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\textsubscript{H}a\textsubscript{2} 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')\textsubscript{2} fragments, especially those bearing V\textsubscript{H}a\textsubscript{2} 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')\textsubscript{2} fragments selectively bind CD5 molecules in appendix cell lysates. Interactions of V\textsubscript{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\textsubscript{H} may have the potential to significantly skew the composition of the B cell repertoire (10–12).

In normal rabbits of the V\textsubscript{H}a\textsubscript{2} haplotype, the majority of peripheral B cells that have undergone a productive V\textsubscript{H}–D\textsubscript{H}–J\textsubscript{H} gene rearrangement use the V\textsubscript{H}a\textsubscript{2} allotype-encoding V\textsubscript{H}1 gene (13–15). The V\textsubscript{H}a\textsubscript{2} specificities were found to correlate with consistent differences in the amino acids at certain positions in FR1 and FR3 (13, 16). Alicia (ali) V\textsubscript{H} mutant 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 polyclonal anti-rabbit IgM (Southern Biotechnology Associates, Birmingham, AL), biotin-conjugated mouse anti-rabbit CD4 and mouse anti-rabbit CD8 (Spring Valley Laboratories Inc., Woodbine, MD), biotin conjugated polyclonal anti-rabbit IgM (Southern Biotechnology Associates, Birmingham, AL), biotin-conjugated mouse anti-rabbit CD4 and mouse anti-rabbit CD8 (Spring Valley Laboratories Inc., Woodbine, MD), biotin conjugated polyclonal anti-rabbit IgM (Southern Biotechnology Associates, Birmingham, AL), biotin-conjugated mouse anti-rabbit IgM 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).

Materials and Methods

Animals, Reagents, and Antibodies. Rabbits of the V\textsubscript{H}a\textsubscript{2} (F-I) or V\textsubscript{H} mutant 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., MD), biotin conjugated polyclonal anti-rabbit IgM (Southern Biotechnology Associates, Birmingham, AL), biotin-conjugated mouse anti-rabbit 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).
**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 V\(_H\) fragments was described previously (21). The purified F(\(ab\)')\(_2\) and V\(_H\) were biotinylated with a biotinylation kit using NHS-LC-biotin (Pierce Chemical Co., Rockford, IL). Semithin 7-μm serial sections of mutant V\(_H\)-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, RCD5 (b and c) or isotype-matched control, normal mouse IgG2a (a) followed by V\(_H\)-biotin (a and c) or biotin-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) (b). In (d), the tissue section was stained with V\(_H\)-biotin and in (e) with V\(_H\) followed by anti-CD5 (T1) antibody and then with biotin-conjugated goat anti-mouse IgG. The sections were then incubated with ABC-GO and labeled cells visualized by nitro blue tetrazolium in conjunction with BCIP.

**Cell Attachment Assay.** Immulon 4 flat-bottom plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated with V\(_H\)-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. CD4/CD8 depleted appendix cells (isolated as described above) were incubated with BSA-biotin, mutant V\(_H\)-2 F(ab')\(_2\)-biotin, V\(_H\)-2 F(ab')\(_2\)-biotin, or unlabeled anti-CD5 antibody (clone T1) followed by V\(_H\)-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 μg anti-CD5-PE for 40 min at 4°C.

**CD5 Isolations from Appendix Cell Lysates.** 5 × 10\(^6\) appendix cells were isolated from 2-wk-old V\(_H\)-mutant (alb/alb) rabbits with no endogenous V\(_H\)-2 molecules. Isolated cells were biotinylated and cell lysates prepared with a cellular labeling and immunopre-
cidation 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 × 10⁸ 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 V₅₆₂⁻ F(ab')₂-coated Dynabeads M-280 for 5 h at 4°C on a rocking platform and then incubated with V₅₆₂⁺ 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 of B cell surface immunoglobulin, we purified and biotinylated F(ab')₂ fragments from rabbit IgG and used them as well as V₅₆₂ fragments (lacking an associated VL) to assess binding to appendix germinal center cells by immunohistochemistry. Biotin-labeled F(ab')₂ or V₅₆₂ 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 RC5 (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 inhibited the binding (Fig. 1 c). Thus, the interaction of CD5 and V₅₆₂ does not require V₅₆₂. 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-V₅₆₂ interaction is framework region specific and does not depend on antibody specificity, although changes in V₅₆₂ sequences can alter or eliminate binding.

V₅₆₂⁺ F(ab')₂ Binds to IgM⁺ Appendix B Cells and the Binding Is Specifically Inhibited by anti-CD5 Antibody. To determine which cell subpopulation binds to F(ab')₂, we used a cell attachment assay. IgM-depleted cells, enriched for non-lymphoid cells, did not bind to V₅₆₂⁺ or V₅₆₂⁻ F(ab')₂ coated plates (Fig. 2). In contrast, CD4/CD8-depleted appendix cells, mainly IgM⁺ B cells, showed binding to F(ab')₂ fragments, especially to those bearing V₅₆₂ determinants. By flow cytometry, we confirmed that F(ab')₂-biotin stained IgM-positive appendix B cells (Fig. 3 a). CD4/CD8-depleted appendix B cells stained positively with biotinylated V₅₆₂⁺ F(ab')₂ (solid thick line) but not with mutant V₅₆₂⁻ F(ab')₂ (Fig. 3 b, solid thin line). In addition, anti-CD5 mAb was able to inhibit the interaction between biotinylated F(ab')₂ and appendix B cells (dotted line, Fig. 3 b). This is consistent with the immunohistochemistry data and again indicates that F(ab')₂ binds to CD5 on B cells. Under conditions of competitive inhibition, F(ab')₂ fragments were unable to inhibit binding of 10 μg of anti-CD5-PE to CD5 although unlabeled anti-CD5 antibody inhibited the binding (Fig. 3 c). Thus F(ab')₂ has a lower relative avidity compared to the anti-CD5 for the site on CD5 recognized by this mAb.

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 2. Attachment of appendix B cells to F(ab')₂-coated plates. The plates were coated with V₅₆₂⁺ (■ and ◆) or mutant V₅₆₂⁻ (■ and ◆) F(ab')₂ fragments. CD4/CD8-depleted (■ and ◆) or IgM-depleted (■ and ◆) appendix cell suspensions in PBS (10⁷/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).
purified F(ab')₂ fragments to Dynabeads. Coated beads were then used to isolate F(ab')₂ ligand from lysates of surface biotinylated appendix cells. Similarly, anti-CD5 antibodies (T1 and RCD5) were coupled to the Dynabeads. Anti-CD5 coated beads isolated two molecules (Fig. 4 and data not shown), one migrating at the position corresponding to its expected relative molecular mass of 67,000 (67,000 Mₒ) on SDS-PAGE and one of 56,000 (56,000 Mₒ) most likely representing a differently glycosylated form of CD5 (23). V₁₂a₂⁺ F(ab')₂-coupled beads also isolated two molecules from the cell lysate precleared with V₁₂a₂⁺ F(ab')₂-coupled beads. These had the same molecular mass as those from anti-CD5 coated beads, the major one of 67,000 (67,000 Mₒ) and another one of 56,000 (56,000 Mₒ). The 56,000 molecule was isolated by both anti-CD5 and F(ab')₂ coupled beads but not by control uncoupled beads; thus it is unlikely to represent a nonspecifically bound molecule. Furthermore, when cell lysates from biotinylated appendix cells were first preincubated with anti-CD5-coated beads, these two molecules were no longer isolated by V₁₂a₂⁺ F(ab')₂ beads. In addition, some affinity-purified rabbit antibodies also isolated CD5 molecules from cell lysates (data not shown) arguing against that CD5-V₅ interaction is framework region specific and is not dependent on antibody specificity.

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 V₁₂a₂⁺ structures in the first and third framework regions. V₁₂a₂⁺ 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 V₁₂a₂⁺ surface IgM.

Most dark zone B cells in appendix germinal centers express high levels of CD5 (Fig. 1 b) and the majority of B cells in normal animals bear V₁₂a₂ framework regions encoded by the V₁₂ gene (13, 15). The presence of both CD5 and V₁₂a₂ 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}2 stimulating CD5, CD5 stimulating the V_{\mu}2-associated B cell receptor (BCR) or both. Selective expansion of V_{\mu}2^{+} 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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