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A HUMAN CD5+ B CELL CLONE THAT SECRETES AN IDIOTYPE-SPECIFIC HIGH AFFINITY IgM MONOCLONAL ANTIBODY[1]

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We previously demonstrated the occurrence of a naturally arisen human anti-idiotypic B cell clone, that we transformed with EBV (EBV383). We show evidence that EBV383 not only expresses the CD5 surface Ag, but also contains the 2.7-kb mRNA transcript encoding this protein. In addition, we show the presence of the 3.6-kb mRNA precursor. Most Ig produced by CD5+ B cells are polyreactive natural IgM antibodies encoded by unmutated copies of germline V_H genes. However, in this study we present data demonstrating the monoreactive high affinity character of the anti-idiotypic antibody (mAb383) produced by EBV383. These data are in agreement with our previous observations, showing that the V_H chain of mAb383 is encoded by an extensive somatically mutated V_HV gene in a way that is consistent with an Ag-driven immune response. A possible role for this remarkable anti-idiotypic antibody in the maintenance of B cell memory is discussed.

Analysis of the B cell compartment of mouse and man has revealed evidence for the existence of B cell subpopulations/lineages with different phenotypical and functional characteristics (1–6). First a population of conventional CD5− B cells (Ly-1 in the mouse and Leu 1 in humans) exists that undergoes clonal expansion in response to T-dependent Ag and establishes acquired humoral immunity and immunological memory (7–11). Activation of memory B cells upon re-encounter with Ag results in the production of specific antibody with high affinity for the Ag. Somatic hypermutation acting selectively on rearranged antibody V region genes and Ag-driven selection of mutations are at the basis of affinity maturation of this specific antibody response (7–10). As opposed to these conventional CD5− B cells, CD5+ (IgM+ > IgD+) B cells apparently do not participate in conventional immune response but instead produce low affinity multireactive IgM antibodies with self-reactivity and with reactivity towards a variety of microbial Ag (1, 11–22). For the reactivity of their IgM antibodies, their strategic anatomical distribution in peripheral lymphoid tissues and their relative predominance in early ontogeny, it is likely that CD5+ B cells make an important contribution to the establishment of "natural immunity" (1, 9, 11–25).

So far available data on the expression of V genes by CD5+ B cells in the mouse and humans suggested, concurrent with their proposed function, preferential usage of V_H and V_L germline genes in the absence of somatic mutation (26–31).

Our previous work described the molecular characterization of a human monoclonal anti-idiotypic IgM antibody produced by an EBV-transformed B cell clone (EBV383) expressing CD5 as demonstrated by cytofluorometric analysis (32). The V_H chain of this antibody was derived from the V_HV family of human V region gene segments. Parallel studies on the germline V_HV gene isolated from the same adult, healthy donor revealed extensive somatic mutation in the expressed V_HV gene leading to amino acid replacements accumulating in CDR I, CDR II, and framework III (33).

In the present report we extend our previous studies suggesting an Ag-driven immune response by CD5+ B cells. In addition to surface expression of CD5, we show that the EBV-transformed B cell clone EBV383 contains the 2.7-kb mRNA transcript coding for the CD5 protein as well as the 3.6-kb mRNA precursor. In agreement with the previously observed extensive somatic variability of its V region, we find that the IgM antibody secreted by EBV383 shows a monospecific and high affinity binding ($K_d = 8.67 \times 10^{-9}$ mol/liter) to its putative ligand, i.e., the Id 6-15C4.

These studies, together with our previous work have consequences for our understanding of repertoire selection in at least the human CD5 B cell compartment. We discuss the possibility that somatically mutated antibody V regions of anti-idiotypic CD5+ B cells function as "persistent Ag" to maintain the function of long-lived conventional memory B cells in the absence of Ag.

MATERIALS AND METHODS

Virus strains and peptides. The Pitman-Moore strain of rabies virus was propagated in dog kidney cells. Rabies virus Ag (dog kidney cell vaccine) was prepared as described previously (34). Synthetic peptides corresponding to certain parts of the rabies virus glycoprotein (35, 36) were obtained from the European Veterinary Laboratory (Amsterdam, The Netherlands).

Rabies virus neutralizing mAb (mAb6-15C4). The establishment

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and characterization of mAb6-15C4 has been described in detail previously (37). For the experiments described in this study, mAb6-15C4 was isolated from tissue culture supernatant by protein A-Sepharose affinity chromatography (Pharmacia Fine Chemicals, Uppsala, Sweden). Subclass determination (IgG2b), the L chain composition (κ), virus neutralizing activity, and its delineation of a sequential determinant on the rabies virus glycoprotein have also been reported elsewhere (37).

EBV transformation and cloning of cells. A healthy adult human donor was vaccinated intramuscularly with one dose of dog kidney cell vaccine. This procedure was repeated on days 7 and 21. After 2 yr the donor was revaccinated and 2 yr after the last vaccination, PBMC were isolated, EBV transformed, and cloned as described before (32).

ELISA. Microtiter plates (Titertek type III, Flow Laboratories, Irvine, Scotland) were coated overnight with antibodies in 0.1 M carbonate buffer pH 9.6. All other incubations were carried out in PBS containing 1% BSA (Organon Teknika, Oss, The Netherlands), 0.05% Tween 80, and 1 M NaCl. Incubations with antibodies or Ag were carried out at 37°C for 1 h. To determine antibody reactivity against mAb6-15C4, microtiter plates were coated with mAb6-15C4 or with a panel of control murine monoclonal antibodies of the same isotype (IgG2b,κ), at a concentration of 3 μg/ml. Culture supernatant of EBV383 was titrated on the test plate or control plate and the bound antibody was detected with horseradish peroxidase-conjugated rabbit anti-human IgM Ig (Dakopatts, Glostrup, Denmark). All plates were developed for 10 min at room temperature using 100 μl/well of tetramethylbenzidine substrate solution (38). This reaction was stopped with 1 M H_2SO_4 and at 450 nm absorbance was read by a Titertek Multiscan (Flow Laboratories, McLean, VA). Results of triplicate samples were expressed as mean absorbance value.

Inhibition ELISA was carried out as follows. After overnight incubation at room temperature of 10 ng of mAb383 with 0, 0.15, 1.5, 15, and 150 μg, respectively, of mAb6-15C4, rabies virus Ag, peptide G5, an irrelevant control peptide E125, and several irrelevant control murine mAbs with the same isotype as mAb6-15C4, these incubation mixtures were placed on microtiter plates coated with mAb6-15C4 as described above. mAb383 activity in these incubation mixtures was detected with horseradish peroxidase-conjugated rabbit anti-human IgM Ig (Dakopatts). The inhibition ELISA was developed and absorbance was measured as described above. Results of triplicate samples were expressed as mean percentage inhibition value according to the formula:

$$\text{percentage inhibition} = \frac{(\text{OD450 nm uninhibited} - \text{OD450 nm test})}{\text{OD450 nm uninhibited}} \times 100.$$

To detect a possible multireactivity of mAb383, the antibody was analyzed in a direct binding ELISA for reactivity with dsDNA, ssDNA, thyroglobulin, cardiolipin, insulin, TNP, DNP, human IgG Fc, OVA, and cytochrome C. The direct binding ELISA was carried out exactly as reported elsewhere (39). Briefly, microtiter plates were coated with the Ag mentioned above. These plates were incubated with supernatants containing mAb383 and the positive controls, respectively. Binding of any of these antibodies to any coat was detected with alkaline phosphatase-conjugated goat anti-human IgM. The ELISA was developed with substrate (2.3 mM *p*-nitrophenylphosphate in 1 M diethanolamine, 0.25 mM $MgCl_2$, pH 9.8) and the reaction was stopped by adding 2.4 N NaOH. Absorbance was read at 405 nm by a Titertek multiscan (39).

FMF.[4] FMF was performed on EBV383 and a CD5$^-$ EBV-transformed human B cell, EBV655, that produced an IgM,κ antibody (40). EBV-transformed B cells were incubated in 0.1 ml of ice cold PBS, containing 1% BSA with: FITC-labeled mouse IgG2a anti-human CD5 mAb (anti-Leu 1, Becton Dickinson Immunocytometry Systems, Mountain View, CA); phycoerythrin-labeled mouse IgG1 anti-human CD19 mAb (anti-Leu 12, Becton Dickinson); FITC-labeled goat F(ab')$_2$ anti-human λ-chain (Tago Inc., Burlingame, CA); FITC-labeled goat F(ab')$_2$ anti-human κ-chain (Tago Inc.). Phycoerythrin-labeled mouse IgG1 anti-human CD3 mAb (anti-Leu 4, Becton Dickinson) and FITC-labeled mouse IgG2a anti-human CD2 mAb (anti-Leu 5B, Becton Dickinson) were used as isotype controls for anti-Leu 12 and anti-Leu 1, respectively. Cells were analyzed with a FMF scan (Becton Dickinson) and results of double labeling experiments are depicted as contourgraphs.

Northern blotting and probes. Total RNA was extracted from 10^7 EBV transformed B cells or 10^7 human T cells as described previously (41). Total RNA was separated on 1% agarose gel, blotting to Genescreen Plus membranes (NEN Research Products, Boston, MA) and hybridization was done according to the manufacturers specifications. Membranes were washed under high stringency conditions (2× SSC, 65°C for 30 min (twice), 0.1 × SCC, room temperature for 30 min). Human CD5 specific cDNA probe (42) (kindly provided by Dr. N. Jones, Dana-Farber Institute, Boston, MA) was ^{32}P-labeled by random hexamer priming as described previously (43). Membranes were exposed on Kodak XAR film (Eastman Kodak, Rochester, NY) by using amplifying screens.

Inhibition studies of human mAb383 binding to solid phase murine mAb6-15C4 by soluble mAb6-15C4 and calculation of the K_d. Aliquots of PBS (0.05 ml) containing 0.05% Tween 20 and 0.1% BSA (PBS-Tween-BSA) and 1×10^{-10} mol/liter mAb383 were mixed with aliquots of PBS-Tween-BSA with increasing amounts of soluble mAb6-15C4 (6.7×10^{-10} to 1×10^{-5} mol/liter). After an 18-h incubation at room temperature, the mixtures were transferred into ELISA plates coated with 3 μg/ml mAb6-15C4 as described above. After 1-h incubation and subsequent washing with PBS-Tween, the amount of mAb383 bound to the solid phase mAb6-15C4 was detected with HRPO-conjugated rabbit anti-human IgM Ig as described above. mAb383 binding in the presence of soluble ligand was expressed as percentage of its binding activity measured under identical conditions but in complete absence of any soluble ligand. The mAb K_d values were calculated according to Friguet et al. (44).

RESULTS

Clone EBV383 is CD5$^+$ B cell. The phenotype of clone EBV383 was determined by dual color flow cytometric analysis. Cells were stained with pE-labeled anti-CD5 mAb and with FITC-labeled goat F(ab')$_2$ anti-human λ or κ L chain-specific antibody. Staining profiles are illustrated in Figure 1. Clone EBV655, derived from the same cloning event, was used as a control. Although both clones show expression of CD19, clone EBV383 bears λ L chain and expresses CD5, whereas clone EBV655 bears κ L chain and appears to be CD5$^-$. Repeated analysis at several occasions during a period of 15 mo of culture of EBV383 shows identical results indicating a stable expression of CD5 on EBV383.

To demonstrate the mRNA transcript for the CD5 molecule, Northern blot analysis was performed on total RNA isolated from EBV383, EBV655, and human T cells. The cDNA probe used, was obtained from the human CD5/T1/Leu 1 cDNA clone pT1-2 (42). Both the 3.6-kb mRNA precursor and the 2.7-kb mRNA transcript are detected in total RNA isolated from EBV383 (Fig. 2, *lanes 1* and *2*) and from human T cells (*lanes 5* and *6*). CD5 surface Ag encoding mRNA is lacking in EBV655. A small RNA fragment with unknown specificity is also visible in Fig. 2, *lanes 1* and *2* and *5* and *6*. Taken together these results characterize EBV383 as a CD5$^+$ B cell clone.

EBV383 secretes a high affinity monoreactive anti-idiotypic antibody (mAb383). Non-T cells of a healthy adult individual, who had been deliberately immunized with rabies vaccine, were transformed with EBV. Clone EBV383 was selected after EBV transformation and limiting dilution twice on basis of the anti-idiotypic reactivity of its culture supernatant with the murine Id 6-15C4. mAb6-15C4 is a rabies virus neutralizing antibody directed against a linear epitope on the rabies virus glycoprotein (37). Inasmuch as it has been demonstrated that the pool of natural IgM antibodies produced by CD5$^+$ B cells contains a high proportion of multireactive specificities including self-specificities, experiments were performed to substantiate the anti-idiotypic specificity of mAb383.

Culture supernatants of EBV383 were tested for binding to exogeneous Ag, haptens, autoantigens, Id other than 6-15C4 rheumatoid factor activity. Culture supernatants of EBV-transformed B cells from neonatal or

[4] Abbreviations used in this paper: FMF, flow microfluorimetry; Ars, hapen *p*-azophenyl arsonate.

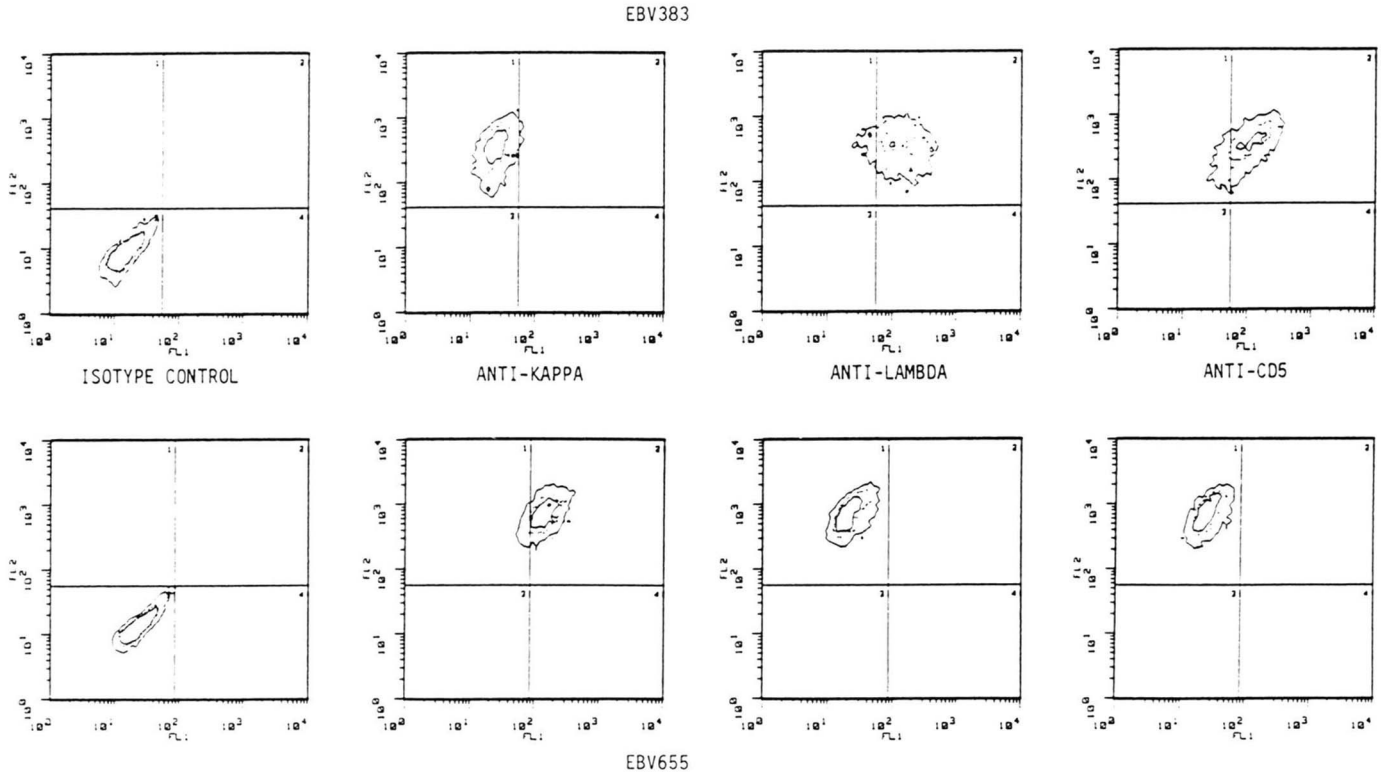

Figure 1. FMF of EBV383 (*upper row*) and EBV655 (*lower row*). Isotype control, fluorescence 1 (*FL1*): mouse IgG2a anti-human CD2 FITC; fluorescence 2 (*FL2*): mouse IgG1 anti-human CD2 PE. Anti-κ, FL1: goat F(ab′)$_2$ anti-human κ FITC; FL2: mouse IgG1 anti-human CD19 PE. Anti-λ, FL1: goat F(ab′)$_2$ anti-human λ FITC; FL2: mouse IgG1 anti-human CD19 PE. Anti-CD5, FL1: mouse IgG2a anti-human CD5 FITC; FL2: mouse IgG1 anti-human CD19 PE.

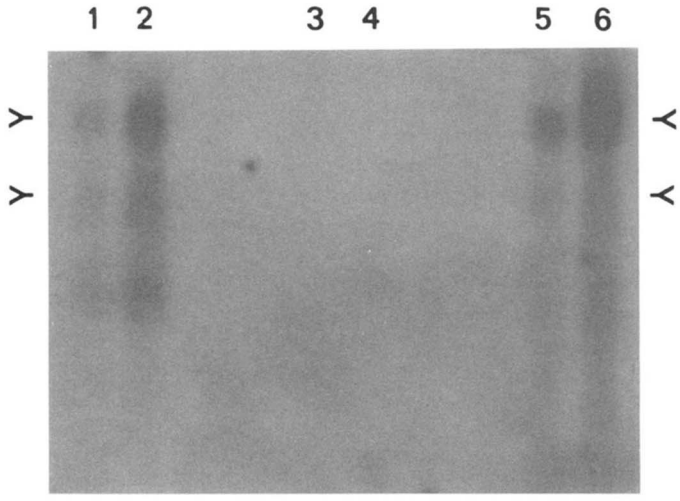

Figure 2. Autoradiogram of Northern blot probed with a CD5-specific probe (as described in *Materials and Methods*). *Lanes 1* and *2*, 7.5 and 15 μg, respectively, of total RNA isolated from EBV383; *lanes 3* and *4*, 7.5 and 15 μg respectively of total RNA isolated from EBV655 (an irrelevant human IgM producing CD5$^-$ EBV-transformed B cell); *lanes 5* and *6*, 7.5 and 15 μg, respectively of total RNA isolated from a human T cell clone as a positive control. *Upper* and *lower arrows* show the 3.6-kb precursor and the 2.7-kb mRNA transcript respectively. Also visible in *lanes 1* and *2* and *5* and *6* is a lower unknown mRNA band.

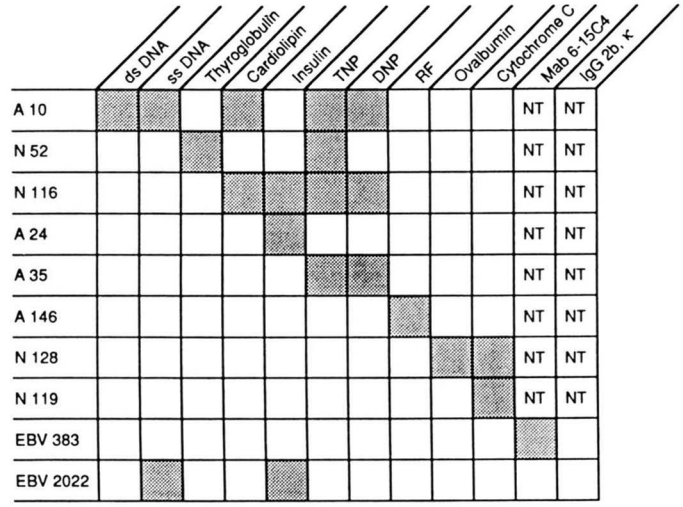

Figure 3. Binding of exogeneous Ag, haptens, autoantigens, murine IgG2b,κ Id other than mAb6-15C4 and human IgFc to human IgM antibodies produced by EBV transformed B cell lines of neonatal (*N*) or adult (*A*) origin, by EBV2022 (producing a human IgM antibody with an expressed V_HV gene, kindly provided by Dr. J. Thomas) and by EBV383. Binding is presented by the *shaded areas*.

adult origin containing IgM antibody with known activities for one or more of the Ag or idiotypes were used as controls. The results summarized in Figure 3 show that mAb383 is devoid of multireactive specificity and it shows only binding to the Id 6-15C4. This monoreactivity of mAb383 was further confirmed by the results of inhibition ELISA experiments in which the binding of mAb383 to the idiotope of 6-15C4 was measured in the presence of various ligands and antibodies as inhibitors. In Figure 4 it is demonstrated that the binding of mAb383 to plate-bound mAb6-15C4 is inhibited in a dose-dependent fashion by either rabies virus or a 21 amino acid peptide G5 representing the epitope of the viral glycoprotein that is recognized by mAb6-15C4, but not by an irrelevant 21 amino acid peptide E125 (32). Furthermore, the binding of mAb383 to Id 6-15C4 could only be inhibited by mAb6-15C4, but not by other mAb of the IgG2b, κ isotype. Based on the latter results the K_d of mAb383

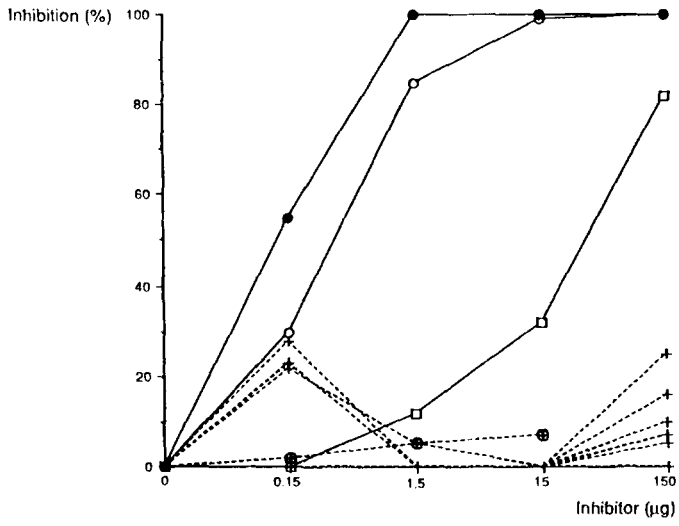

Figure 4. Inhibition ELISA to demonstrate the recognition of the idiotype 6-15C4 by EBV383. Binding of mAb383 to mAb6-15C4-coated microtiter plates is inhibited by peptide G5 (○), rabies virus Ag (□), murine IgG2b, κ mAb from different origin (+), an irrelevant peptide E125 (⊕) and the homologous inhibitor mAb6-15C4 (●). Percentage inhibition was calculated as described in *Materials and Methods*.

for the Id 6-15C4 was calculated and revealed a value of 8.67×10^{-9} mol/liter. Thus, collectively, these data suggest that mAb383 is a monoreactive antibody that binds with high affinity to an idiotypic determinant located within or near the Ag-binding site of mAb6-15C4.

DISCUSSION

Human CD5+ B lymphocytes belong to a subpopulation of cells that is endowed with the production of so-called natural antibodies (1–6). Multireactivity, self-reactivity, a low affinity binding to Ag, and a biased use of unmutated copies of germline V_H genes are distinctive features of these, mostly IgM antibodies (1, 9, 11–31).

In the light of these data, the combination of the phenotypical, functional, and molecular characteristics of clone EBV383 is remarkable. Our data clearly show that EBV383 is a CD5+ B cell (Figs. 1 and 2). The possibility that CD5 expression may have been induced by our EBV transformation protocol is highly unlikely as EBV383 is the only B cell clone out of fifty clones characterized by FMF analysis that shows expression of CD5. Furthermore, most human CD5+ chronic lymphocytic leukemia analyzed thusfar are EBV−. In addition to the phenotypical characterization we showed that clone EBV383 produces an IgM antibody that specifically reacts with the Id of mAb6-15C4 only and fails to react with any of the foreign Ag, haptens, or auto-Ag tested (Figs. 3 and 4). Thus, unlike most human CD5+ B lymphocytes, clone EBV383 produces a monoreactive IgM antibody which binds with high affinity ($K_d = 8.67 \times 10^{-9}$ mol/liter) to its putative ligand, i.e., the Id 6-15C4. This finding is consistent with our previous observation showing that the V_HV gene expressed by EBV383 displays extensive changes from the germline sequence leading to amino acid replacements accumulating in CDR I, CDR II, and framework III (33).

Though the relative contribution of the antibody H and L chains of mAb383 in the high affinity binding to the Id 6-15C4 remains to be established, our findings suggest that EBV383 results from an affinity maturation process brought about by the concerted action of somatic mutation and Id affinity-based selection. This would imply the use of an Id similar to the murine 6-15C4 Id in the human immune response against rabies virus, which possibility is being investigated. Alternatively, but less likely given the K_d of mAb383, EBV383 may have been selected by an epitope of an as yet unknown foreign Ag that is strongly cross-reactive with the 6-15C4 Id. For the first time, our present and previous results show that normal human CD5+ B cells not unlike conventional B cells may mature to B cells expressing monospecific high affinity and somatically mutated antibody V regions. Our data are reminiscent of the data by Harindranath et al.[5] who showed that high affinity IgM rheumatoid factors produced by EBV-transformed CD5+ human B cells display a number of somatic mutations in a way consistent with an Ag-driven immune response. Thus, in contrast to the situation in the mouse, IgM-secreting CD5+ B cells in man undergo somatic hypermutation and affinity maturation as conventional B cells do in response to Ag. However, it should be noted that in the mouse the possibility that V genes of Ly-1+ B cells can be subject to somatic hypermutation in an immune response to T cell-dependent Ag by deliberate immunization, has not been directly addressed. Experiments of Naparstek et al. (45) showed that the unmutated V_H segment used by CRI-A+ antibodies of A/J mice in response to the Ars also encoded IgM antibodies with multireactivity for ssDNA and cytoskeletal fibers. After immunization with Ars, CRI-A+ antibodies expressing somatic mutations in V_H showed high affinity binding to Ars but lost their reactivity to autoantigens. However, thusfar, direct evidence for the production of CRI-A+ somatically mutated anti-Ars antibodies by Ly1+ B cells in A/J mice remains to be demonstrated.

With regard to a physiologic role of CD5+ B cells displaying characteristics similar to EBV383, we envisage the following possibility. The finding that the high affinity binding of mAb383 to Id 6-15C4 can be completely inhibited by rabies virus Ag or peptide G5, that represents an epitope of the viral glycoprotein, suggests that the antibody V region of mAb383 may structurally resemble this G5 epitope (Fig. 4). Determination of the contribution of H and L chains of mAb383 in the binding to mAb6-15C4, elucidation of the nucleotide sequence of the V_L of mAb383 and immunization studies with mAb383 as surrogate Ag are required to test this assumption. If indeed mAb383 would appear to bear an image of the G5 epitope, the interesting possibility arises that upon immunization with Ag (i.e., rabies virus vaccine), an Ag-specific Id-bearing B cell clone (i.e., 6-15C4-like) due to its increased frequency signals and selects a somatically mutated anti-idiotypic B cell clone (i.e., EBV383). Such an anti-idiotypic clone, because its somatically mutated antibody V region now mimics the epitope (i.e., G5), may be dependent on the threshold of the antibodies that it produces, either allow the co-existence of idiotypic long-lived memory B cells specific for the mimicked epitope or signal and select other idiotypic B cells with specificity for the epitope to develop to memory B cells. In both cases B cell

[5] **N. Harindranath, I. S. Goldfarb, H. Ikematsu, S. E. Burastero, R. L. Wilder, A. L. Notkins, and P. Casali.** Complete sequence of the genes encoding the V_H and V_L regions of low and high affinity monoclonal IgM and IgA rheumatoid factors produced by CD5+ B cells from a rheumatoid arthritis patient. Submitted for publication.

memory will be maintained in the absence of Ag. If one considers the evidence showing that Id can be processed and presented in the form of idiopeptides in association with MHC class II molecules, the presence of Ag-specific Th cells would be sufficient to activate both Ag-specific idiotypic and anti-idiotypic B cells (46–50).

The idea that B cell memory may be subject to idiotypic control has been proposed earlier by others (51–53). However, our version of this general idea contains some key elements, i.e., images of Ag carried by somatically mutated V regions of anti-idiotypic antibodies of CD5+ B cells that would support the views that immunological memory is a systemic rather than a clonal property (52, 54).[6]

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