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Autoreactivity of Human VH Domains from cDNA Libraries: Analysis with a Bacterial Expression System

Jean-Michel Lecerf, Yan Chen, Pascale Richelet-Sécordel, Xiaowei Wang, and B. David Stollar

Previous studies showed that VH domains of several anti-DNA Abs can bind DNA in the absence of VL. In the current work, we tested the VH autoreactive potential more generally, examining VH domains that did not come from known autoantibodies. Using a bacterial expression system, we produced 11 fusion proteins, each containing a VH domain and a B domain of staphylococcal protein A. The VH domains were coded in cDNA libraries from circulating B cells of healthy young adult humans. Thus, binding properties of the Ig molecules from which they came were unknown. The B cells had not been stimulated in vitro. Seven cDNA clones combined the frequently expressed \( \text{V}_{\text{H}}\text{-}3-23 \) gene segment with varied \( \text{D}_{\text{H}} \) and \( \text{J}_{\text{H}} \) segments. The other clones contained unmutated \( \text{V}_{\text{H}}\text{-}3-7, \text{V}_{\text{H}}\text{-}3-9, \text{V}_{\text{H}}\text{-}3-53, \) and \( \text{V}_{\text{H}}\text{-}4-39 \) segments. We compared these bacterial expression products with single-chain Fv, VH and VL domains of IgM mAb 18/2, a \( \text{V}_{\text{H}}\text{-}3-23 \)-encoded, DNA-binding autoantibody. Submicromolar concentrations of 5 of the 11 VH domains bound to ssDNA. Those and one more also bound to immobilized poly(dT), and two bound to circular plasmid dsDNA. Soluble poly(dT) was the most potent inhibitor in competitive ELISA. Seven of the VH domains also bound to immobilized nuclear ribonucleoprotein, four to histone and none to thyroglobulin. Two interacted with the matrix of a Sephacryl S-100 column.

The polyreactive autoantigen-binding properties of these VH domains raise the question of whether these properties may play a role in the formation of the VH repertoire of circulating B cells. 

\text{A}utoantibody formation gained recognition in its association with disease (1, 2), an aberration from normal function. Normally, B cell tolerance of autologous substances is established through deletion or anergy of autoreactive B cells and the absence of T cell help (3–7) or receptor editing that pairs potentially autoreactive Ig H chains with L chains that preclude autoreactivity (8, 9). Potentially autoreactive B cells do exist in healthy individuals (3). They are generally silenced, but they can be activated if T cell help becomes available (10) or if nonspecific B cell stimulants are encountered (11). Thus, endogenous substances can be effective haptens when assembled with foreign protein carriers (e.g., see Ref. 10), and a significant fraction of hybridomas from LPS-stimulated B cells of newborn mice (12) or immunized young adult animals (13) produce Abs that react with self Ags such as nucleic acids, thyroglobulin, and cytoskeletal proteins.

Not all autoreactive B cells are eliminated or silenced in normal subjects. Natural autoantibodies are formed and secreted at all stages of life (14, 15). Indeed, because some serum components inhibit autoantigen binding (16–19), the abundance of natural autoantibodies is greater than what is estimated with serologic assays of whole serum. In experimental models also, autoantibody production can coexist with expression of the corresponding autoantigen (20, 21), and autoreactive B cells may be the first to be stimulated by exposure to a related foreign Ag (22).

Frequent natural autoantibody activity among IgM molecules with unmutated V region gene segments has led to the suggestion that autoreactivity could help to shape the naive B cell repertoire in a positive way (23, 24) as well as by negative selection (4) and that VH domains play an important role in part of this process (24). Nucleic acid binding is a relatively frequent property of natural autoantibodies (13). H chains play a dominant role in DNA binding by several autoantibodies as well as structurally related anti-DNA Abs induced by immunization, and in many cases the VH domain alone can bind DNA (25–29). Perhaps H chains with nucleic acid-binding activity arise even more frequently than whole Ig anti-DNA but are paired with L chains that block autoreactivity. To test this possibility, we have analyzed autoantigen binding by VH domains that were not chosen for study on the basis of known activity. Rather, they were coded in cDNA made from circulating B cells of healthy young adult humans. The cells were not stimulated in vitro before cDNA was prepared. We could not know the ligand-binding properties of the whole Ig in which these domains occur. The cDNA clones were described previously (30). For the present study, we have emphasized clones that have few V region mutations and contain the frequently expressed \( \text{V}_{\text{H}}\text{-}3-23 \) gene segment, which has been associated with autoantibodies such as the IgM anti-DNA mAb 18/2 (31). We have used bacterial expression vectors, described previously (32), to prepare VH domain proteins from the cloned cDNAs, along with VH, VL, and single-chain Fv (scFv) domains of IgM anti-DNA Ab 18/2. Expression of recombinant V domains as fusion proteins with the B domain of staphylococcal protein A facilitated secretion of soluble protein, assay, and purification.

\text{Abbreviations used in this paper: scFv, single-chain Fv; CDR, complementarity-determining region; AP, alkaline phosphatase; RNP, ribonucleoprotein; VH, domain comprising \( \text{V}_{\text{H}}\text{, D}_{\text{H}}, \) and \( \text{J}_{\text{H}} \) segments; VL, domain comprising \( \text{V}_{\text{L}}, \text{J}_{\text{L}} \) segments.}
Materials and Methods

Antigens for serologic assay

Poly(dT), poly(dU), poly(dA), and poly(dC) were purchased from Pharmacia, Piscataway, NJ. Circular plasmid was used for dsDNA; a scFv-containing expression vector (25) was expanded in DH5α cells and purified with a Maxiprep column (Qiagen, Chatsworth, CA). Native calf thymus DNA (Sigma, St. Louis, MO) was boiled for 10 min and quickly chilled to form ssDNA. Core histones were acid-extracted from H1-depleted calf thymus chromatin (33). Human thyroglobulin was purchased from Dako, Carpinteria, CA, and partially purified nuclear ribonucleoprotein (RNP) from Immunovision, Springdale, AZ.

V region cDNA

Two human VH cDNA libraries in M13 phage DNA (34) served as sources of 11 clones. GenBank accession numbers and names for all clones are listed in Table I, along with information on corresponding germline genes and VH segment mutations. Seven of these clones have VH segments with 96 to 100% base sequence identity to that of VH3-23, three have other unmutated VH3 family members, and one has an unmutated VH4 family member. VH and VL cDNAs for mAb 18/2 were originally amplified from hybridoma mRNA (31) and cloned into M13 phage (34). MAb 18/2 is a polyreactive DNA-binding IgM with an unmutated VH3-23 gene segment (31).

Amino acid sequences of the V domains are aligned in Figure 1. As expressed, 18/2 VH and two cDNA clones have an unmutated VH3-23 amino acid sequence. One of these two clones has a single JH replacement. Five cDNA clones have VH3-23 segments with 1, 2, 3, or 11 amino acid replacements. The other VH domains have germline VH3 or VH4 family gene segments. Complementarity-determining regions 3 (CDR3s) of the cDNA clones are all different in sequence and length.

The V region inserts in M13 DNA were amplified by a PCR in which primers (Table II) contained unique restriction sites allowing insertion into pIg20-based expression vectors (32). Oligonucleotides were synthesized by...

![FIGURE 1](http://www.jimmunol.org/) Amino acid sequences of the expressed VH domains, translated from cDNA. The reference VH sequence, 18/2, uses unmutated VH3-23, 21 bases of Dxp1, and unmutated Jγ5. Amino acids added by the restriction site-containing primers are in italics: R or RAQQA residues preceding the initial VH codon, and SRHHGL or AAAHGL following residue 113. The latter amino acids are followed by a thrombin cleavage sequence (THROMBIN) and a single B domain of staphylococcal protein A (SPA).
the Protein Chemistry Facility at Tufts University School of Medicine (Boston, MA).

**Plasmids, bacterial strains, and media**

The expression vectors plg20, plg20H, plg20L, and plg20H (32) were used for expression of VH, VL, or scfV fused to the B domain of staphylococcal protein A. The plasmid constructions were introduced into Escherichia coli SURE cells (Stratagene, La Jolla, CA) by electroporation for further manipulations and into E. coli strain BL21(DE3)pLysE cells (35) by chemical transformation for expression of recombinant proteins. Bacterial strains were grown in Luria broth medium (10 g/L Bacto-yeast extract, and 10 g/L NaCl, pH 7.0). Livingston, NJ) for growth of SURE cells and with 100 μg/ml ampicillin (Jersey Lab & Glove Supply, Livingston, NJ) for growth of SURE cells and with 100 μg/ml ampicillin and 20 μg/ml chloramphenicol (Sigma) for growth of BL21 (DE3)pLysE cells.

**Bacterial expression and purification of recombinant proteins**

Bacterial expression of recombinant proteins and their purification with columns of IgG-Sepharose were described previously (36). Recombinant proteins were eluted from the human IgG-Sepharose column with 0.1 M acetic acid, pH 3.4. Fractions with an OD280 above 0.05 were pooled and adjusted to pH between 5 and 6 by addition of an equal volume of 0.1 M sodium acetate, pH 5. The recombinant proteins were purified further with a 1-m1 cation exchange column. The resin, carboxymethyl-Bio-Gel A (Bio-Rad, Hercules, CA), was poured into a column, washed with 3 to 4 bed volumes of a solution of 0.5 M NaOH and 2 M NaCl, and then equilibrated with 10 mM sodium acetate, pH 5. The ionic strength of the recombinant protein solution was reduced by addition of distilled water to give a final concentration of 10 mM acetate, and the protein sample was loaded onto the column. The loaded resin column was washed with 10 mM acetate, pH 5, and then stepwise with five buffers containing 10 mM acetate and increasing concentrations of NaCl (50, 100, 150, 200, 300 mM). Proteins were collected in 1-m1 fractions and quantified by UV absorbance spectra. The program Peptidesort, from the Genetics Computer Group (Madison, WI), was used to calculate the m.w. and the extinction coefficient of each protein, based on the amino acid sequence.

**Detection of recombinant proteins**

Coomassie blue staining or Western blotting was used for detection of proteins separated by SDS-PAGE (25, 36). Samples of 2 μg of purified protein were used for Coomassie blue staining and ~200 ng for Western blotting. The protein samples were mixed with an equal volume of 2× SDS loading buffer (100 mM Tris-HCl (pH 6.8), 200 mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol) and boiled for 5 min before being loaded onto a 12% or 15% SDS-polyacrylamide gel. For Western blotting, protein was transferred electrophoretically from the gel to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA). The transfer buffer was 20 mM Tris, 200 mM glycine, and 20% (v/v) methanol. After transfer, the membrane was incubated in blocking solution (PBS containing 5% nonfat dry milk and 0.1% Tween). The blotted membrane was incubated with 1 μg/ml rabbit IgG (Sigma) for detection of the fusion protein through its protein A domain, and bound IgG was detected with AP-conjugated goat anti-rabbit IgG. Between incubations, the membrane was washed three times in washing buffer (blocking solution diluted 1:10 with PBS); each incubation was for 1 h at room temperature. Finally, the membrane was washed three times in washing buffer and once with AP buffer (100 mM NaCl, 5 mM MgCl2, 100 mM Tris-HCl, pH 9.5) and developed with 0.33% nitro blue tetrazolium and 0.165% 5-bromo-4-chloro-3-indolyl phosphate in AP buffer.

ELISA was used to measure Ag binding activity. Wells of UV-treated (37) polystyrene microtiter plates (Immulon 1, Dynatech, Alexandria, VA) were coated with 100 μl of nucleic acid, nucleoprotein, or protein Ag at a concentration of 2 μg/ml. The wells were blocked with 1.4% BSA in PBS and incubated with various concentrations of recombinant proteins. Then the plates were washed and 2 μg/ml rabbit IgG in PBS were added to detect the recombinant protein. Bound rabbit IgG was detected with AP-labeled goat anti-rabbit IgG (Boehringer Mannheim, Indianapolis, IN), diluted 1:3000.

**Analytical gel filtration**

A 1.4- × 48-cm column of Sephacryl S-100 (Pharmacia) was used to test for the size distribution among monomers, dimers, and higher aggregates. Purified recombinant protein samples of 20 μg in 1 ml of PBS were applied to the column, which was then washed with PBS. Fractions of 1 ml were collected. Recombinant protein was detected by ELISA, through recognition of its protein A domain. For this purpose, samples of 100 μl of each fraction were used to coat wells of an Immulon 2 microtiter plate (Dynatech). Wells were then blocked with 1.4% BSA in PBS and washed. Rabbit IgG (1 μg/ml) was added to each well. After a 1-h incubation, bound rabbit IgG was detected with AP-labeled goat anti-rabbit IgG.

**Thrombin cleavage of VH-protein A fusion proteins**

A 100-μg sample of recombinant VH-protein A fusion protein in 400 μl of PBS with 2.5 mM CaCl2 was incubated with 1.75 μg of thrombin (Hematologic Technologies, Essex Junction, VT) at 25°C for 2 h. The reaction was stopped by addition of EDTA to a final concentration of 5 mM. Separated protein A and any undigested protein were removed by addition of IgG-Sepharose beads. The suspension was mixed on a rotator overnight at 4°C, and the beads were removed by centrifugation. PAGE and Coomassie blue staining revealed that the supernatant fluid contained only cleaved VH domain and a small amount of thrombin. Protein A was eluted from the IgG-Sepharose beads at pH 3.4. For ELISA on nucleic acid-coated microtiter plates, the bound VH domain was detected with a rabbit serum in-
Life Technologies (Gaithersburg, MD). kD, kilodaltons. SDS-polyacrylamide gel. Markers are the low m.w. prestained set from were boiled in Tris buffer with SDS and DTT and separated on a 15% on IgG-Sepharose followed by cation-exchange chromatography. Proteins recombinant proteins were aggregated under nondenaturing con-

... from amino acid sequences (22,000). To determine whether the proteins migrated at the position expected for the m.w. calculated ... recovered after this second step. Final yields varied from 0.1 to 2.0 mg/L of bacterial culture.

The cDNAs for V and scFv domains were cloned into pIg20-based vectors and expressed in E. coli BL21(DE3)pLysE cells. Recombinant proteins were harvested from the bacterial culture supernatant and affinity-purified with IgG-Sepharose. A second step of purification on a cation-exchange column provided more highly purified protein preparations, eliminating mainly higher m.w. proteins. From 50 to 70% of the Ig affinity-purified proteins were recovered after this second step. Final yields varied from 0.1 to 2.0 mg/L of bacterial culture.

When analyzed by SDS-PAGE (Fig. 2), the recombinant proteins migrated at the position expected for the m.w. calculated from amino acid sequences (~22,000). To determine whether the recombinant proteins were aggregated under non-denaturing conditions, we passed them through a Sephacryl S-100 column calibrated with globular proteins of known m.w. The recombinant protein solutions had very little aggregated material (Fig. 3). Some had a small amount of what appeared to be dimer, but in each case most protein emerged in a large peak, near the point of emergence of cytochrome c (m.w. 12,400) (Fig. 3, A and C). On SDS-PAGE, the protein in this large peak behaved as the major band in Figure 2. Thus, flow of the proteins through the Sephacryl S-100 column was slightly slower than expected from predicted m.w.s, indicating the proteins may have interacted weakly with the column matrix. Such behavior was especially marked for two proteins, 1f6-3, which emerged 10 fractions later (Fig. 3B), and Amu10-2, which emerged in fractions 100 to 110 (not shown). Interaction of these two VH domains with the polysaccharide-containing column matrix is reminiscent of the behavior of a human IgM paraprotein, WEA, which bound to a Sepharose column and reacted with polysaccharide determinants of Klebsiella (43) as well as with ssDNA and polynucleotides (44).

As a further test of integrity of recombinant domains, we determined whether 18/2 VH was able to associate with 18/2 VL to form an Fv. The two proteins were mixed at equal concentrations and loaded onto a Sephacryl S-100 size exclusion column. They emerged as one major peak corresponding in size to a VH-VL heterodimer (Fig. 4A). Fractions 40 and 41, with the highest protein concentration, were pooled and analyzed by Western blotting (Fig. 4B). The pooled fractions contained an approximately equimolar mixture of 18/2 VH and 18/2 VL, consistent with assembly of the two V domains into a 1:1 complex. This pooled fraction is not likely to consist of VH-VH and VL-VL dimers because the separate VH and VL preparations, analyzed with the same column, emerged mainly as expected for a monomer in each case (not shown). Furthermore, 18/2 VH-VL association was previously measured with surface plasmon resonance, which revealed a $K_a$ of $\sim 6 \times 10^5$ M$^{-1}$ for the interaction (32).

Nucleic acid binding by VH domains

We then tested the autoreactive potential of separate VH domains obtained from the cDNA libraries. Five of the recombinant VH proteins reacted, at concentrations between 0.1 and 1 $\mu$M, with immobilized ssDNA (Fig. 5). Although 18/2 VH bound to immobilized poly(dT), it did not bind to immobilized ssDNA. The same was true for the VH domain 1g5-3; and two others (1f6-3 and Amu6-3) gave weak and variable binding to poly(dT). Competitive ELISA revealed that 18/2 scFv and the library VH domains bound to soluble as well as to immobilized polynucleotides and that both the Fv and VH domains reacted more strongly with poly(dT) than with ssDNA or dsDNA (Fig. 6).

The 5 VH domains that bound ssDNA were expressed with: an unmutated V$\gamma_3$-23 gene product (Amu10-2); a V$\gamma_3$-32 product with 1 (Agamma6-3), 2 (Agamma34-1), or 11 (Agamma21-3) substitutions; or an unmutated V$\gamma_3$-53 gene product (Amu1D4-3). The presence of a V$\gamma_3$-23 gene product was not sufficient for ssDNA binding, however, because other V$\gamma_3$-32 coded proteins (1g5-3 and Amu1D11-3 or 18/2 VH) did not bind to it. Some distinction was noted in the CDR3 Arg content even though the clones were not chosen on that basis (Fig. 1). The two domains that bound both ssDNA and dsDNA (Amu10-2 and Amu1D11-3) each had two CDR3 Arg residues. Four of the six nonbinders had no CDR3 Arg and two had one Arg each.

To determine whether these interactions with nucleic acids depended simply on binding to charged polymers or whether there was selectivity for portions of the pyrimidine or purine bases, we
tested reactions of VH domains with other polynucleotides. Examples are shown in Figure 7. For all but Ig5-3, reactions with poly(dC) were nearly equal to those with poly(dT). Poly(dU) reactivity was variable. None of the VH domains bound to poly(dA) (Fig. 7). In simultaneous control experiments, a mAb to poly(dC) reacted only with poly(dC). A human SLE serum, diluted 1/1000, reacted strongly with poly(dT), poly(dU), and poly(dC) but only very weakly with poly(dA) (not shown).

Several of the proteins did not bind ssDNA; therefore, it was unlikely that the protein A portion of the fusion protein was responsible for binding of the positive VH domains. To test this issue further, the Agamma21-3 fusion protein was digested with thrombin to release the free VH domain. In ELISA performed with an anti-recombinant scFv serum as a detector, the VH retained nucleic acid-binding activity (Fig. 8); the protein A domain did not bind to immobilized nucleic acid (not shown).

Affinity measurements

Affinities of two VH domains for poly(dT) were measured using the Bioevaluation software 3.0 (BIACore), with sensor chips bearing d(T)25. Assessment of the on-rate constant requires knowledge of the active VH protein concentration. The concentration was determined with the BIACore for VHAgamma21-3 and by passage of a mixture of VH protein and poly(dT) through a Sephacryl S-100 column such as that shown in Figure 3 for both VHAgamma21-3 and VH1g5-3. Results with both procedures were consistent for Agamma21-3. Based on these measurements, concentrations of 10 to 100 nM active VHAgamma21-3 and VH1g5-3 were used in the BIACore measurements of affinity. Agamma21-3 and Ig5-3 had association rate constants (K_a) of 1.2 (± 0.8) × 10^4 and 2.7 (± 0.3) × 10^3 s⁻¹ M⁻¹. Their dissociation rate constants (K_d) were 3.1 (± 0.3) × 10⁻⁴ and 1.3 (± 0.2) × 10⁻³ s⁻¹; and their K_d were

FIGURE 3. Gel column filtration of VH-SPA fusion proteins Agamma21-3 (A), Igf6-3 (B), and Tmu69 (C) through a Sephacryl S-100 HR size exclusion column monitored by ELISA. The column (1.5 cm × 48 cm; total volume, 72 ml) was calibrated with relative m.w. standards: β-amylase (M_r 200,000); BSA (M_r 66,000); carbonic anhydrase (M_r 29,000); and cytochrome c (M_r 12,400); high m.w. Z-DNA marked the void volume. Samples of 20 μg of purified recombinant protein were applied to the column, and fractions of ~1 ml were collected. For analysis by ELISA, 100 μl of each fraction were placed in a well of a microtiter plate to allow protein adsorption. Adsorbed protein was detected through the SPA domain by sequential incubations with rabbit γ-globulin and AP-conjugated goat anti-rabbit IgG Ab.
Western blot analysis of 18/VH assays with immobilized ligands are summarized in Table III. Results of available in solution. In addition, the RNP binding depends on a determinants available in the immobilized preparation are not either the affinity of this interaction with soluble Ag is very low or nRNP or poly(dT) did not inhibit the binding to immobilized RNP. The question arises whether the reactions that we observed are coincidental and unrelated to in vivo processes. The Ags that we tested are frequently reactive with natural autoantibodies and Igs from hybridomas of newborn or unimmunized animals as well as with Abs from patients with autoimmune disease; therefore, it is possible that there is a link with in vivo reactions. Prominent among our positive test Ags was poly(dT), which also reacted most strongly with the IgM form (45) as well as the scFv form (Fig. 6) of Ab 18/2. Oligo(dT) was the first epitope defined for SLE anti-ssDNA Abs in serum of a patient with active disease (46). Subsequently, poly(dT) was found to be prominent in reactions of other human SLE sera (47) and monoclonal autoantibodies from humans (45) and mice (48, 49). Poly(dT) was a prominent ligand for a crystallized Fab from lupus mouse autoantibody Hed-10 (50), and (dT)₆ was a component in the crystal structure of ligand-bound anti-ssDNA autoantibody BV04-01 (51). Reaction with poly(dT) appears to be an amplified version of binding to ssDNA and seems to have the same significance as a marker for both natural autoantibodies and some disease-related anti-DNA Abs.

The frequent reactivity of poly(dT) may reflect its structural flexibility, which allows many different three-dimensional interactions with Abs from patients with autoimmune disease; therefore, it is possible that there is a link with in vivo reactions. Prominent among our positive test Ags was poly(dT), which also reacted most strongly with the IgM form (45) as well as the scFv form (Fig. 6) of Ab 18/2. Oligo(dT) was the first epitope defined for SLE anti-ssDNA Abs in serum of a patient with active disease (46). Subsequently, poly(dT) was found to be prominent in reactions of other human SLE sera (47) and monoclonal autoantibodies from humans (45) and mice (48, 49). Poly(dT) was a prominent ligand for a crystallized Fab from lupus mouse autoantibody Hed-10 (50), and (dT)₆ was a component in the crystal structure of ligand-bound anti-ssDNA autoantibody BV04-01 (51). Reaction with poly(dT) appears to be an amplified version of binding to ssDNA and seems to have the same significance as a marker for both natural autoantibodies and some disease-related anti-DNA Abs.

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with diverse Ab binding sites. For example, the structure of (dT)₃ in the binding site of Ab BV04-01, determined from the crystal structure of the Ag-Fab complex, is not one that could be predicted (51). The adopted conformation allowed side chains of Ab BV04-01 to associate with bases, pentoses, and phosphates of (dT)₃ through hydrogen bonding, ring stacking, and ionic interactions. Our serologic tests with several polynucleotides indicated that VH domain binding to poly(dT) is not simply a charge interaction with a polyanion, because there was no binding to similarly charged poly(dA) and binding to dsDNA was weak or absent. There was variable dependence on the hydrophobicity of the thymine methyl group, as seen in the

![Figure 6](image1)

**FIGURE 6.** Competitive immunoassays. Various concentrations of soluble poly(dT) (), ssDNA () or dsDNA (■) were incubated with 18/2 scFv-SPA (300 nM) (A), Amu10-2 VH-SPA (450 nM) (B), Agamma21-3 VH-SPA (450 nM) (C), or 1g5-3 VH-SPA (450 nM) for 30 min (D), and the mixture was added to wells coated with 2 µg/ml poly(dT). Bound recombinant protein was detected with rabbit IgG and AP-labeled goat anti-rabbit IgG. Each point is the average of duplicate wells.

![Figure 7](image2)

**FIGURE 7.** Reactions of VH domains with polynucleotides. Varying concentrations of recombinant 18/2 scFv (A), Amu10-2 (B), Agamma21-3 (C), or 1g5-3 (D) were added to wells coated with 2 µg/ml poly(dT) (), poly(dU) (), poly(dC) (), or poly(dA) (■) or wells that were simply blocked with BSA (). Bound recombinant protein was detected with rabbit IgG and AP-labeled goat anti-rabbit IgG. Duplicate points are plotted.
relative reactions of poly(dU) and poly(dT) with different VH domains. Measured affinities for (dT)\textsubscript{25} were 3.8 \times 10^{-7} \text{M}^{-1} and 2.0 \times 10^{-7} \text{M}^{-1} for demonstrably active protein in two of the VH domain preparations. These values are within the range of many in vivo interactions. However, it is possible that nucleic acids are not the critical physiologic ligands for these VH domains and Abs in vivo and that polynucleotides mimic a feature of a different endogenous ligand, such as a protein. Known examples of such mimicry are cross-reactions of DNA with Abs to Sm (52) or ribosomal proteins (53).

Another question that requires further investigation is whether the conformation of the recombinant VH truly resembles that of a native protein. The VH domains were not aggregated, and stoichiometric 18/2VH-18/2VL association (Fig. 4) suggested that these recombinant forms can adopt a conformation related to native Fv. Comparable VH and VL domains of a monoclonal anti-Z-DNA Ab reassociated to form a functional Fv as reflected in both specific Ag binding and Id expression (25). However, only part of the purified VH protein folds into a polynucleotide-binding conformation as judged by the measurement of stable complexes detected by gel exclusion chromatography. With purified VH preparations of various Abs, we have found from 5 to 40% of the protein active by this criterion (Y. Chen and B. D. Stollar, unpublished data). In affinity-purified samples of Agamma21-3 and Ig5-3 stored frozen at −20°C, <5 to 6% of the protein was active. In supernatants containing other unpurified VH domains, >40% was active in poly(dT) binding as measured by BIAcore experiments (P. Richalet-Sé cordel, unpublished data). Studies on factors affecting folding and stability of recombinant VH are in progress.

**VH binding activity and natural autoantibodies: relation to the circulating B cell repertoire**

Because the VH domains that we tested were from B cells that spontaneously produced IgM mRNA, without in vitro stimulation, and were from adult subjects, it is possible that the cDNA libraries were shaped by the history of Ag exposures of these individuals. On the other hand, several of the clones had unmutated V\textsubscript{H} and J\textsubscript{H} segments, characteristic of IgM\textsuperscript{+}IgD\textsuperscript{−} "naive" B cells (54), which make up about two-thirds of circulating IgM-producing cells (30, 54).

The autoreactive potential of VH chains, including several coded by unmutated germline genes, may be related to the fact that autoreactivity is not a unique feature of autoimmune diseases but is also part of the normal Ab repertoire of circulating B cells (55, 56). Many natural autoantibodies are polyreactive (able to bind to several kinds of structures) IgM molecules. Although the V regions of IgM natural autoantibodies may be coded by unmutated V gene segments, the CDR3H sequences are diverse (57), appear to have been ligand selected (58, 59), and are important for autoantigen binding and polyreactivity (59–62).

It is not known how B cells producing natural autoantibodies have been selected before encountering foreign Ag. The human
V_{H}3-23 gene segment, which is expressed frequently (63, 64) and is found in autoantibodies, in Abs to exogenous Ags (65, 66), and in many functional Fv domains in phage libraries (66, 67), may facilitate binding of various Ags, including autoantigens. Raising a different option, Silverman et al. (68) have observed that framework sites of V_{H}3-containing IgM bind to bacterial Ags such as staphyloccocal protein A, suggesting that superantigen interactions may underlie the frequent expression of certain V_{H}3 segments.

In the bone marrow, major losses of developing B cells, by apoptosis, occur after expression of the μ-δ-R receptor between pro-B and pre-B cell stages and soon after expression of the complete Ig receptor on immature B cells (69). Removal of strongly autoreactive cells explains some of the loss (70–72), but it is also possible that many cells die for lack of a corresponding ligand in the marrow environment. Perhaps low affinity binding (or binding with low receptor occupancy) to self ligands provides a survival signal, yielding circulating B cells that are both modestly autoreactive and polyreactive. H chain could play a prominent role in some of these interactions and L chains in others (IgM rheumatoid factors are associated with a particular κ chain (55)). Polyreactivity of these IgM molecules would make them very suitable in screening for invaders such as bacteria and viruses; the modest affinity of interaction at a single site could be greatly amplified by multivalence.

The ability of self ligands to yield either negative or positive selection can depend on the state of the cell and its intracellular signal transduction systems (73). Immature B cells are particularly sensitive to inactivation by reagents that bind strongly and cross-link B cell IgM receptor assemblies (74). On the other hand, autoreactive B cells can be positively selected by autoantigen when intracellular signaling from the B cell receptor is attenuated, as demonstrated in mice lacking CD45 (75). The outcome may also depend on the concentration of Ag, mode of presentation, affinity of interaction between autoantigen and receptor, and the time of autoantigen expression (21, 76, 77). Further analyses of the binding and functional properties of B cell receptors at various stages of ontogeny may help to clarify the physiologic processes.

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References


