to 1+ population. Enhanced expression of λ chains was not observed in B cells stimulated with LPS alone that did not lead to RAG expression (Fig. 1A and B). B cells cultured without stimuli showed similar flow cytometric patterns to those of the original cells (not shown). When purified κ− cells (>99% pure) were stimulated with LPS/IL-4 for 4 d, 3–4% of the total cells became positive for λ chains (our unpublished result).
The formation of coding joints, and may require the cell cycle.

It has been reported that the joining of signal ends is slower than the broken signal ends and hairpin coding ends. It has been observed in B cells stimulated with LPS alone that progresses much lower level of the circular excision product was detected in B cells stimulated by LPS plus IL-4 for 2 d, and assessed for RAG-2 expression by RT-PCR.

As shown in Fig. 2A, LPS plus IL-4 stimulated B cells in which RAG-2 expression was assessed by RT-PCR. Thymocytes or bone marrow cells were used as a positive control. (B) Confirmation of an Apal restriction site in the PCR-amplified segment of Vα1-Jα1 excision product. (C) Comparison of proliferative response to LPS/IL-4 between purified λ+ B cells and κ+ B cells. Purified λ+ B cells, κ+ B cells, or unseparated B cells were cultured with or without LPS/IL-4 for 2 d followed by incubation with [3H]TdR for an additional 12 h. Data were presented as the mean of triplicate assays.

Detection of DNA Excision Product Derived from Vα1-Jα1 Rearrangement. If the expressed λ chains are produced by newly rearranged λ genes, circular DNA excision product derived from Vα-Jα rearrangement might be detected (5). Fig. 2A shows that this is the case. PCR amplification of the extracted DNA followed by Southern blotting with the probe depicted in Fig. 2B revealed that LPS/IL-4-stimulated B cells in which RAG-2 was expressed, were shown to contain a definitely higher level of DNA excision product reflecting Vα1-Jα1 rearrangement compared with unstimulated or LPS-stimulated B cells. It has been reported that the signal joint formation generates a restriction site cleavable with ApalI (18). The amplification product (302 bp) was cleaved by ApalI into two fragments with expected lengths of 229 and 73 bp, respectively (Fig. 2B), thus confirming the identity of the amplified product. The initial stage of V(D)J recombination is the generation of broken signal ends and hairpin coding ends. It has been reported that the joining of signal ends is slower than the formation of coding joints, and may require the cell cycle progression (19). Vα1-Jα1 excision product detected in LPS/IL-4-stimulated B cells, however, may not be due to the mere joining of the preexisting signal breaks since a much lower level of the circular excision product was detected in B cells stimulated with LPS alone that progresses the cell cycle, but does not induce RAG expression (11).

As shown in Fig. 2C, purified λ+ B cells and κ+ B cells showed a comparable [3H]TdR uptake either in the presence or absence of LPS/IL-4. Thus, LPS/IL-4-induced increase of λ+ B cells is not considered to be due to the preferential expansion or survival of a small number of λ+ cells present in the original B cell preparation during the culture. Taken together, λ chains expressed in response to LPS/IL-4 are thought to be generated from newly rearranged λ chain genes.

As shown in Fig. 1B, λ chains were predominantly expressed on κ+ B cells. It remains unclear whether λ gene rearrangement is undertaken more efficiently after isotype switching, or whether these two events apparently proceed in a synchronous manner due to their similar IL-4 dependence. It is interesting to note that Ku, a DNA-dependent protein kinase activator protein complex that is involved both in isotype switching (20) and in V(D)J recombination (21), was induced in mouse spleen B cells stimulated by surface Ig engagement and IL-4 (22).

Detection of Vα1-Jα1 Rearrangement in GC B Cells In Vivo. Do mature B cells also undergo λ chain gene rearrangement in vivo? We have reported that RAG-1 and RAG-2 are induced in the draining LN cells of immunized mice (11, 12). It was confirmed that popliteal LN cells expressed RAG-2 transcripts on days 6 and 8 after immunization when mice were immunized in the footpads with TNP-KLH and alum (Fig. 3A). Very interestingly, DNA excision product derived from Vα1-Jα1 rearrangement was detected by PCR in the same LN cells as those in Fig. 3A on
Table 1.

|          | Day 0 | Day 6 | Day 8 |
|----------|-------|-------|-------|
| RAG-2    | -     | -     | -     |
| GAPDH    | 472bp | 576bp | -     |

Days 6 and 8 after immunization, but not on day 0 (Fig. 3 B), suggesting that the induced \( \text{RAG} \) proteins are functional and able to mediate \( \lambda \) chain gene rearrangement in vivo. Further, the localization in the LN of the cells that underwent \( \text{V}_{\lambda}1-\text{J}_{\lambda}1 \) rearrangement was examined in the LN section by in situ PCR. On day 8 after immunization, well-developed GCs were observed in LN sections (Fig. 3 C, a and b). A majority of the cells possessing DNA excision product of \( \text{V}_{\lambda}1-\text{J}_{\lambda}1 \) rearrangement were found within GCs in which \( \text{RAG} \)-expressing B cells have been reported to be localized (11-13; Fig. 3 C, b and c). Intracellular deposition of the amplified DIG-labeled DNA fragments was observed when in situ PCR was performed in the presence of primers, but not in their absence. The primers for human IL-2 gene did not generate the DIG-labeled product either (not shown), thus indicating the specificity of the method. Thus, data presented here strongly suggest that GC B cells undergo receptor editing in parallel with \( \text{RAG} \) gene expression.

These findings and recent observations made by other investigators (14, 15) provide a new aspect in immunology that \( \text{V(D)J} \) combination established in a given premature B cell clone can be revised even at mature stages. What is a biological role of \( \text{RAG} \) gene products expressed in GC B cells? GC is a microenvironment in which somatic hypermutations, isotype switching, and clonal selection for affinity maturation of antibodies are undertaken (23, 24). Somatic hypermutations in GCs may produce not only high affinity antibodies, but also generate autoreactive B cell clones. The high affinity clones will be selected positively through interaction with follicular dendritic cells retaining immune complexes on their surface, thus leading to affinity maturation of antibodies (25). On the other hand, autoreactive clones must be either deleted or rendered anergic to maintain self tolerance. Receptor editing in mature B cells suggests another possible way to extinguish autoreactivity in GCs. We have observed that a majority of \( \text{RAG} \)-expressing B cells in GCs are apoptotic and present in tingible bodies (12). This may reflect, at least in part, a result of \( \text{RAG} \)-dependent revision of antigen receptors and their subsequent selection in GCs. Further elucidation of the role of \( \text{RAG} \) gene products in GCs will provide valuable clues to understanding the onset of autoimmune diseases, lymphomas, and other immune disorders.
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