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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 (FR[1]1 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_H-J_H 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 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 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).

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

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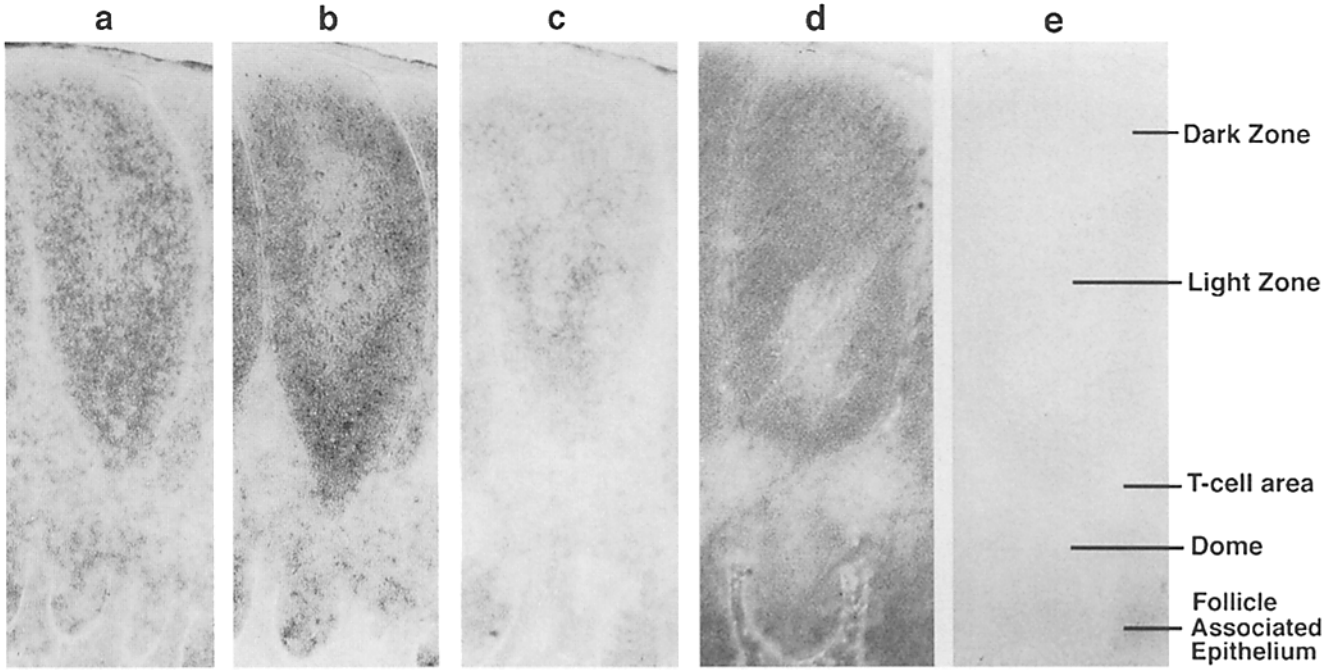

Figure 1. Biotinylated $F(ab')_2$ fragments stain dark zone cells of appendix germinal center and the staining is inhibited by anti-CD5 antibody. Appendix tissue sections were stained with (*a*) V_Ha2^+ $F(ab')_2$-biotin; (*b*) anti-CD5 antibody RCD5; (*c*) anti-CD5 antibody RCD5 followed by V_Ha2^+ $F(ab')_2$-biotin; (*d*) V_H-biotin; (*e*) purified V_H followed by anti-CD5 (T1) antibody.

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_Ha2^- 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 IgG_2a (*a*) followed by V_Ha2 $F(ab')_2$-biotin (*a* and *c*) or biotin-conjugated goat anti–mouse IgG (Jackson ImmunoResearch Laboratories) (*b*). In (*d*) the tissue section was stained with 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_Ha2^+ or mutant V_Ha2^- $F(ab')_2$ 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 μl of 2% BSA was added to each well for 2 h at room temperature to block plates. After another wash with PBS 100 μl 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–rabbit 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 μl of formalin solution (10% in PBS) for 30 min at room temperature followed by addition of 50 μl of toluidine blue (1% [wt:vol] in 10% formalin solution) and incubation for another 30 min. After extensive washing with deionized water, plates were air dried, cells solubilized by addition of 100 μl 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_Ha2^+ $F(ab')_2$-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_Ha2^- $F(ab')_2$-biotin, V_Ha2^+ $F(ab')_2$-biotin, or unlabeled anti-CD5 antibody (clone T1) followed by V_Ha2^+ $F(ab')_2$-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 (ali/ali) rabbits with no endogenous V_Ha2 molecules. Isolated cells were biotinylated and cell lysates prepared with a cellular labeling and immunopre-

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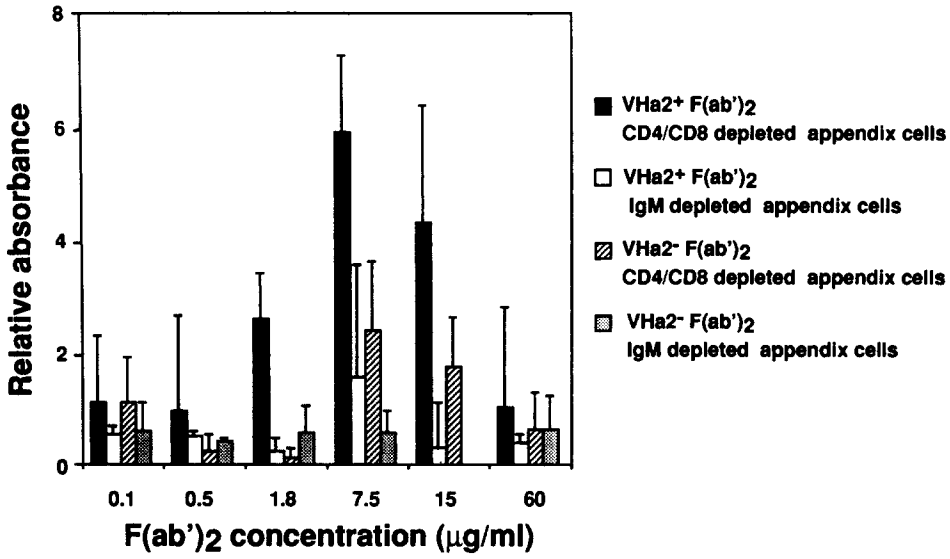

Figure 2. Attachment of appendix B cells to $F(ab')_2$-coated plates. The plates were coated with V_Ha2^+ (■ and □) or mutant V_Ha2^- (▨ and ■) $F(ab')_2$ fragments. CD4/CD8-depleted (■ and ▨) or IgM-depleted (□ and ■) appendix cell suspensions in PBS (10^7/ml) were added. After incubation, washing, 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).

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 (4×10^8 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 precleared with V_Ha2^- $F(ab')_2$-coated Dynabeads M-280 for 5 h at 4°C on a rocking platform and then incubated with V_Ha2^+ $F(ab')_2$-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 V_H 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 V_H fragments (lacking an associated V_L) to assess binding to appendix germinal center cells by immunohistochemistry. Biotin-labeled $F(ab')_2$ or V_H 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–rabbit CD5 mAb RCD5 (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 V_H (Fig. 1 *e*). Thus, the interaction of CD5 and V_H does not require V_L. 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–V_H interaction is framework region specific and is not dependent on antibody specificity, although changes in V_H sequences can alter or eliminate binding.

V_Ha2^+ $F(ab')_2$ 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 V_Ha2^+ or V_Ha2^- $F(ab')_2$ 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 V_Ha2 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 V_Ha2^+ $F(ab')_2$ (*solid thick line*) but not with mutant V_Ha2^- $F(ab')_2$ (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

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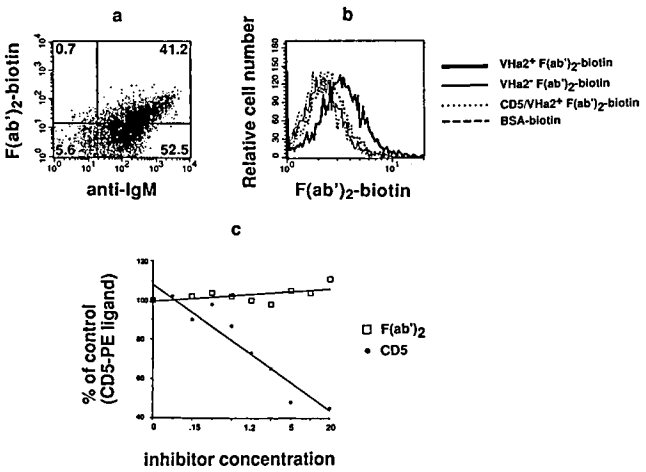

Figure 3. V_Ha2^+ $F(ab')_2$ binds to IgM^+ appendix B cells and the binding is specifically inhibited by anti-CD5 antibody. (*a*) Total appendix cells were first incubated with V_Ha2^+ $F(ab')_2$-biotin then washed with PBS and stained with fluorescein-conjugated goat anti-rabbit IgM and streptavidin-PE 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_Ha2^- $F(ab')_2$-biotin (*solid thin line*), V_Ha2^+ $F(ab')_2$-biotin (*solid thick line*), or unlabeled anti-CD5 antibody (clone T1) followed by V_Ha2^+ $F(ab')_2$-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.

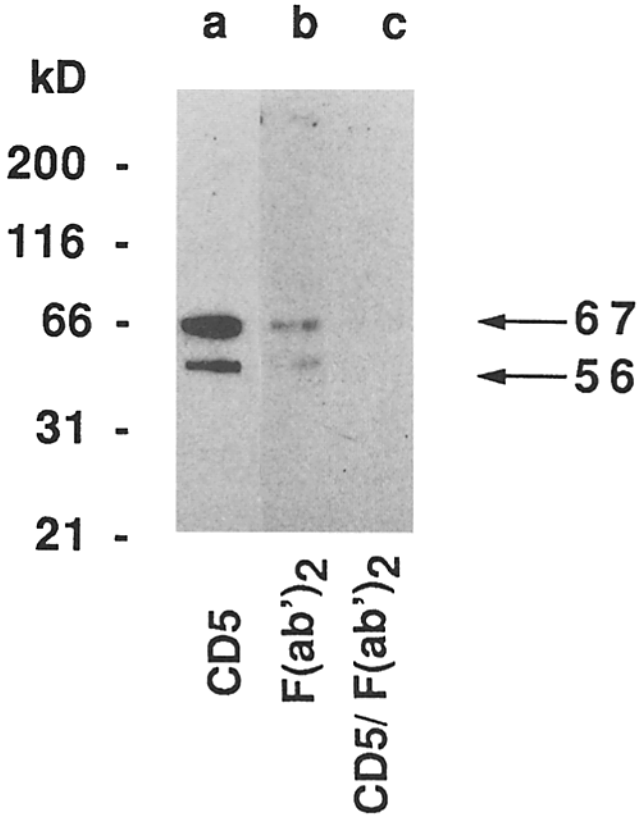

Figure 4. Immobilized $F(ab')_2$ fragments isolate CD5 molecules from appendix cell lysates. SDS-PAGE shows that 67-kD and 56-kD CD5 proteins were isolated by (*a*) anti-CD5 antibody T1 and (*b*) V_Ha2^+ $F(ab')_2$-coated Dynabeads. CD5 molecules were not isolated by V_Ha2^+ $F(ab')_2$ if the cell lysate was first preincubated with anti-CD5 coated beads (*c*).

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 M_r) on SDS-PAGE and one of 56,000 (56,000 M_r) most likely representing a differently glycosylated form of CD5 (23). V_Ha2^+ $F(ab')_2$-coupled beads also isolated two molecules from the cell lysate precleared with V_Ha2^- $F(ab')_2$-coupled beads. These had the same molecular mass as those from anti-CD5 coated beads, the major one of 67,000 (67,000 M_r) and another one of 56,000 (56,000 M_r). 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 V_Ha2^+ $F(ab')_2$ beads. In addition, some affinity-purified rabbit antibodies also isolated CD5 molecules from cell lysates (data not shown) arguing again that CD5-V_H 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 bearingV_Ha2^+ structures in the first and third framework regions. V_Ha2^+ 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_Ha2^+ 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 V_Ha2 framework regions encoded by the V_H1 gene (13, 15). The presence of both CD5 and V_Ha2 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_Ha2 stimulating CD5, CD5 stimulating the V_Ha2-associated B cell receptor (BCR) or both. Selective expansion of V_Ha2$^+$ 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 V_H 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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