Regulatory Role for Complement Receptors (CD21/CD35) in the Recombination Activating Gene Expression in Mouse Peripheral B Cells

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A population of peripheral B cells have been shown to express recombination activating gene products, RAG-1 and RAG-2, which are considered to be involved in revising the B cell antigen receptor (BCR) in the periphery. BCR engagement has been reported to turn off RAG expression in peripheral B cells, whereas the same treatment has an opposite effect in immature B cells in the bone marrow. In contrast to receptor editing that is involved in the removal of autoreactivity in immature B cells, it has been shown that secondary V(D)J rearrangement in peripheral B cells, termed receptor revision, contributes to affinity maturation of antibodies. Here, we show that RAG-2 expression in murine splenic B cells was abrogated by the coligation of BCR with complement receptors (CD21/CD35) much more efficiently than by the engagement of BCR alone. On the other hand, the same coligation augmented proliferation of anti-CD40-stimulated B cells. Consistent with these observations, RAG-2 expression was lower in the draining lymph nodes of the quasi-monoclonal mice when they were immunized with a high-affinity antigen than with a low-affinity one. These findings suggest a crucial role for CD21/CD35 in directing the conservation or the revision of BCRs in peripheral B cells.

1. Introduction

Recently, secondary V(D)J rearrangement (V, variable; D, diversity; J, joining) of Ig genes has been shown to occur in the spleen or the lymph node (LN) B cells of immunized mice, as a consequence of increased expression of the V(D)J recombinase, RAG-1 and RAG-2 (1-7). Several human B cell clones have been identified that are considered to have undergone secondary V(D)J rearrangement in the periphery, probably in GC (8,9). BCR engagement of immature B cells in the bone marrow results in the enhancement of RAG expression, and leads to immune tolerance by editing autoreactive BCRs, a process termed receptor editing (10). In contrast, RAG expression in peripheral B cells may not be tolerance-driven because BCR cross-linking has been shown to turn

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off its expression (11,12). Thus, V(D)J rearrangement in peripheral B cells, recently referred to as receptor revision, is predicted to contribute to the generation of high-affinity antibodies (Abs) (13,14). Recently, we have demonstrated that L chain rearrangement in murine LN B cells that occurs in the course of immune responses contributes to the generation of high-affinity Abs (15), thus suggesting strongly that receptor revision along with somatic hypermutation is responsible for affinity maturation by diversifying BCR repertoire in the periphery.

In germinal centers (GCs), B cells that acquired high-affinity BCRs are positively selected through interaction with follicular dendritic cells (FDC) that bear immune complexes, thus leading to affinity maturation of Abs (16,17). Murine B cells and FDC express the complement receptor type 1 (CR1; CD35) and type 2 (CR2; CD21) (18). B cells with high-affinity BCRs that interact strongly with immune complexes retained on FDC via their BCR and CD21/CD35 have been shown to receive survival signals (19). On the other hand, it has been reported that RAG expression was induced in murine spleen B cells by immunization with a low-affinity antigen, but to a lesser extent with a high-affinity antigen (11). In this context, it appears reasonable if RAG expression is turned off in B cells that acquired high-affinity BCR, because the high-affinity BCRs must be conserved for affinity maturation to occur. In the present report, we show that coligation of BCR with CD21/CD35 leads to abrogation of RAG expression more efficiently than BCR engagement alone in murine splenic B cells. Based on these findings, role for CD21/CD35 in the regulation of receptor revision is discussed.

2. Materials and methods

2.1. Mice
Male C3H/HeN mice (7-9 weeks of age) were purchased from Japan Charles River (Kanagawa, Japan).

2.2. Abs and other reagents
Abs and other reagents used in the present work were obtained from the following sources: F(ab')$_2$ fragment of goat anti-mouse IgM antibody (anti-μ), Organon Teknika Co. (Durham, NC); anti-CD21/CD35 mAb (7G6, rat IgG2b) and a control rat IgG2b mAb (A95-1), PharMingen (San Diego, CA); anti-mouse CD40 mAb (LB429) Seikagaku Kogyo (Tokyo, Japan); avidin, and Chicken γ-globulin (CGG) (St. Louis, MO).   Anti-μ, 7G6 and an isotype-matched control Ab of 7G6 were biotinylated using a biotinylation kit (American Qualex, San Clemente, CA) (1).

2.3. Cell Culture
B cells were prepared by treating spleen cells from C3H/HeN mice with anti-Thy 1.2 mAb plus rabbit complement as described previously (20). The B cells (3 x 10^6/ml) were cultured with 1 μg/ml anti-mouse CD40 mAb and 10 ng/ml mouse recombinant IL-4 or IL-7 (PeproTech, Princeton, NJ) for 3 days in 1 ml of RPMI-1640 medium

containing 10% fetal bovine serum (GIBCO, Grand Island, NY), 1 x 10^{-5} M 2-mercaptoethanol, 100 U/ml penicillin G and 50 μg/ml streptomycin.

2.4. Proliferative response of B cells

B cells (6 x 10^5) were cultured in quadruplicate with anti-CD40 plus IL-7 as described above in 0.2 ml of the culture medium for 48 h. Then, the cells were pulsed with 20kBq of [^{3}H]-thymidine (TdR)(Amersham Pharmacia Biotech., Tokyo, Japan) for 16 h.

2.5. Assessment of RAG-2 expression by Reverse transcriptase-dependent-PCR(RT-PCR)

Total RNA was extracted from 1x10^6 cells from cultures or isolated LNs by the RNA Zol B method, as described (1). The extracted RNA preparations were reverse transcribed and resultant cDNA was amplified by PCR using following sense and antisense primers that span an intron; 5'-CACATCCACAAGCAGGAAGTACAC-3' and 5'- GGTTCA-GGGACATCTCCTACTAAG-3' for RAG-2, and 5'-CCATCACCATCTTCCAGGAG-3' and 5'-CCTGCTTCACCACCTTCCTTG-3' for GAPDH, respectively. Igβ was amplified using sense 5'-ATGGCCAGGCTGGCGTTGTCTC-3' and antisense 5'-GAGGCGCTGTTCATGTAGCAGTG-3' as reported by Meffre et al. (12). PCR was performed using the polymerase, AmpliTaq Gold (Perkin Elmer, Foster City, CA) as reported previously (6). The amplified products were electrophoresed on 7.5% polyacrylamide gel and visualized by staining with SYBER Green I (FMC BioProducts, Rockland, ME). The level of the amplified product of RAG-2 was estimated by Southern blotting using a [^{32}P]-labeled probe, the PstI-HinfI 124 bp internal fragment of RAG-2 cDNA as described previously (6,21).

RT-PCR analysis of IL-7Rα expression was done in a similar fashion using following primers; sense 5'-CGAGTGAAATGCCTAACTC-3' and antisense 5'-GCGTCCAGTTGCTTTCAC-3' (21).

2.6. Imunization of mice

Mice were immunized in the footpad with an antigen emulsified in complete Freund adjuvant as described previously (1,2). Popliteal lymphnode (LN) cells were examined for the expression of RAG-2 as described above. Serum antibody titer was assessed as described (20).

3. Results

3.1. Downregulation of RAG-2 by coligation of CD21/CD35 with BCR.

Isolated murine splenic B cells usually expressed a low but significant level of RAG-2, which varied in each mouse. Culture of the B cells with or without anti-CD40 for 3 days resulted in a complete loss of RAG-2(21). The RAG-2 expression was, however, maintained or more frequently augmented when B cells were stimulated with anti-CD40

in the presence of IL-7.   IL-7 alone did not enhance RAG expression as reported previously (21).   IL-4, as well as IL-7 was effective in augmenting RAG expression in anti-CD40-activated mouse slenic B cells (1,2,7).

To investigate the regulatory role for the complement receptor (CD21/CD35) in the regulation of receptor revision, it was examined how the level of RAG-2 in anti-CD40/IL-7-stimulated B cells is modulated by engagement of BCR or by coligation of BCR with CD21/CD35.   Mouse CD21 and CD35 are isoforms that are encoded by a single gene, and generated by an alternative splicing (18).   7G6 is a mAb raised against a C3d-binding site present on both isoforms (18).   The coligation of BCR with CD21/CD35 was performed by treating B cells first with anti-μ and 7G6, both of which were biotinylated, followed by the addition of avidin.   RAG-2 expression enhanced by anti-CD40/IL-7-stimulation was partially reduced when either BCR or CD21/CD35 alone were engaged with anti-μ (5 μg/ml) or 7G6, respectively.   Further ligation of these bound Abs with avidin did not augment the suppression (Fig. 1A, lane 2-lane 6).   When anti-μ was increased to 10 μg/ml, RAG-2 expression was inhibited to a greater extent, but was not diminished totally (Data not shown).   Simultaneous addition of anti-μ and 7G6 did not show additive inhibitory effects (Fig. 1A, lane 7).   However, very interestingly, coligation of BCR with CD21/CD35 by avidin resulted in a stronger suppression of RAG-2 expression than the engagement of either BCR or CD21/CD35 alone (Fig. 1A, lanes 4,6 and 8).   These results were reconfirmed by comparing the level of the RAG-2 transcript among lanes 2, 4 and 8 in Fig. 1A by semi-quantitative RT-PCR that was performed with varying PCR cycles or with varying dilutions of cDNA.   Similar observations were made in the RAG expression in B cells stimulated with anti-CD40 + IL-4 (Data not shown).

The coligation-dependent suppression of RAG-2 expression was similarly observed when BCR were engaged weakly with a lower concentration of anti-μ (1 μg/ml).   RAG-2 expression was markedly suppressed when BCR and CD21/CD35 were coligated while the engagement of BCR alone showed weak inhibitory effects (Fig. 1C, lanes 2, 3, and 6).   On the other hand, the coligation-induced suppression was abrogated when 7G6 was replaced by an isotype-matched control mAb (Fig. 1C, lanes 3 and 4), thus indicating that the coligation of BCR with CD21/CD35 but not with other molecules on B cells including FcγRII may be responsible for the suppression.

We have reported that isolated IgD$^+$ splenic B cells are originally IL-7R-negative, but a part of the B cells become positive for IL-7R in response to anti-CD40/IL-7 in vitro (21).   Inhibition of the RAG-2 expression by coligation of BCR with CD21/CD35 may not be due to the suppression of IL-7R expression, because expression of IL-7Rα was not significantly affected by the coligation (Fig. 1B).   It was confirmed that the γ common chain, another subunit of IL-7R, was constitutively expressed in these B cells (Data not shown).

Collectively, RAG-2 expression in splenic B cells was more strongly downregulated by the coligation of BCR with CD21/CD35 when the engagement of BCR alone caused only partial inhibition.
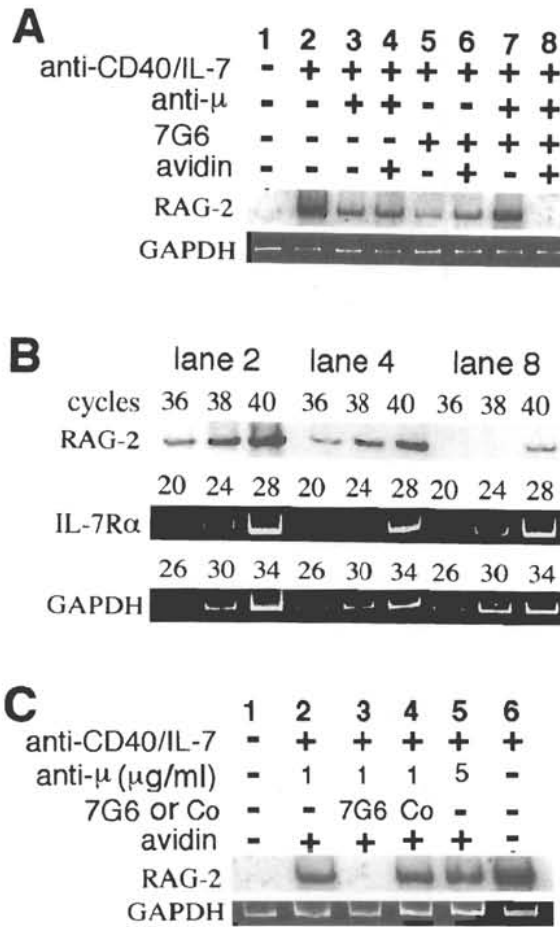
Fig. 1. (A) Inhibition of RAG-2 expression in anti-CD40/IL-7-stimulated B cells by coligation of BCR with CD21/CD35. Anti-μ (F(ab')$_2$ fragment), anti-CD21/CD35 mAb (7G6) and its isotype-matched control mAb were used as biotinylated forms. Murine B cells were cultured with anti-CD40/IL-7 for 3 d. Where indicated, 5 μg/ml anti-μ, 5 μg/ml 7G6 and 5 μg/ml avidin were added, alone or in combination, to the culture. RAG-2 expression was assessed on day 3 of the culture by RT-PCR/Souther blotting. (B) RAG-2 expression was inhibited much more strongly by coligation of BCR with CD21/CD35 than by engagement of BCR alone. The expression of RAG-2 and IL-7Rα was compared among lanes 2, 4 and 8 in panel A. (C) RAG-2 expression was inhibited efficiently by coligation of BCR with CD21/CD35 even when BCR were engaged with a lower concentration of anti-μ. B cells were stimulated with anti-CD40/IL-7 in the presence of 1 or 5 μg/ml anti-μ, 5 μg/ml 7G6 or an isotype-matched control mAb (Co), and 5 μg/ml avidin. All results are representatives of five experiments.

3.2. Enhanced proliferation of splenic B cells by the coligation of CD21/CD35 with BCR.

It has been reported that coligation of CD21/CD19 coreceptor with BCR enhances signal transduction and effectively reduces the affinity threshold for BCR-mediated B cell activation (18,22,23). Next, it was investigated how the activation status of anti-CD40/IL-7-stimulated B cells is modulated when RAG expression is suppressed. [^{3}H]-thymidine uptake was induced in B cells that were stimulated with anti-CD40/IL-7, and was further was augmented when BCR of the B cells were coligated with CD21/CD35 (Fig. 2). On the other hand, anti-μ plus 7G6 had no enhancing effects when these two Abs were not coligated with avidin. Avidin itself had no effects on the anti-CD40/IL-7-induced proliferation (Data not shown). Thus, it is considered that coligation of BCR with CD21/CD35 delivers a stimulatory signal for B cell proliferation concomitant with an inhibitory signal for RAG expression.
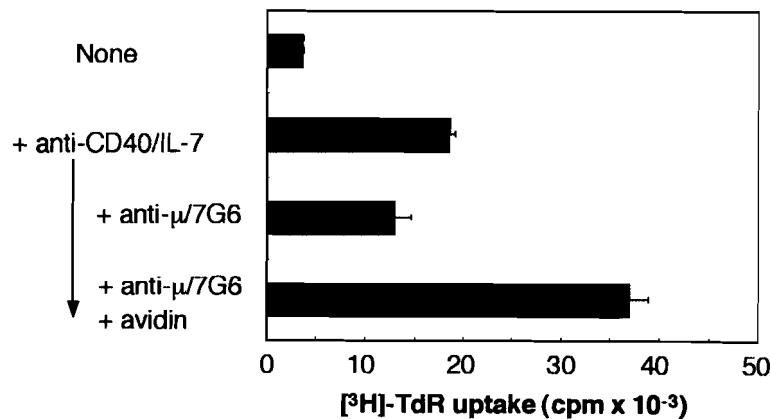

Fig. 2. Augmentation of proliferative response of anti-CD40/IL-7-stimulated B cells by coligation of BCR with CD21/CD35. B cells (6 x 10^5) were stimulated with 1 μg/ml anti-CD40 and 10 ng/ml IL-7 for 48 h, followed by incubation with [^{3}H]-TdR for 16 h. Biotinylated anti-μ (1 μg/ml) and 7G6 (5 μg/ml) with or without avidin (5 μg/ml) were added to the culture as indicated. Data are mean cpm ± SD from quadruplicate wells. Representative data from two experiments.

3.3 A low-affinity Ag induces a higher level of RAG-2 in vivo.

In vitro experiments described above strongly suggest that mutated B cells generated during GC reaction will conserve their BCR by inhibiting RAG expression if their BCR and CD21/CD35 are coligated strongly with Ag-C3d complexes on FDC. If this is also the case in vivo, GC B cells with low-affinity BCR for the immunized antigen will express a higher level of RAG-2 than the high-affinity clones. We attempted to confirm this assumption using the QM mouse, which is an anti-4-hydroxy-3-nitrophenylacetyl (NP) IgH-knock-in strain with the genotype VHDJH17.2.25/JH$^-$, κ^-/κ^-, λ^+/λ^+ (24). We selected two NP-related ligands for anti-NP BCR in QM mice. A hapten, 3-nitro-4-hydroxy-5-iodophenylacetyl (NIP) is a high-affinity ligand that has ~11 fold higher

association constant than that of NP as reported previously (15). On the other hand, we selected p-nitrophenylacetyl (pNP) as a low-affinity ligand, which showed more than ~100 fold lower affinity than NIP (Fig. 3A,15). QM mice were immunized with either NIP- or pNP-conjugated chicken-γ-globulin (CGG) in the footpad, and the expression of RAG-2 in popliteal LN cells was assessed by RT-PCR. RAG-2 expression was not detected on day 0. An increased level of RAG-2 transcript was observed on day 10 in the LN of QM mice that were immunized with pNP-CGG, whereas RAG-2 expression was only marginal when immunized with NIP-CGG (Fig. 3B). This may not be due to that QM mice are unresponsive to NIP-CGG because anti-NP IgM in the serum increased similarly after immunization with either antigen (Fig. 3C). Thus, these findings together with those in in vitro experiments strongly suggest that RAG expression is regulated by combined signals mediated by BCR and CD21/CD35.
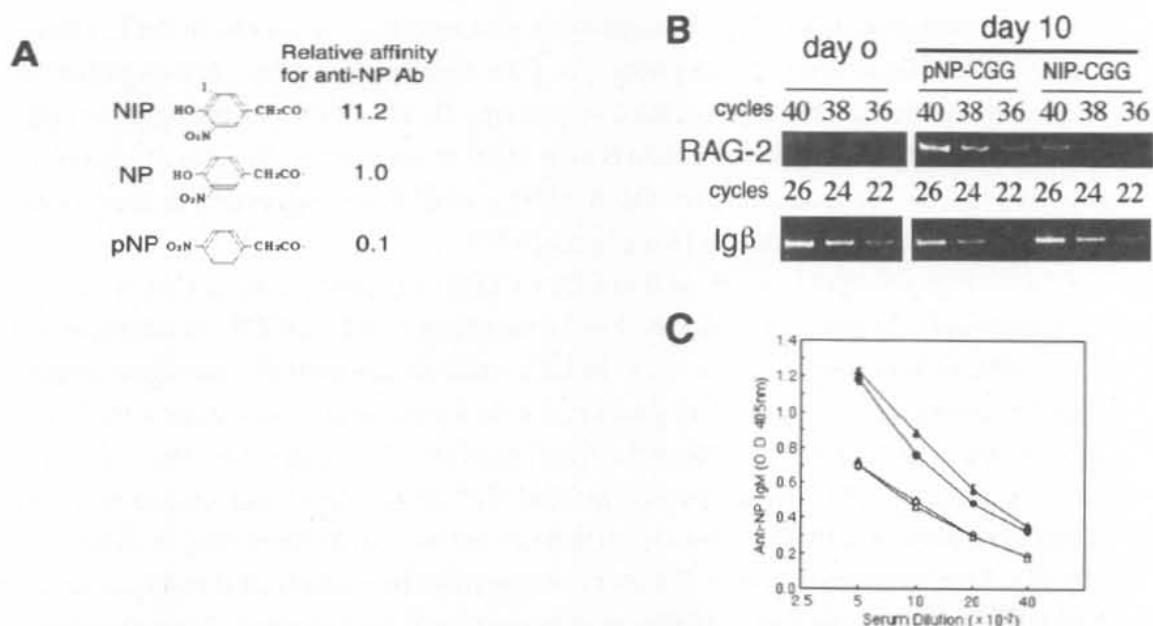

Fig. 3. RAG-2 expression in QM mice that were immunized with either a high-affinity or a low-affinity Ag. (A) Structures of a high-affinity hapten (NIP) and a low-affinity hapten (pNP). The affinity for anti-NP IgM from QM mice was presented as a relative association constant where the affinity of NP hapten was defined as 1.0 (association constant, ~ 2 x 10^6/M). (B) RAG-2 expression and (C) anti-NP IgM response in QM mice immunized with either pNP-CGG or NIP-CGG. Male QM mice (N = 3) were immunized with either NIP-CGG or pNP-CGG in the footpad. On day 0 and day 10 after immunization, RAG-2 expression was assessed in pooled popliteal LN cells by RT-PCR with the varying reaction cycles indicated. Anti-NP IgM levels in pooled sera that were collected from mice immunized with NIP-CGG (triangle) or pNP-CGG (circle) on day 0 (open symbol) or day 10 (closed symbol) were assayed by ELISA using a plate coated with NP-BSA and peroxidase-conjugated goat anti-mouse IgM. Data are mean ± SD from triplicate assays. Representative data from two experiments.

4. Discussion

V(D)J rearrangement in peripheral B cells, termed receptor revision has been implicated to contribute to the generation of high-affinity Abs (13,14). Our recent experiments have shown that this is the case. We have observed that new rearrangement of λ gene that occurs in the draining LN B cells after immunization contributes to the generation of high-affinity IgG bearing λ chains (15). Therefore, receptor revision along with somatic mutation may be involved in affinity maturation of Abs. Using knockin mice whose RAG-2 expression can be monitored by a green fluorescent protein (GFP) reporter, it has been suggested that an increase in RAG^+ B cells in the spleen after immunization is largely due to migration of immature-type B cells ($B220^{low}HSA^{high}493^+$) from the bone marrow (25,26). In contrast to these observations made in the spleen, we did not observe an increase of immature B cells in the draining LN after immunization in the foot pad (15), consistent with the fact that only mature B cells enter LNs (27). A majority of RAG-expressing B cells in the LN were $B220^{high}493^-GL-7^+$, a GC-like phenotype (Our unpublished data). Although further studies are needed to characterize RAG-expressing B cells in the periphery, these cells are qualitatively different from immature B cells in the bone marrow, because it has been reported that BCR engagement results in turning off of RAG expression in the former (11,12), but in its augmentation in the latter (9,10).

In the present report, we show that CD21/CD35 may play a crucial role in the downregulation of RAG expression. The involvement of CD21/CD35 is interesting in the context of B cell selection in GCs. In GCs, centroblasts actively undergo somatic hypermutations of V regions of Ig genes in the dark zone, and differentiate into centrocytes with diversified BCR in the light zone (16,17). Centrocytes that interact strongly with Ag-C3d complexes presented on FDC are positively selected to become memory or plasma cells, whereas low-affinity or autoreactive clones may be deleted (16,17). One hypothesis is that receptor revision must be shut off in B cells that acquired high-affinity BCR, otherwise affinity maturation would be disturbed. This regulatory mechanism may be operative because our experiments showed that coligation of BCR with CD21/CD35 led to turning off of RAG expression. In human tonsil B cells, RAG expression was abrogated by cross-linking BCRs with anti-κ Abs (12). In 3-83 (anti-H-2K^k) transgenic mice, V(D)J recombination in splenic B cells were induced by immunization with a low-affinity recombinant antigen, but not with a high-affinity antigen (11). We made a similar observation using the quasi-monoclonal mouse, who expresses a site-directed IgH gene specific for NP hapten (24). RAG expression was much higher in the draining LN B cells when the mice were immunized with p-NP, a low-affinity analog of NP than with NIP, a high-affinity hapten. Thus, it is suggested that RAGs in peripheral B cells may be downregulated when the cells acquire high-affinity BCRs.

On the other hand, the same coligation delivers stimulatory signal(s) for the proliferation of B cells (Fig. 3). When CD21 complexed with CD19 is coligated with BCR, it has been shown that signals induced through each receptor may synergize and

provide a stronger stimulus for inducing B cell proliferation (22,23). Therefore, CD21/CD35 is considered to play a critical role in directing the conservation or the revision of acquired BCRs, thereby supporting affinity maturation.

Acknowledgments

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