SYNGENEIC ANTIIDIOTYPIC IMMUNE RESPONSES TO A B CELL LYMPHOMA

Comparison Between Heavy Chain Hypervariable Region Peptides and Intact Ig as Immunogens

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The idiotypic (Id)[1] determinants of surface Ig of B cell lymphoma are excellent examples of tumor-specific markers (1–3). Anti-Id raised against such tumor-derived Id react almost exclusively with the malignant B cell clone and not with normal cells in the host (4). Consequently, such antibodies have been useful both as therapeutic agents and as reagents for the study of the biology of these tumors (4–11). The mechanism of the antitumor effect of passively administered anti-Id has been assumed to involve the direct binding of the antibodies to the tumor cells in vivo, followed by cell destruction by host effector mechanisms such as complement, natural killer cells, and macrophages. However, some observations (12, 13) suggest the possibility of indirect mechanisms, such as the induction of immunoregulatory Id networks involving tumor-specific suppressor T cells.

Immunization with intact Id proteins, isolated light (L) chains, or even the variable (V) domain of the L chain (V_L) has been shown (14–18) to induce immune responses that are Id-specific, and that are capable of regulating the differentiation of tumor cells. These immune responses also can confer a state of resistance to tumor growth. Recently (19–22), synthetic peptides comprising the amino acid sequences of the hypervariable regions of heavy (H) or L chains have been reported to be capable of inducing the production of antibodies reactive with Id determinants of intact Ig molecules. Herein, we explore the feasibility of using synthetic peptides comprising hypervariable region sequences of an H chain to generate syngeneic antibodies specific for the Id determinants on the surface of a B cell tumor.

We have determined the nucleic acid sequence of the V_H gene expressed by a B lymphoma, synthesized peptides corresponding to the second and third hypervariable regions, and produced both polyclonal and monoclonal antibodies (mAb)

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; C, constant region of Ig; cDNA, complementary DNA; CDR, complementarity-determining region; D, diversity-generating region of Ig; ELISA, enzyme-linked immunosorbent assay; H, heavy chain of Ig; Id, idiotype; J, joining region of Ig; L, light chain of Ig; mAb, monoclonal antibody; mRNA, messenger RNA; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

against these peptides. We demonstrate that polyclonal antisera raised against the peptides failed to react with the intact IgM. However, a small percentage of mAb made against one complementarity-determining region (CDR) peptide (CDR3) did react with an Id determinant expressed both by the isolated μ chain and the intact IgM molecule. Three such mAb were characterized. They bound the intact IgM with markedly lower affinity than did anti-Id that had been produced against the intact protein. Vaccination of animals with the CDR3 peptide did not reproduce the immunity to tumor challenge that could be obtained by vaccination with the intact IgM protein.

Materials and Methods

Mice. C3H/HeN and C3H/HeN × BALB/c F_1 mice were purchased from Simonsen Laboratories (Gilroy, CA), or bred at the Laboratory Animal Facility at the Stanford University Medical School.

Tumor and Cell Lines. 38C13, a carcinogen-induced B cell tumor, was serially transplanted in syngeneic C3H mice (23). This tumor and its in vitro adapted cell line express IgM (κ) on the cell membrane but do not secrete Ig. A high Id-secreting cell line was established by fusion between the 38C13 tumor and the nonsecreting BALB/c myeloma cell line P3X63Ag8.653 (24).

Sequence of the 38C13 μ Chain. The sequence of FR4, CDR3, FR3, and part of CDR2 of the 38C13 μ chain was obtained by direct messenger RNA (mRNA) sequencing (25, 26) using a synthetic oligonucleotide primer. Poly A^+ mRNA was isolated from the high Id-secretor cell line following a described protocol (27). Based on the known sequence of the constant (C) region of the μ chain of mouse IgM (28), the following primer was synthesized: d(CAGGAGACGAGGGGGAA). This sequence was designed to hybridize to the C region of μ chain mRNA near the V-C region junction; more precisely, 24 bases into the C region where the nucleotide sequence for murine and human mRNA (29) is identical. Hence, the same primer can be used to sequence V regions of mRNA of murine and human source.

A second method was used to confirm the sequence determined from the RNA and to extend the sequence to include the entire V_H region. A complementary DNA (cDNA) library of 38C13 B cell tumor poly A^+ mRNA, cloned into the expression vector λ gt 11, was kindly provided by Dr. Tom St. John (Stanford University). A clone containing a 2.2 kilobase (kb) cDNA insert coding for the μ chain was isolated by immunologic screening (30). This clone was hybridized to a ^{32}P-labeled cDNA probe obtained by reverse transcription of the mRNA using the synthetic oligonucleotide as primer. This confirmed the immunologic screening. A 550 base pair (bp) Eco RI–Sma I fragment containing the entire V region was isolated from this cDNA and subcloned in M13 mp 10 (31), using JM 101 as host. The DNA sequence was then determined (32).

Peptide Synthesis and Conjugates. Peptides were synthesized by solid-phase technique on a Beckman Model 990B peptide synthesizer using commercially available amino acid polystyrene resins and t-Boc protected amino acids (Peninsula Laboratories Inc., Belmont, CA) by methods that have been described previously (33, 34). All couplings were >99% complete, as determined by the reaction of the resin with ninhydrin (35). The purity of the crude product was determined by high pressure liquid chromatography on a C-18 reverse-phase column (Merck and Co., Rahway, NJ), and by amino acid analysis. The peptides used in these experiments were not purified further, since they all contained >90% of the desired product. The peptides were conjugated to bovine serum albumin (BSA) using succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Pierce Chemical Co., Rockford, IL) as described (34). They were conjugated to thyroglobulin (Sigma Chemical Co., St. Louis, MO) for immunization using a slightly different crosslinker, *m*-maleimidobenzoyl *N*-hydroxysuccinimide ester (Pierce Chemical Co.) (36). By using both a different carrier protein and crosslinker when using the peptide as an immunogen or as an antigen, we sought to minimize interference by antibodies generated against either the

carrier protein or the crosslinker. The degree of conjugation was quantitated by comparing the amino acid composition of the carrier protein before and after reaction with the peptide. The conjugates used in this study all contained between 15 and 25 peptides per molecule of BSA, and 25 to 35 peptides per 100 kilodaltons (kD) of thyroglobulin.

Immunization and Fusion. C3H/HeN mice were immunized intraperitoneally with 50 μg peptide-thyroglobulin conjugate in complete Freund's adjuvant, and boosted weekly in incomplete Freund's adjuvant for 4 wk. 3 d before fusion, an intravenous injection was given. Nucleated spleen cells were fused with P3X63Ag8.653 myeloma cells, as described (37).

Antibody Assay. An enzyme-linked immunosorbent assay (ELISA) was performed to determine the reactivity of polyclonal sera and mAb against the peptides and the 38C13 IgM protein. Briefly, individual wells were coated by incubation with a solution of 10 μg/ml of either the peptide-BSA conjugates, whole μ chains, or intact IgM proteins. For screening of hybridoma supernatants, this panel of different targets was extended to include seven unrelated mouse IgM proteins. Before use, the coated microtiter plates were washed extensively with phosphate-buffered saline (PBS) containing 0.05% Triton X 100 and were quenched with PBS containing 5% nonfat dry milk for at least 1 h at room temperature.

The 38C13 IgM protein was isolated from ascites fluid of a C3H/HeN × BALB/c F_1 mouse inoculated with the high Id-secreting cell line. This IgM protein was purified by ammonium sulfate precipitation (45% saturation) followed by size fractionation over a column of Sephadex G-100. Four unrelated IgM mAb, 4C5, G10, G4, and G3, were similarly isolated from ascitic fluid induced by these hybridomas, respectively. MOPC 104E and TEPC 183 myeloma proteins were purchased from Bionetics Laboratory Products (Charleston, SC), and used without further purification. Isolated H chains from 38C13 IgM and 4C5 IgM were prepared as follows: IgM proteins were dialyzed against 0.5 M Tris, 0.15 M NaCl, pH 8. Mercaptoethanol was added to a final concentration of 0.4 M. The mixture was stirred in the dark at 37°C for 45 min, then alkylated by adding a slight molar excess of 2-iodoacetamide. After 30 min at room temperature, solid urea was added to a final concentration of 6 M. H and L chains were separated by chromatography using a column of ACA 44 Ultragel (LKB Instruments, Gaithersburg, MD).

Hybridoma supernatants or sera from mice immunized with the thyroglobulin conjugates of the peptides or the 38C13 IgM were serially diluted with PBS and 5% nonfat dry milk, and added to the wells of a microtiter plate precoated with target antigens. After incubation for 8 h at 4°C, the plates were washed, alkaline phosphatase–labeled goat antibodies specific for mouse γ chain (Southern Biotechnology Associates, Birmingham, AL) were added, and the plates were incubated for 2 h at room temperature. Enzyme substrate (*p*-nitrophenyl phosphate, disodium salt) was added to each well, and the absorption at 405 nm (A_{405}) was measured by an ELISA reader.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Transfer. The various monoclonal IgM proteins were separated by SDS-PAGE on a 10% gel, under reducing conditions. The proteins were electrophoretically transferred to nitrocellulose (Millipore, Bedford, MA) in a Trans-Blot Cell (Bio-Rad Laboratories, Richmond, CA) and treated with PBS with 5% nonfat dry milk at 37°C for several hours to saturate the matrix. The paper was incubated with antipeptide hybridoma culture supernatant or control supernatant for 2 h at 4°C, washed and treated with 5×10^5 cpm/ml of ^{125}I-labeled sheep anti–mouse Ig (Amersham Corp., Arlington Heights, IL). The nitrocellulose filters were washed and exposed to x-ray film (XAR-2, Eastman Kodak, Rochester, NY). The electrophoretic transfer of the μ Ig chains was confirmed by treating the nitrocellulose with a 1:100 dilution of rabbit anti–mouse IgM (Bionetics Laboratory Products) in PBS with 5% skim milk, followed by exposure to 5×10^5 cpm/ml of ^{125}I-labeled protein A.

Tumor Challenge. The 38C13 B lymphocyte cell line to be used in vivo was maintained in culture in RPMI 1640 medium containing 10% fetal calf serum, glutamine, antibiotics, and 5×10^{-5} M mercaptoethanol. Groups of 10 mice each were immunized with either peptide-thyroglobulin or 38C13 IgM–thyroglobulin conjugate. The latter reagent was prepared by the addition of 5 mg of solid thyroglobulin to 5 mg of 38C13 IgM in 1 ml

of 0.15 M NaCl, pH 4.5, followed by an 8-h incubation at room temperature in the presence of 5 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Pierce Chemical Co.).

Animals were injected intraperitoneally five times, with 50 μg of thyroglobulin conjugate per injection, essentially as described for the immunization of animals before hybridoma production. The mice were then challenged with 10^2 38C13 cells; a dose lethal to 100% of animals by day 30. The presence of tumor and the day of death were recorded.

Results

Nucleic Acid Sequencing of V_H from the 38C13 B Cell Tumor. mRNA was isolated from the cytoplasmic RNA of the high Id-secreting cell line using an oligo-dT column. Direct sequencing of mRNA was performed using a synthetic primer corresponding to the amino terminal portion of the first C domain of the μ chain. This provided the nucleic acid sequence coding for amino acid residues 60 to 124 (Fig. 1). The remainder of the V_H sequence was derived by M13 sequencing of a 550 bp cDNA fragment containing the 5′ untranslated, leader, and V_H coding regions. The resultant nucleotide sequence determined by these two methods, as well as the corresponding amino acid sequence is shown in Fig. 1. This μ chain has a V region of 126 residues, including a diversity (D) region of 9 amino acids and a J_2 (joining) region of 15 amino acids.

Synthesis of Peptides Corresponding to the Hypervariable Regions. Four peptides corresponding to the second and third hypervariable regions of the 38C13 μ chain were synthesized. They were chosen based on consideration of secondary structure predictions. The positions of the hypervariable regions are shown in Fig. 1, whereas the predicted secondary structure of this polypeptide chain is shown in Fig. 2. Two peptides were synthesized to span the second hypervariable

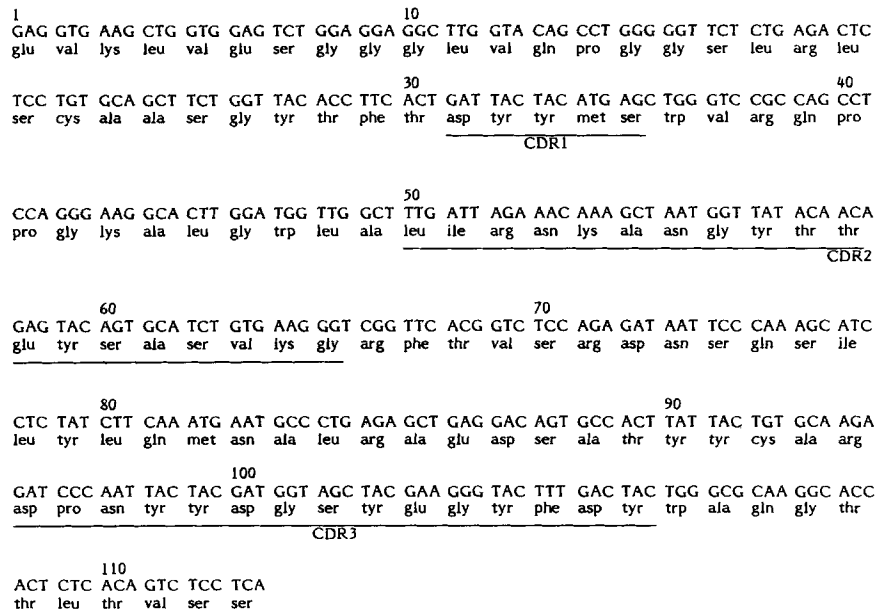


FIGURE 1. Nucleotide and translated amino acid sequence of the 38C13 μ chain, CDR and amino acid number are according to Kabat (38).

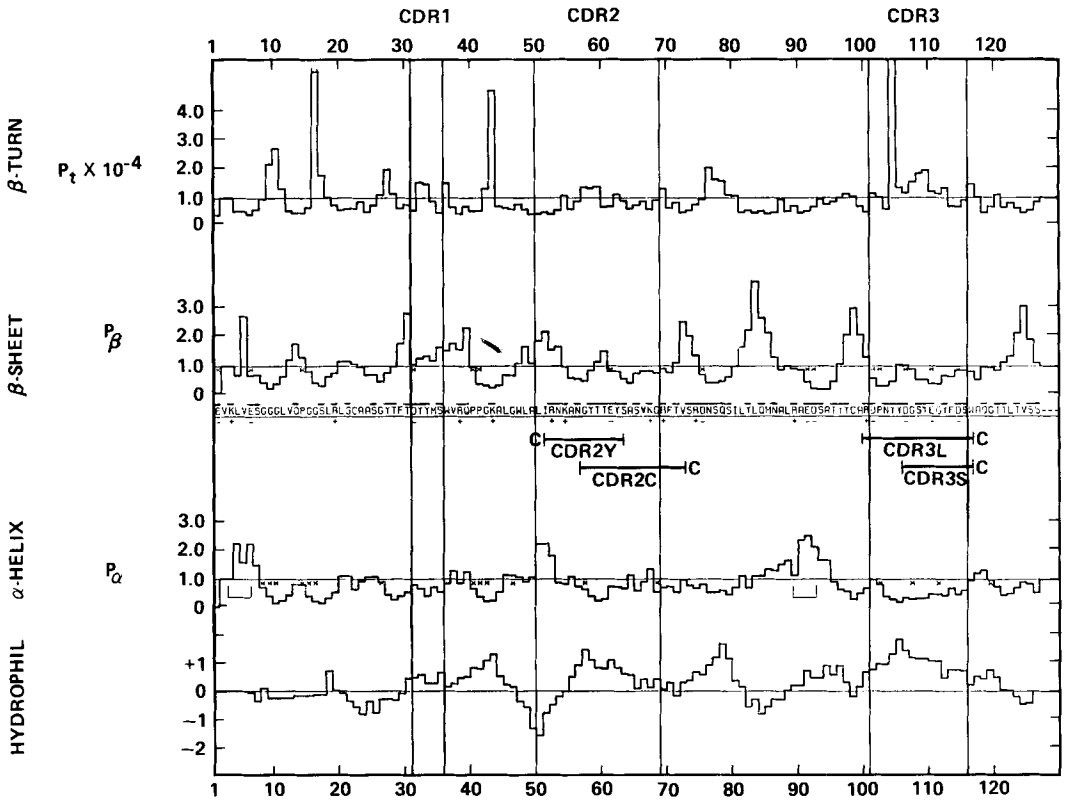


FIGURE 2. Location of the four oligopeptides within the sequence of the 38C13 μ chain, their predicted secondary structure (39), and their relative hydrophilicity. The relative hydrophilicity of the protein was calculated by a moving average over six residues using the values assigned by Kyte and Doolittle (40). Positive values correspond to the hydrophilic regions. Lines are written over polar amino acids; either positive or negative signs indicate charged amino acids.

region, CDR2Y (NH_2-CIRNKANGYTTEY-COOH) and CDR2C (NH_2-GYTTEYSASVHGRFTC-COOH) (using one-letter amino acid code). Each contains a predicted reverse turn distal to its attachment to the carrier protein. Similarly, CDR3S (NH_2-YDGSYEGYDYC-COOH) and CDR3L (NH_2-ARDPNYYDGSYEGYFDYC-COOH) correspond to the residues within the third hypervariable region. The longer sequence includes a highly predicted reverse turn in its amino terminal region that is absent in the shorter analogue.

Polyclonal Anti-CDR3 Sera Bind to Peptide and Isolated H Chains but Fail to Bind the Native IgM. Mice were immunized by five intraperitoneal injections of either the CDR3L- or CDR3S-thyroglobulin conjugate. Pooled immune sera were analyzed for their ability to bind the immunizing peptide and to crossreact with either 38C13 μ chain or intact 38C13 IgM. These sera were compared with the sera from animals immunized with intact 38C13 IgM conjugated to thyroglobulin, and with an irrelevant peptide conjugate as a control.

As shown in Fig. 3, CDR3L-thyroglobulin elicited a strong antipeptide response in the animals (Fig. 3*A*), as well as a response against the isolated H chains

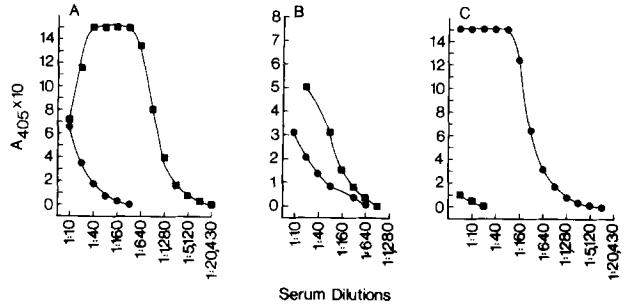


FIGURE 3. Binding of anti-CDR3L (solid squares) and anti-38C-13 IgM (solid circles) pooled antisera to wells precoated with CDR3L-BSA conjugate (*A*), 38C-13 μ chain (*B*) and 38C-13 IgM (*C*).

of 38C13 IgM (Fig. 3*B*). However, this serum failed to bind intact IgM (Fig. 3*C*). As expected, the sera from animals immunized with the intact 38C13 IgM showed a strong reaction against the intact protein (Fig. 3*C*). However, this serum reacted with the isolated H chain to approximately the same degree as the antipeptide sera (Fig. 3*B*). Interestingly, the antiserum raised against 38C13 IgM did react slightly with the peptide (Fig. 3*A*). Neither anti-CDR3L nor anti-38C13 immune serum reacted with the isolated H chain of an irrelevant IgM (4C5), demonstrating their Id specificity (data not shown). Sera from mice immunized with the control peptide-thyroglobulin conjugate showed no reactivity with any of these antigens.

Similarly, the mice immunized with the CDR3S-thyroglobulin conjugate generated a significant antipeptide response. This immune serum also reacted, although to a lesser extent than the anti-CDR3L immune serum, with the isolated μ chain. However, there was no reactivity with the 38C13 IgM protein detectable above background (data not shown).

mAb Elicited by CDR3L Bind to the μ Chain, and to the Native IgM. The antipeptide immune response was dissected further by the production of hybridomas. The spleens of mice hyperimmunized with either CDR3L-, CDR3S-, CDR2Y-, or CDR2C-thyroglobulin conjugates were removed, and the cell suspension was fused to P3X63Ag8.653 myeloma cells. Hybrids were selected for growth in HAT medium (hypoxanthine, aminopterin, thymidine). The supernatants of growing hybrids were tested for antipeptide and antiprotein activity after at least two changes of the original growth medium.

Hybridomas producing antibodies against the immunizing peptide were found in each fusion with an overall frequency of 9–15% of the obtained hybrids (30–80% of the seeded wells). However, antibodies crossreacting with intact 38C13 IgM were obtained only when CDR3L-thyroglobulin was used as immunogen. In this case, 65% of the seeded wells were positive for hybrid growth. 53 hybridoma supernatants (14% of growing hybrids) were reactive with the CDR3L peptide. 23 of these supernatants reacted with the isolated H chain of 38C13 IgM. The supernatants of 10 of these hybridomas also reacted with the intact 38C13 IgM. These supernatants were unreactive with plates coated with the irrelevant 4C5 μ chain or IgM.

The frequencies given above are likely to be overestimates, because the

screening assays were performed on hybrids that had not been subcloned. For instance, ≥25% of these supernatants could be shown to contain more than one IgG subclass. On the other hand, because of the necessity to use antibodies specific for mouse γ chains as the detecting reagent, we have excluded antibodies of the IgM isotype from the analysis.

10 of the hybridomas that were stable in producing antibody against the CDR3L peptide were subcloned and subjected to further analysis (Table I). 7 of these 10 mAb reacted with the isolated 38C13 H chain. Of these, only three also reacted with the intact 38C13 IgM. All of these antibodies showed Id specificity because they did not react with any of the other six IgM proteins. Variable background is seen in this assay due to slight contamination of the IgM preparations with IgG.

The crossreactivity of these hybridomas with the isolated μ chains, and the Id specificity were further examined using immunoblots. The same IgM proteins that had been used for the ELISA assays were subjected to SDS-PAGE under reducing conditions, electrophoretically transferred to nitrocellulose, probed with each of the 10 antipeptide antibodies, and detectd with an ^{125}I-labeled anti-mouse γ chain reagent (Fig. 4). The same mAb that had reacted with the isolated μ chain in ELISA assay also reacted in this assay. Once again, the reaction showed Id specificity for 38C13. Variable IgG contamination of the different irrelevant IgM preparations is once again evident, accounting for the spots in the γ chain region of the gel.

To ascertain that the crossreactivity of the mAb was indeed peptide induced, we performed the inhibition tests shown in Fig. 5. The reactivity of the antipeptide antibodies with 38C13 IgM, 38C13 μ chains, and CDR3L-BSA conjugate could be inhibited by preincubation of the antibodies with free CDR3L-BSA. Preincubation of these antibodies with the irrelevant peptides such as CDR2Y (Fig. 5) did not affect binding to any of these targets. These inhibition curves

TABLE I

Induction of Anti-Id Using CDR3L Peptide

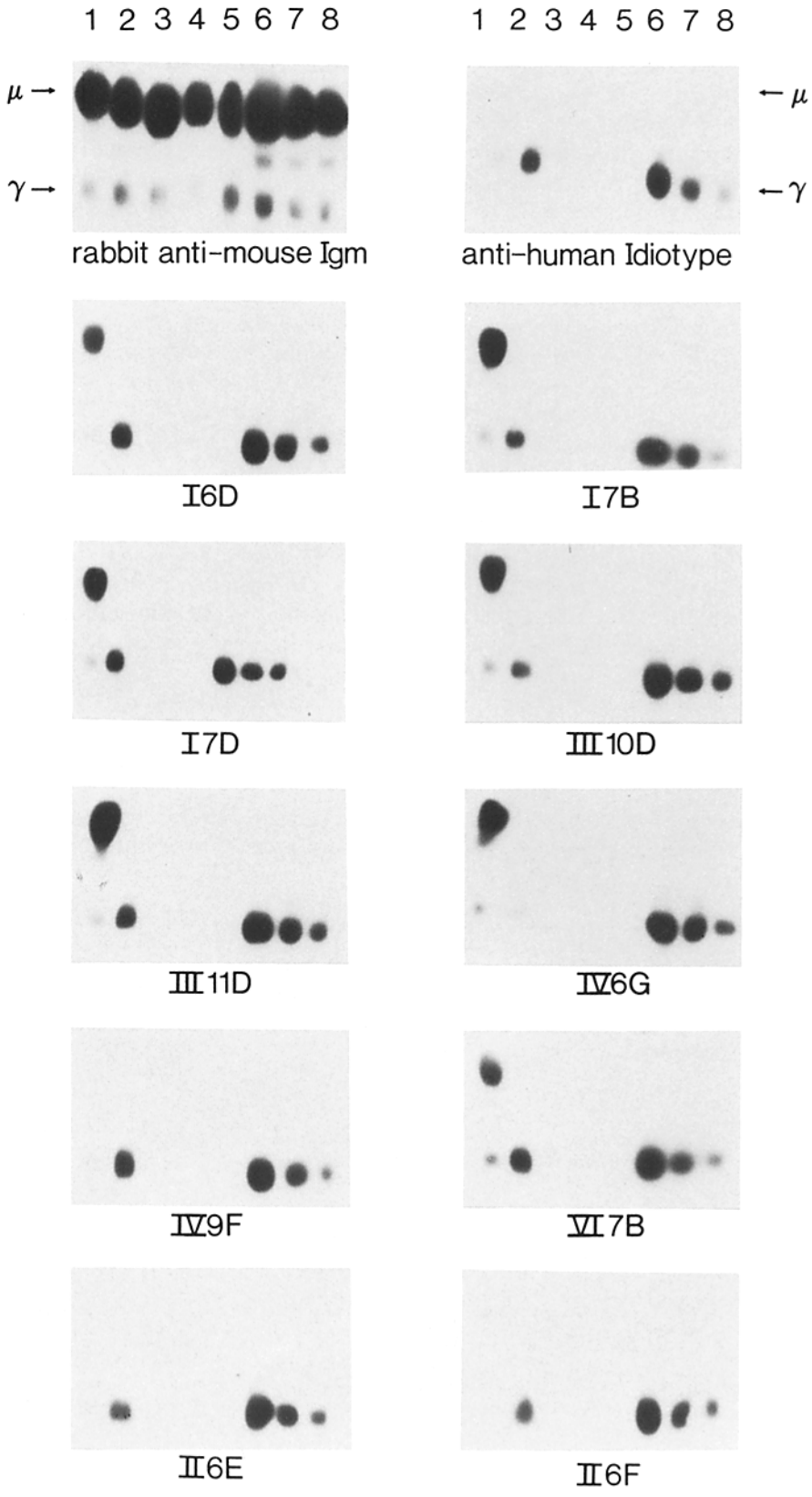
	A_{405} (× 10^3) using various antibodies*									
	CDR3L BSA	38C13 H chain	38C13 IgM_κ	4C5 H chain	4C5 IgM_κ	MOPC 104E IgM_λ	TEPC 183 IgM_κ	G4 IgM_λ	G3 IgM_λ	G10 IgM_λ
Anti-human Id‡	0	3	0	0	17	0	14	15	20	50
16D	1,410	290	53	6	28	0	25	30	46	120
17B	1,500	943	316	6	32	0	24	85	63	120
17D	1,500	1,012	393	7	32	0	28	81	54	120
III 10 D	1,500	630	20	3	29	0	23	91	59	130
III 11 D	1,500	1,092	161	3	34	0	14	87	60	131
IV 6 G	1,500	1,016	521	1	33	0	13	43	62	126
IV 9 F	742	42	0	0	37	0	14	50	68	128
VI 7 B	1,100	163	0	0	39	0	11	40	95	130
II 6 E	1,500	14	0	0	37	0	15	24	88	128
II 6 F	1,500	36	5	7	16	0	17	30	73	120
Goat anti-mouse IgM§	—	343	853	175	1,500	730	1,500	1,500	640	534

Antipeptide mAb were assayed as undiluted culture supernatants. Bound antibodies were detected with alkaline phosphatase–conjugated goat anti-mouse IgG and substrate. The numbers represent A_{405} measured after 60 min incubation at room temperature. All supernatants were tested in triplicate.

* Antigens were coated at 10 μg/ml.

‡ A monoclonal anti-human Id was used as negative control.

§ Alkaline phosphatase–conjugated goat anti-mouse IgM was used to detect the IgM and isolated H chains.


1 2 3 4 5 6 7 8
μ →
γ →
rabbit anti-mouse Igm
1 2 3 4 5 6 7 8
← μ
← γ
anti-human Idiotype
I6D
I7B
I7D
III10D
III11D
IV6G
IV9F
VI7B
II6E
II6F

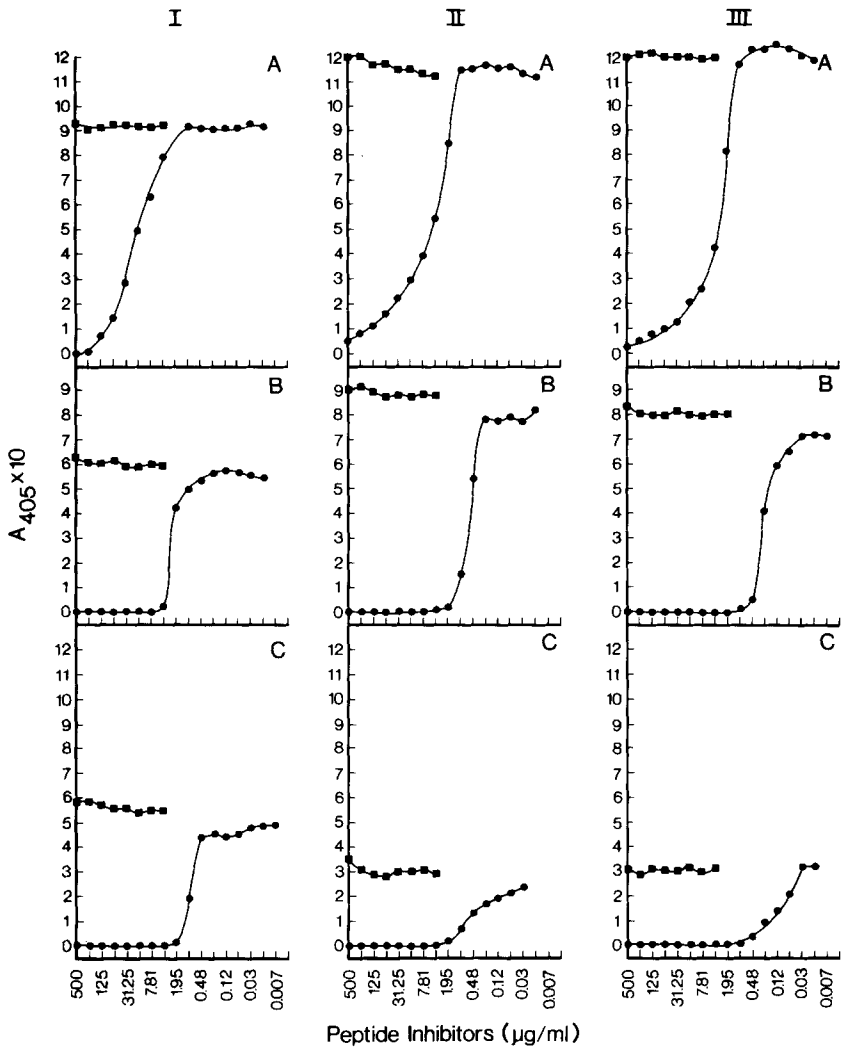


FIGURE 5. Inhibition by BSA-conjugated peptides of antipeptide antibody binding to antigen-coated plates. Decreasing amounts of CDR3L-BSA (solid circles) or CDR2Y-BSA conjugate (solid squares) were mixed with a fixed amount of mAb (*I*, IV6G; *II*, 17D; and *III*, 17B). After 1 h incubation at room temperature, 50 μl of this reaction mixture was transferred to microtiter plates coated with CDR3L-BSA (*A*), isolated 38C-13 μ chain (*B*), or 38C-13 IgM (*C*). After overnight incubation at 4°C, enzyme-labeled goat anti-mouse IgG was added to each well. Enzyme substrate was added, and A_{405} measured 1 h later.

FIGURE 4. Nitrocellulose blots of reducing SDS-PAGE probed with antipeptide mAb. The region of the gel containing the H chains is shown. The upper two panels show the control blots indicating the position of the eight different μ chains probed with rabbit anti-mouse IgM (upper left; positive control) and the reactivity of ^{125}I-labeled sheep anti-mouse IgG after incubation with an irrelevant mouse mAb (upper right; negative control). Lanes 1–8 represent different murine μ chains: 1, 38C-13; 2, 4C5; 3, RBC; 4, MOPC104E, 5; TEPC183; 6, G10; 7, G3; 8, G4. The antipeptide mAb used to probe the nitrocellulose blots is indicated under each panel.

demonstrated that minimal amounts of CDR3L peptide could inhibit the binding of the antibodies to the peptide-conjugate, isolated H chains, or intact IgM. However, there was a significant difference in the inhibitor concentration required; ~10 times the amount of inhibitor peptide was necessary to inhibit 50% of antibody binding to the peptide conjugate as compared with the intact IgM. The varying inhibitor requirement could be due either to variation in the concentration of the epitope on the plate in the three cases, or to a difference in affinity of the antibody for the different targets. The antipeptide antibodies were compared to anti-Id mAb raised against the intact Ig, 4C8, and 3H5 (Fig. 6). The idiotope(s) recognized by these antiprotein antibodies require the association of both H and L chains. Identical amounts of each of the antibodies were added to the wells of a microtiter plate precoated with 38C13 IgM, assuring that the anti-Id and the idiotope concentration was identical for the antibodies being compared. Fig. 6 shows that the antiprotein antibodies 4C8 and 3H5, raised against the intact protein, showed at least two orders of magnitude higher avidity (the concentration required to occupy half the antigenic sites) than the cross-reacting antipeptide antibodies.

Immunization with IgM Induces Tumor Immunity but Immunization with CDR Peptide Does Not. Normal C3H/HeN mice injected weekly with 38C13 IgM–thyroglobulin conjugate produced demonstrable anti-Id within 3 wk. When these mice were challenged with a lethal dose of tumor cells, a high fraction were resistant to tumor growth for observation periods up to 100 d after tumor inoculum. In a series of five experiments, the degree of tumor protection ranged from 60 to 100% of the immunized mice. As long as the 38C13 IgM protein was coupled to an immunogenic carrier, adjuvant was unnecessary, and a single injection of the conjugate was sufficient to produce long-lasting immunity. A typical experiment is shown in Fig. 7 (Maloney, Kaminski, and Levy, in preparation).

In contrast, animals immunized with the CDR3L peptide conjugate developed tumors and died with a time course identical to the control animals (Fig. 7). The progressing tumors in the animals were all Id^+, since all these animals had developed increasing amounts of the 38C13 IgM protein in their sera before death.

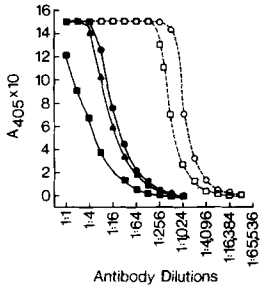


FIGURE 6. Comparison of conventional anti-Id antibodies with antipeptide antibodies. Purified anti-Id antibodies, 4C8 (open circles) and 3H5 (open squares), and antipeptide antibodies 17B (closed circles), 17D (closed triangles), and IV6G (closed squares) were added to wells precoated with 38C-13 IgM. Starting at 10 μg/ml, the antibody solution was diluted over 16 wells. Bound antibody was detected by addition of goat anti–mouse IgG. The color development (A_{405}) was measured 30 min after the addition of the enzyme substrate.

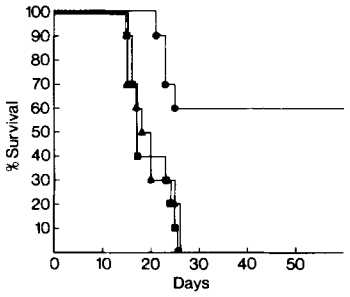


FIGURE 7. Tumor immunity induced by immunization with idiotype. 10 C3H/HeN mice were immunized five times at weekly intervals with thyroglobulin conjugates of 38C-13 IgM (closed circles), control peptide (closed triangles), and CDR3L peptide (closed squares). 7 d after the last immunization, mice were inoculated with 10^2 38C13 tumor cells.

Discussion

Several reports (19–22) have appeared recently in which synthetic peptides were used to induce antisera that react with Id determinants of Ig. Peptides of 13 to 16 amino acids in length, spanning CDR3 of the H chains (19, 20), CDR2 of the L chain (22), and J_H (21) have all led to antibody responses that could recognize not only the appropriate isolated protein chains, but the specific assembled native Ig molecules. Typically, these antisera have been characterized by sensitive ELISA assays or immunoblotting procedures.

We have extended the basic observation that peptides can induce an anti-Id immune response in a system in which the biologic relevance of these responses can be tested. We have used a B cell tumor in which syngeneic anti-Id immune responses can be induced against the intact Id protein produced by the tumor. These immune responses include anti-Id antibodies, as well as immunity to tumor challenge, which is presumably based on a T cell mechanism.

The amino acid sequence of the Ig H chain produced by the B cell tumor was deduced from nucleic acid sequencing. This was done by a combination of direct mRNA sequencing and the sequencing of a cloned V_H cDNA fragment. Both of these methods provided the sequence of the third hypervariable region. The RNA sequencing method had the advantage of speed and direct applicability to human tumors, since it was based on a primer homologous to a sequence in the first C μ exon, which is identical in mouse and man. However, this method is critically dependent on the quality of the RNA preparation. Also, a single sequencing reaction did not allow determination of the sequence beyond the second hypervariable region (150–200 nucleotides upstream from the primer). This method could be enhanced by the use of a second, upstream primer based on the sequence determined in the first reaction. Instead, we chose in this case to complete the sequence using a cloned cDNA fragment.

The homology of the 38C13 variable region with a murine V_H region of subgroup III allowed for the identification of the three hypervariable regions. Linear peptides of CDR2 and CDR3 were chosen based on the predicted secondary structure of these areas. Only if the peptide adopts a similar conformation to what it maintains in the intact protein will the peptide successfully stimulate the production of crossreactive antisera. Of the three common second-

ary structures in proteins, α-helices, strands of β-pleated sheets, and reverse turns, we believe reverse turns have the greatest probability to exist in a short linear peptide, because of the energy requirements in their formation. Turns are thought to be formed in Ig and other globular proteins by the association of two regions of hydrophobic amino acids located on either side of the turn. Consequently, the majority of residues involved in turn formation can be included in a linear peptide. In contrast, sheet and helix formation and subsequent stabilization rely far more on through-space interactions. Such associations as the packing of a hydrophobic face of a helix or sheet on the hydrophobic core of a globular protein or the interstrand hydrogen bonds in a β-pleated sheet will not be present in a short linear peptide. We do not assume that the synthesized peptides will exist exclusively as reverse turns, but rather that they will have the lowest energy requirements to assume that conformation.

We synthesized two peptides corresponding to CDR2 and one to CDR3 (CDR2Y and CDR2C), (CDR3L). Each of these peptides contains a reverse turn distal to its site of attachment to the carrier protein. To test our ideas on the importance of turns in a peptide immunogen, we also synthesized CDR3S, which is identical to CDR3L except that it lacks the predicted reverse turn. All of the peptides elicited a roughly equal antipeptide response. Antibodies against the two CDR2 peptides failed to recognize the isolated H chain. Antisera against both CDR3S and CDR3L reacted with the isolated μ chain, but those elicited by CDR3L reacted to a greater degree. However, only the antibodies against CDR3L reacted with the intact assembled IgM protein, thereby providing support for the importance of the predicted reverse turn in this region. The antibodies that crossreacted with the intact IgM were present in low concentration in polyclonal serum. Such antibodies were detected only by isolating individual hybridoma clones. Their relative reactivities against the peptide, the isolated H chain, and the intact IgM were compared. Not surprisingly, their ability to bind the intact Ig was much less than their ability to bind the peptide against which they were induced. All of the interactions were shown to be specific, because they could be inhibited by the immunizing peptide. Comparison of the antipeptide mAb with those elicited by the intact Ig revealed that the two classes of antibodies dramatically differed in their ability to bind the intact IgM. The anti-Id generated against the intact IgM bound the protein with at least two orders of magnitude greater avidity than the antipeptide antibodies.

We believe that both the low number of crossreactive antibodies and their low avidity relative to antiprotein antibodies is due to the lack of a well-defined conformation in the peptide. Linear peptides are capable of stimulating a large number of B cell clones to produce antibody, only a small fraction of which will crossreact with the intact protein. The percentage of the antibodies that will crossreact will depend on the characteristics of the corresponding region in the protein. The highest percentage of crossreactive sera will result when antibodies are directed against a region lacking a well-defined conformation, i.e., a region that resembles a peptide, best exemplified by the amino or carboxyl termini of the protein.

A peptide can adopt the identical conformation that it maintains in the intact protein either by a stochastic or an inductive mechanism. The stochastic model

predicts, (*a*) the percentage of antipeptide antibodies that crossreact with the intact protein depends on the statistical likelihood that the peptide exists in that particular conformation, and (*b*) a peptide is capable of occasionally stimulating the identical B cell clones that are elicited by the linear epitopes of the intact protein. If a peptide triggers a humoral response by this mechanism, some of the antipeptide mAb produced should crossreact with the intact protein with equal avidity as those elicited by the linear immunogenic regions of the protein.

In contrast, if the conformation of the peptide is induced by the surface Ig, then (*a*) the fraction of the antipeptide antibodies that crossreact with the intact protein is dependent on the free energy necessary to induce the peptide to adopt that particular conformation, and (*b*) the energy necessary to induce the peptide will be reflected in the surface Ig having a lower affinity constant for the peptide relative to the same peptide with a defined conformation. If the energy necessary for the induction becomes too great, the clone will not be stimulated, and antibody recognizing the peptide in that conformation will be absent from the humoral response. This mechanism predicts that linear peptides lacking a single, well-defined conformation will always elicit lower-avidity crossreactive antibodies than those engendered by the intact protein.

Although we have not screened enough antipeptide clones to allow us to distinguish between the two mechanisms, the results of the experiments in this report indicate that high-affinity, crossreactive anti-Id clones, if not impossible to generate, will be very difficult to obtain by using peptides lacking a defined secondary structure as immunogens. Moreover, the low-affinity anti-Id antibodies such as those elicited by the peptides are unlikely to play an important role in immune regulation. CDR3L was incapable of inducing tumor immunity in syngeneic animals, although native 38C13 IgM was very effective in doing so. This biologic test is, admittedly, extremely stringent. Peptides with fixed configurations similar to epitopes in the native hypervariable regions might be able to induce higher affinity anti-Id antibodies with greater potential physiological significance.

Summary

The nucleic acid sequence of the heavy chain variable region (V_H) expressed by 38C13, a B cell tumor of C3H origin, was determined by a combination of direct (messenger RNA) mRNA sequencing by primer extension and complementary DNA (cDNA) isolation and sequencing in M13. The V_H amino acid sequence was deduced, and hypervariable regions were identified. From an analysis of predicted secondary structure, regions of predicted antigenicity were chosen, and a series of synthetic peptides corresponding to CDR2 and CDR3 (complementarity-determining region) were produced. These peptides were coupled to protein carriers and used to immunize syngeneic C3H mice. All peptides gave rise to a vigorous antibody response. However, only the CDR3 peptides induced antibodies that crossreacted with the isolated H chain protein. Only one CDR3 peptide induced antibody-producing clones, isolated as hybridomas, that reacted with the intact IgM protein. However, the appearance of these clones was a low-frequency event. All antibodies reacting with the H chain or the intact IgM protein were idiotypically specific for 38C13. These monoclonal

antiidiotype (anti-Id) antibodies, raised against CDR3 peptides, gave strong reactions in enzyme-linked immunosorbent assays and immunoblots, but they were of low affinity compared to syngeneic anti-Id raised against the intact IgM protein. Moreover, while the intact IgM was capable of inducing tumor immunity, the CDR peptides were not able to do so.

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