CD5 Is a Potential Selecting Ligand for B Cell Surface Immunoglobulin Framework Region Sequences

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Summary

In rabbits nearly all B lymphocytes express the glycoprotein CD5, in contrast to mice and humans, where only a small proportion of B cells express this molecule (Raman, C., and K.L. Knight. 1992. J. Immunol. 149:3858–3864). CD5+ B cells appear to develop early in ontogeny and be maintained throughout life by self-renewal. The function of CD5 on B cells is still unknown. We showed earlier that “positive” selection occurs during B lymphocyte development in the rabbit appendix. This selection favors B cells expressing surface immunoglobulins with V_Ha2 structures in the first and third framework regions (Pospisil, R., G.O. Young-Cooper, and R.G. Mage. 1995. Proc. Natl. Acad. Sci. USA. 92:6961–6965). Here we report that F(ab')2 fragments, especially those bearing V_Ha2 framework region determinants, specifically interact with the B cell-surface glycoprotein CD5. This interaction can be inhibited by anti-CD5 antibodies. Furthermore, immobilized F(ab')2 fragments selectively bind CD5 molecules in appendix cell lysates. Interactions of V_H framework region structures with CD5 may affect maintenance and selective expansion of particular B cells and thus contribute to autostimulatory growth of autoimmune or transformed cells.

During B cell development a rigorous selection process acts on newly formed B cells. Those bearing self-reactive Ig molecules can be eliminated (1, 2), undergo receptor editing (3, 4) or develop clonal anergy (5–7). In addition, B cells appear to receive positive signals for survival (8–10). Superantigens or self-antigens interacting with evolutionarily conserved “family-specific” sequences in the first and third framework regions (FR1 and FR3) of the V_H may have the potential to significantly skew the composition of the B cell repertoire (10–12).

In normal rabbits of the V_Ha2 haplotype, the majority of peripheral B cells that have undergone a productive V_H-D_JH gene rearrangement use the V_Ha2 allotype-encoding V_H1 gene (13–15). The V_Ha2 specificities were found to correlate with consistent differences in the amino acids at certain positions in FR1 and FR3 (13, 16). Alicia (ali) V_H mutants (17) have a small deletion encompassing the V_H1 gene at the 3' end of the V_H cluster (13, 15) thus most of the B cells in young ali/ali rabbits are V_Ha2 negative. We showed earlier that B cells producing surface immunoglobulin with FR1 and FR3 V_Ha2 allotypic structures are preferentially expanded and positively selected during their development in the appendix (18). The antigen or ligand(s) responsible for this selection, however, were not determined. In this study we provide evidence for CD5-V_H framework region interaction. The interaction between CD5 and B cell surface immunoglobulin may affect maintenance and selective expansion of particular B cells and may be a promoting factor in the evolution of autoimmune or transformed cells.

Materials and Methods

Animals, Reagents, and Antibodies. Rabbits of the V_Ha2 (F-I) or V_H mutan ali (F-I) haplotype were bred and raised in our own National Institute of Allergy and Infectious Diseases allotype-defined pedigreed colonies. The antibodies used in this study were mouse mAbs to rabbit CD5, RCD5 (19) and human CD5, T1 or T1-RD1 (Coulter Corp., Hialeah, FL), biotin-conjugated mouse anti-rabbit CD4 and mouse anti-rabbit CD8 (Spring Valley Laboratories Inc., Woodbine, MD), biotin-conjugated goat anti-mouse IgG and FITC-labeled normal goat IgG (Jackson ImmunoResearch Laboratories, Inc., West Chester, PA), avidin conjugated to biotinylated glucose oxidase (ABC-GO; Vector Laboratories, Inc., Burlingame, CA), nitro blue tetrazolium in conjugation with 5-bromo-4-chloro-3-indolyl phosphate (Sigma Chemical Co., St. Louis, MO), Dynabeads M-450 and M-280 streptavidin (Dynal Inc., Great Neck, NY).

1Abbreviations used in this paper: ali, Alicia; BCR, B cell receptor; FR, framework region.

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Tissue Staining and Immunohistochemistry. The preparation and purification of F(\(ab\)')2 fragments was described previously (20). Briefly, a globulin fraction of rabbit serum was first prepared by precipitation with ammonium sulfate (50% saturation). The precipitated proteins were dialyzed against acetate buffer, pH 4.5, and digested with pepsin (2 mg/100 mg protein) for 18 h at 37°C. Digests were dialyzed against PBS. The residual undigested IgG was removed with protein A-Sepharose. The isolation of VH fragments was described previously (21). The purified F(\(ab\)')2 and VH were biotinylated with a biotinylation kit using NHS-LC-biotin (Pierce Chemical Co., Rockford, IL). Semithin 7-\(\mu\)m serial sections of mutant V\(\alpha\)2- rabbit appendix collected at 6 wk of age were cut and incubated as described (18). In Fig. 1, tissue sections were stained with the primary reagent, mouse anti-rabbit CD5 mAb, RCI)5 (b and c) or isotype-matched control, normal mouse IgG2a (a) followed by V\(\alpha\)2- F(\(ab\)')2-biotin (a and c) or biotin-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) (b). In (d) the tissue section was stained with VH-biotin and in (e) purified VH followed by anti-CD5 (T1) antibody. The sections were then incubated with ABC-Go and labeled cells visualized by nitro blue tetrazolium in conjunction with BCIP.

Cell Attachment Assay. Immunon 4 flat-bottom plates (Dyne
tech Laboratories, Inc., Alexandria, VA) were coated with V\(\alpha\)2+ or mutant V\(\alpha\)2- F(\(ab\)')2 fragments diluted in 0.1 M NaHCO\(_3\) and incubated at 4°C overnight (according to the directions from Gibco BRL, Gaithersburg, MD). NaHCO\(_3\) alone was added to the control wells. The plates were washed three times with Dulbecco's PBS and 100 \(\mu\)l of 2% BSA was added to each well for 2 h at room temperature to block plates. After another wash with PBS 100 \(\mu\)l of CD4/CD8-depleted or IgM-depleted appendix cell suspensions in PBS (10^7/ml) were added and incubated for 1 h at 37°C. To isolate CD4/CD8 or IgM-depleted appendix cells, cells were first incubated with biotin-conjugated mouse anti-rab
bit CD4 and mouse anti-rabbit CD8 mAbs or biotin-conjugated polyclonal anti-rabbit IgM. After washing with PBS-1% BSA, the cells were incubated with Dynabeads M-280 streptavidin and bound cells were removed using a magnet (MPC; Dynal Inc.). The plates were rinsed very gently three times with PBS and cells fixed with 100 \(\mu\)l of formalin solution (10% in PBS) for 30 min at room temperature followed by addition of 50 \(\mu\)l of toluidine blue (1% [wt:vol] in 10% formalin solution) and incubation for another 30 min. After extensive washing with destained water, plates were air dried, cells solubilized by addition of 100 \(\mu\)l 2% SDS and incubation for 15 min at 37°C, and absorbance was measured at 650 nm using a microtiter plate reader (Molecular Devices Corporation, Menlo Park, CA). The relative absorbance was calculated as a ratio of each sample to the control.

Flow Cytometry. Total appendix cells were first incubated with V\(\alpha\)2+ F(\(ab\)')2-biotin then washed with PBS and stained with fluo
coresein-conjugated goat anti-rabbit IgM and streptavidin-PE conjugate. Cells stained with BSA-biotin and FITC-labeled normal goat IgG served as a negative control. CD4/CD8 depleted appendix cells (isolated as described above) were incubated with BSA-biotin, mutant V\(\alpha\)2- F(\(ab\)')2-biotin, V\(\alpha\)2+ F(\(ab\)')2-biotin, or unlabeled anti-CD5 antibody (clone T1) followed by V\(\alpha\)2+ F(\(ab\)')2-biotin for 30 min at 4°C. The cells were washed and incubated for 30 min at 4°C with streptavidin-fluorescein conjugate. For competitive inhibition studies, total appendix cells were incubated with a nonsaturating amount of PE-conjugated anti-CD5 (CD5-PE; clone T1-RD1). PE-conjugated mouse IgG2a served as a negative control. Different concentrations of F(\(ab\)')2 or nonconjugated anti-CD5 were incubated with the 10 \(\mu\)g anti-CD5-PE for 40 min at 4°C.

CD5 Isolations from Appendix Cell Lysates. 5 \(	imes\) 10^6 appendix cells were isolated from 2-wk-old V\(\alpha\)2-mutant (ab/ab) rabbits with no endogenous V\(\alpha\)2 molecules. Isolated cells were biotinylated and cell lysates prepared with a cellular labeling and immunopre-
cipitation kit using biotin-7-NHS (Boehringer Mannheim, Indianapolis, IN). To remove proteins that may bind nonspecifically to the beads, the lysate was first gently rocked with 100 μl of uncoupled beads (4 × 10^9 beads/ml) for 30 min at 4°C and complexes removed with a magnet. This step was repeated three times. The lysate was then divided into two equal aliquots. One aliquot was first incubated on a rocking platform at 4°C overnight with 300 μl of Dynabeads M-450 coupled with anti-human CD5 antibody (T1). The complexes were collected with a magnet and supernatants removed carefully. Both aliquots were first preincubated with μH2-F(ab')2-coated Dynabeads M-280 for 5 h at 4°C on a rocking platform and then incubated with μH2-F(ab')2-coated beads overnight at 4°C. The complexes were collected again with a magnet and supernatants removed. Dynabead complexes were washed twice in buffer 1 (50 mM Tris, 150 mM NaCl, and 0.1% NP-40) then twice in buffer 2 (50 mM Tris, 50 mM NaCl, and 0.1% NP-40) and finally once in 10 mM Tris buffer, pH 7.5. The beads were boiled in SDS gel-loading reducing buffer for 3 min and protein content analyzed by 15% SDS-PAGE. Beads were collected again with a magnet and supernatants removed carefully. Both aliquots were first incubated with μH2-F(ab')2-coated Dynabeads M-280 for 5 h at 4°C on a rocking platform and then incubated with μH2-F(ab')2-coated beads overnight at 4°C. The complexes were collected again with a magnet and supernatants removed. Dynabead complexes were washed twice in buffer 1 (50 mM Tris, 150 mM NaCl, and 0.1% NP-40) then twice in buffer 2 (50 mM Tris, 50 mM NaCl, and 0.1% NP-40) and finally once in 10 mM Tris buffer, pH 7.5. The beads were boiled in SDS gel-loading reducing buffer for 3 min and protein content analyzed by 15% SDS-PAGE Ready Gels (Bio-Rad Laboratories, Hercules, CA) and a streptavidin-peroxidase chemiluminescence technique according to the manufacturer's instructions.

Results

Biotinylated F(ab')2 Fragments Stain Dark Zone Cells of Appendix Germinal Center and the Staining Is Inhibited by anti-CD5 Antibody. To identify a ligand for VH FR1 and FR3 on B cell surface immunoglobulin, we purified and biotinylated F(ab')2 fragments from rabbit IgG and used them as well as VH sequences (lacking an associated VL) to assess binding to appendix germinal center cells by immunohistochemistry. Biotin-labeled F(ab')2 or VH fragments mainly stained germinal centers with high intensity in the dark zones and low intensity in the light zones (Fig. 1, a and d). A similar pattern of staining was observed in appendix follicles stained by either mouse anti-CD5 mAb RCDF5 (1), or a mouse anti-human CD5 mAb, T1 (22) (Fig. 1 b and data not shown). Staining of the germinal centers by biotin-labeled F(ab')2 can be inhibited by prior incubation of tissue sections with anti-CD5 antibodies (Fig. 1 c), suggesting that F(ab')2 fragments bind to the CD5 molecules on dark zone B cells. Similarly, staining of the germinal centers by anti-CD5 antibody can be inhibited by preincubation of tissue sections with purified VH (Fig. 1 c). Thus, the interaction of CD5 and VH does not require VL. In addition, some but not all affinity-purified rabbit antibodies stain dark zones of the appendix and the staining can be inhibited by anti-CD5 antibodies (data not shown). Together these data argue that CD5-VH interaction is framework region specific and is not dependent on antibody specificity, although changes in VH sequences can alter or eliminate binding.

**Figure 2.** Attachment of appendix B cells to F(ab')2-coated plates. The plates were coated with V_H2(F(ab')2 and μH2(F(ab')2 fragments. CD4/CD8-depleted (■ and □) or IgM-depleted (□ and □) appendix cell suspensions in PBS (10^7/ml) were added. After incubation, washing, and staining as described in Materials and Methods, the relative absorbance was calculated as a ratio of each sample to the control wells (buffer + BSA block).

Immobilized F(ab')2 Fragments Isolate CD5 Molecules from Appendix Cell Lysates. To isolate the molecule on B cells that interacts with F(ab')2 fragments, we covalently coupled
Figure 3. V_{\mu}2^+ F(ab')_2 binds to IgM^+ appendix B cells and the binding is specifically inhibited by anti-CD5 antibody. (a) Total appendix cells were first incubated with V_{\mu}2^+ F(ab')_2-biotin then washed with PBS and stained with fluorescein-conjugated goat anti-rabbit IgM and streptavidin-PE conjugate. Cells stained with BSA-biotin and FITC-labeled normal goat IgG served as a negative control (not shown). (b) CD4^+CD8^+ depleted appendix cells were incubated with BSA-biotin (broken line), mutant V_{\mu}2^+ F(ab')_2-biotin (solid thin line), V_{\mu}2^+ F(ab')_2-biotin (solid thick line), or unlabeled anti-CD5 antibody (clone T1) followed by V_{\mu}2^+ F(ab')_2-biotin (dotted line). (c) Total appendix cells were incubated with 10 μg PE-conjugated anti-CD5 antibody and different concentrations of either F(ab')_2 (open squares) or unconjugated anti-CD5 antibody (closed circles). The data were expressed as mean fluorescence minus control and percent inhibition calculated relative to CD5-PE in the absence of any inhibitor.

Discussion

Interaction of FR structures on B cells with previously unidentified ligand(s) was postulated to contribute to antigen-independent signals to survive rather than undergo apoptosis (18, 24). The data reported here demonstrate an interaction between CD5 and B cell surface immunoglobulin, most likely involving framework region sequences. We showed earlier that "positive" selection occurs during B lymphocyte development in the rabbit appendix (18). This selection favors B cells with receptors bearing V_{\mu}a2^+ structures in the first and third framework regions. V_{\mu}a2^+ structures as F(ab')_2 fragments bind IgM^+ B cells irrespective of antibody specificity and the binding can be inhibited by anti-CD5 antibodies. Thus CD5 is a potential selecting ligand that contributes to survival and expansion of B cells with V_{\mu}a2^+ surface IgM.

Most dark zone B cells in appendix germinal centers express high levels of CD5 (Fig. 1b) and the majority of B cells in normal animals bear V_{\mu}a2 framework regions encoded by the V_{\mu}1 gene (13, 15). The presence of both CD5 and V_{\mu}a2 on the same cell raises the possibility of a relationship between the coexpression of these interacting...
proteins and the self-renewing capacity of these cells. Future investigations must determine whether the expansion we observed is mediated through signals transmitted by V_{\mu}\alpha2 stimulating CD5, CD5 stimulating the V_{\mu}\alpha2-associated B cell receptor (BCR) or both. Selective expansion of V_{\mu}\alpha2^{-} B cells in the appendix could occur via CD5-V_{H} interaction either on the same cell or through interactions with nearby cells in a developing cluster.

A role for CD5 as a candidate selecting ligand is further suggested by its physical and functional coupling to the BCR (25). Thus CD5 accessory molecules in the BCR complex on CD5^{-} B cells may have a unique potential to modulate BCR signals after interaction with antigens or superantigens (25, 26). A limited repertoire of V_{H} genes has been observed in the CD5^{-} B cell populations of human and mouse (27, 28). This may also reflect selective B cell expansion during fetal and neonatal B cell development through interactions with autologous antigens or superantigens (10, 29).

Studies of the phenotype of a CD5 knockout mouse suggest that CD5 may play a role in positive selection of developing thymocytes with specific antigen receptors (30).

Similarly, the interaction between CD5 as a surface ligand and its receptor on the same or other B cells may generate distinct activation signals at different stages of B cell development and selection. As B lymphocytic leukemia cells express CD5 (31), and CD5^{-} B1 B cells provide a source of autoantibody-producing cells (25–27), the CD5-framework region interaction might contribute to autostimulatory growth of transformed cells as well as mediate selection of autoreactive repertoires. CD5 may interact directly with a counterreceptor, such as CD72 (32, 33) or VH and transmit modulating signals to the B cell. The amount of signaling and qualitative differences in signaling may determine B cell negative or positive selection (34). CD5-V_{H} interaction alone may induce a signal that is sufficient to promote expansion and/or survival of B cells or may influence the fate of B cell selection in combination with other signals.

Our data provide evidence for CD5-V_{H} framework region interaction and suggest it may affect maintenance and selective expansion of particular B cells. After V_{H} ligand recognition, CD5 stimulation may also be a promoting factor in the evolution of autoimmune or transformed cells.

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