How Well Can an Idiotope Peptide Mimic Replace Its Parent Idiotype in a Synthetic Peptide Vaccine?

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Purpose. To determine whether a vaccine consisting of an idiotope peptide mimic of the third complementarity-determining region of the immunoglobulin heavy chain (CDR-H3) is an effective substitute for its parent idiotype. Such peptide vaccines could ultimately be used for targeting pathological B lymphocytes.

Methods. Hen egg lysozyme (HEL) conjugates of the Fab' fragment of monoclonal anti-fluorescein antibody 9-40 (Fab'-HEL) or a peptide mimic of the 9-40 CDR-H3 (referred to as the "B epitope" or "Bep," the conjugate is referred to as "Bep-HEL") were injected into separate cohorts of B10.A mice. Two additional control cohorts were injected with either the Bep peptide alone or a noncovalent mixture of Bep and HEL. Sera were assayed for both anti-idiotope and anti-idiotype activity by enzyme-linked immunosorbant assay (ELISA). Primary, secondary, and tertiary immune responses were examined.

Results. Both the Bep-HEL idiotope and the Fab-HEL idiotype immunogens elicited homologous, allogenic immune responses. No cross-reactivity was observed between anti-idiotope and anti-idiotype responses after primary immunization. With secondary immunization, 50% of mice immunized with the Bep-HEL conjugate exhibited a cross-reactive anti-idiotype response. Conversely, 100% of mice immunized with the Fab'-HEL conjugate exhibited a marginal, but statistically significant cross-reactive anti-idiotope response. Upon tertiary immunization, 100% of mice immunized with Bep-HEL exhibited a cross-reactive anti-idiotype response, and 55.6% of mice immunized with the Fab'-HEL conjugate exhibited a cross-reactive anti-idiotope response.

Conclusions. Covalent coupling of a xenogenic carrier protein to an idiotype immunogen or its peptide mimic significantly enhances the intensity of homologous, allogenic anti-idiotype or anti-idiotope immune responses. Multiple immunizations are necessary to induce cross-reactivity between the peptide mimic and its parent idiotype.

KEY WORDS: idiotype peptide; peptide vaccine; cross-reactivity; xenogenic carrier protein.

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ABBREVIATIONS: Bep, peptide mimic of 9-40 CDR-H3 with amino acid sequence of GGGCTSYGYHGAYC, forms an intramolecular disulfide bond; CDR-H3, third complementarity-determining region of the immunoglobulin heavy chain; DMSO, dimethylsulfoxide; DSG, disuccinimidylglutarate; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; ELISA, enzyme-linked immunosorbant assay; EtOEt, diethylether; Fab, antigen-binding fragment prepared by papain cleavage; $F(ab')_2$, divalent antigen-binding fragment prepared by pepsin cleavage; Fab', monovalent antigen-binding fragment prepared by reduction of $F(ab')_2$, contains free thiol groups at the C-terminus; HEL, hen egg lysozyme; PBS, phosphate-buffered saline (100 mM NaCl, 50 mM $NaPO_4$, pH 7.4, 0.2% (w/v) NaN_3); SMCC, succinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate; THF, tetrahydrofuran.

INTRODUCTION

Cancer vaccines rely on antigens expressed uniquely or at least predominantly on cancer cells to achieve specificity. Ideally, the cancer antigen should be unique to the cancer cell, which is the case with most lymphomas and leukemias. Typically only one or a few clones are involved in the pathology (1,2), and the malignant lymphocytes express either B cell receptors (BCRs) or T cell receptors (TCRs) on their surfaces. Because of the gene rearrangement involved in the ontogeny of these cell lines, the particular BCR or TCR is unique to the clone that expresses it and thus serves as an operational definition of that clone. Therefore, the idiotype—the unique portion of a BCR or a TCR—presents an ideal target for immunotherapy in lymphocyte cancers.

This idea dates back 25 years to the early work of Stevenson *et al.* (3), Haughten *et al.* (4), and Levy *et al.* (5), which showed that the BCR idiotypes of B cell lymphomas or lymphocytic leukemias were effective tumor-specific antigens. Their pioneering work led to fundamental and preclinical immunotherapy investigations in animal models, followed by experimental immunotherapy in humans (for review, see Refs. 1, 6–8). However, such immunotherapy must be personalized for each patient, and the murine antibodies can elicit a human anti-mouse antibody response (9), although the toxicity was reported to be low (1). Originally, the idea was to use such monoclonal antibodies as molecular homing devices for another therapeutic agent such as a conjugated toxin or radiolabel (1), but if the BCR target is cross-linked by antigen or anti-idiotype antibodies without a second, activating signal, apoptosis ensues and so the antibodies alone proved surprisingly effective. Furthermore, it can be difficult to obtain sufficient quantities of purified patient-specific antigen, so less ideal molecular targets have also been pursued, for example, CD20 (1), but not surprisingly these also target some healthy cells.

Two different approaches to anti-idiotype immunotherapy have been investigated: (i) patient-specific anti-idiotype antibodies generated in mice and used either alone or in combination with other therapy for the treatment of active disease, or (ii) a therapeutic vaccine (either protein- or DNA-based) used to elicit an immune response in the patient against his/her own malignant lymphocytes. In his review of such immunotherapies, Prof. Ronald Levy (Stanford University) suggested that the former approach is more suitable for patients in relapse, whereas the latter approach is more suitable for patients in remission (1).

Yet another possibility is to make a peptide mimic of one or more of the idiotopes that comprises the idiotype, and then use the peptide as an immunogen to generate anti-idiotope antibodies that will cross-react with the parent idiotype. This approach has been undertaken in animal models using linear peptides corresponding to either the first (10,11), second (10–14), or third (10,11,15–17) CDR of the heavy chain, as well as the second CDR of the light chain (18). The third hypervariable region of the heavy chain—the CDR-H3 loop—has been shown to be superior to the other loops with respect to making cross-reactive antisera (11). In general, these approaches found that anti-peptide antisera produced in another species (xenogenic) were cross-reactive with the parent idiotype antibody. However, only a few of these studies (13,17) actually

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compared the cross-reactivity between the idiotype and the idiotope mimic directly, and those comparisons were somewhat flawed because different T cell epitopes were used in the idiotype and idiotope immunogens. The focus of the current study is to directly compare anti-idiotype and anti-idiotope responses elicted by idiotype and idiotope immunogens both containing the same T cell epitope. Furthermore, immune responses will be studied in the same species (allogenic) from which the parent idiotype immunogen was derived.

The particular antibody chosen as the idiotype is 9-40, a murine (BALB/c) anti-fluorescein IgG_1 κ antibody of medium affinity (19) belonging to a family of clonotypically related anti-fluorescein antibodies whose idiotypes have been well characterized (20–24). Because we intended to study a peptide mimic of the idiotype as an allogenic vaccine, it was necessary to derivatize both the peptide mimic and the parent idiotype in order to make them more foreign to the mouse immune system. Consequently we chose chicken hen egg lysozyme (HEL) as a carrier protein because it has been well studied chemically and immunologically (25), and is small (14.3 kDa), inexpensive, and readily soluble. Because it is easier to ascertain whether coupling between the carrier protein and the idiotype has been successful when the relative change in molecular weights is large, we used the Fab' fragment of 9-40 as the idiotype rather than the intact antibody. Additionally, the Fab' fragment's free thiol group(s) facilitated conjugation to HEL. We also used the CDR-H3 as the idiotope peptide mimic (hereafter designated as "B epitope") likewise coupled to HEL.

Another important consideration is whether a linear or cyclic peptide CDR mimic should be used. Though some linear peptide CDR mimics do elicit effective idiotypic cross-reactivity (13,15–18), this was not observed for a linear peptide CDR-H3 mimic of 4-4-20, an anti-fluorescein MAb idiotypically related to 9-40 (Prof. David M. Kranz, University of Illinois at Urbana-Champaign, personal communication). Heretofore, there have been few if any reports of cyclic peptide CDR mimics, although cyclic peptides have successfully been used in synthetic peptide vaccines for HIV (26,27) and malaria (28,29). Linear and cyclic versions of the same epitope were compared in the malaria studies with contrasting results. In one study (28), the cyclic peptide immunogen was clearly superior to its linear counterpart at eliciting antibodies to *Plasmodium falciparum*, whereas cyclic and linear peptides were equally effective in the other study (29). Different malaria epitopes were examined in the two studies, which may account for these differences. Returning to 9-40, a cyclic peptide should be more effective than a linear one at mimicking the tight reverse turn CDR-H3 conformation (24,30,31). For this reason, our peptide mimic was cyclized by adding cysteine residues to its N- and C- termini.

MATERIALS AND METHODS

Preparation of the (9-40)Fab'

The 9-40 $F(ab')_2$ was prepared by the method of Grey and Kunkel (32). Specifically, 9-40 IgG was digested with pepsin (1:33 enzyme to antibody mass ratio) at 37°C for 18 h in 0.1 M acetate buffer (pH 4.2). The reaction was quenched by addition of a few drops of 1.0 M Tris base to pH 8.0. The resulting 9-40 $F(ab')_2$ (approx. 1 mg/ml) was then reduced with DTT in the same reaction vessel to generate 9-40 Fab'. In particular, 0.22 ml of 1.0 M Tris buffer (pH 8) was first added for buffer capacity and then EDTA and DTT (freshly prepared) were added to final concentrations of 5.6 mM and 2.8 mM, respectively. After reaction for 1 h at room temperature in the dark, excess DTT was removed by size exclusion chromatography (SEC) using a PD-10 column (Amersham Biosciences, Piscataway, NJ) equilibrated in 0.1 M sodium phosphate buffer, pH 6.0, 5 mM EDTA.

Preparation of (9-40)Fab'-HEL Conjugate

HEL was dissolved in pH 8.0 PBS and derivatized with a 100-fold molar excess of SMCC (Pierce Biotechnology, Rockford, IL) for several hours at room temperature. Excess SMCC was removed by SEC (Amersham Biosciences PD-10 column equilibrated with pH 7.4 PBS). Next, 0.1 ml of 35 μM HEL-SMCC was added to 1.2 ml of freshly prepared (9-40)Fab' solution (9.22 μM Fab' in 0.1 M sodium phosphate buffer with 5 mM EDTA buffer, pH 6.0) and allowed to react overnight at 4°C. The product was purified by SEC (Amersham Biosciences Superdex 200 column equilibrated in PBS). Chromatogram fractions were assayed for both 9-40 Fab' and HEL by ELISA. Fractions testing positive for both were pooled, concentrated, and then characterized by SDS-PAGE.

SDS-PAGE gels were scanned with a flatbed scanner and analyzed using UN-SCAN-IT (Silk Scientific, Orem, UT, USA). Peak areas were integrated to determine the following approximate species fractions: underivatized Fab' (33.7%), $(Fab')_1$-HEL (34.5%), $(Fab')_2$-HEL (10.0%), and $(Fab')_3$-HEL (19.6%). An average degree of labeling of 1.13 was determined from this distribution. The higher order species ($(Fab')_2$-HEL, $(Fab')_3$-HEL) were expected because of the 3-fold molar excess of Fab' to HEL in the reaction mixture. The presence of underivatized Fab' in the mixture was not considered a problem because this species was not expected to be immunogenic (a postulate that was later confirmed by our control experiments).

Preparation of the Bep-HEL Conjugate

The Bep idiotope peptide mimic contained the amino acid sequence of the 9-40 CDR-H3 (TSYGYHGAY), two flanking cysteine residues for cyclization, and an N-terminal tryglycine conjugation spacer (which was added to the N-terminus after initial conjugation attempts failed). This peptide (GGGCTSYGYHGAYC) was prepared by standard Fmoc solid phase peptide synthesis at the University of Utah's DNA/Peptide Facility. It was characterized by Matrix-assisted laser desorption ionization mass spectroscopy (MALDI-MS) and found to be in agreement with the expected mass (1395.5 expected vs. 1393.47 measured). One milligram of Bep was dissolved in 70 μl of DMSO and then diluted with 20 μl of PBS. Two milligrams of HEL were dissolved in 70 μl of formamide, then diluted with 20 μl of PBS. Two milligrams of DSG were dissolved in 40 μl of DMSO and then transferred to the Bep solution for 5 min. Excess DSG was removed by precipitating the reaction mixture with THF (~2 ml), then EtOEt, and then centrifuging. The DSG-Bep conjugate was next re-dissolved in 40 μl of DMSO, then added to the HEL solution and allowed to react overnight. Characterization was by MALDI-MS and then re-confirmed after a period of storage by SDS-PAGE.

Immunization Protocol

Cohorts of female B10.A mice (H-2^a haplotype; The Jackson Laboratory, Bar Harbor, MN, USA, cat. no. 000469) 3–5 weeks old were injected with 65 pmol of either the Bep-HEL conjugate (Bep-HEL), the (9-40)Fab'-HEL conjugate (Fab'-HEL), a noncovalent mixture of Bep and HEL (Bep + HEL), or Bep alone. The concentrations of Fab'-HEL and Bep-HEL were estimated based on scanned electrophoresis gels or mass spectrometry integrated peak heights using the software Un-Scan-It (Silk Scientific, Provo, Utah, USA) and adjusted molar extinction coefficients calculated from Trp, Tyr, and Phe residues (33,34). For example, 65 pmol of Bep-HEL is the total amount of $(Bep)_1$-HEL + $(Bep)_2$-HEL + $(Bep)_3$-HEL. Mice were bled by tail vein or artery 2 weeks later (primary and secondary; 16 days for tertiary). They were also bled prior to the boosts as described in "Results." All immunogen preparations were mixed 1:1 (v/v) with Freund's complete adjuvant for the primary immunization and with Freund's incomplete adjuvant for the secondary and tertiary immunizations. Each immunization consisted of two subcutaneous injections of 50 μl. The research adhered to the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, revised 1985).

ELISAs

Nunc MaxiSorp polystyrene strips were coated for 1 h with either 9-40 F(ab')$_2$ (500 ng/well, 50 μl/well, in 1.2 M NaCl, 50 mM $NaPO_4$, pH 7.5) or a Bep-BSA conjugate made analogously to the Bep-HEL conjugate described above (or with BSA as control). Strips were post-coated (blocked) with 1 mg/ml casein solution (in ELISA wash buffer: 150 mM NaCl, 10 mM Tris, 1 mM EDTA, 0.01–0.05% Tween 20) for 30–60 min. Serum samples were applied and incubated at room temperature for 1 h. Tracer antibody [goat anti-(mouse Fab) alkaline phosphatase conjugate (Biodesign International), diluted 1:1000] was added and incubated for 1 h. Substrate (Blue Phos, KPL; diluted with water 50%) was added and the absorbance read at 630 nm after 30 min. The specificity of response in the pilot study was measured by ELISA using Fab fragments of either 9-40, 4-4-20, BV04-01, or BDC1 as coating antigen.

RESULTS

Characterization of Bep-HEL Immunogen

As seen from the mass spectra in Fig. 1, peaks were observed for underivatized HEL, as well as $(Bep)_n$-HEL species with n = 1, 2, or 3. Thus, not all of HEL's six Lys residues were derivatized. This is to be expected based on steric considerations, as well as the reported unequal reactivity of HEL's six Lys residues (35).

Peak areas were integrated to determine the following species fractions in the immunogen: underivatized HEL (54.4%), $(Bep)_1$-HEL (36.7%), $(Bep)_2$-HEL (8.0%), and $(Bep)_3$-HEL (0.9%). These fractions were fit with a Poisson distribution to determine an average degree of labeling of 0.61 Bep peptides per HEL molecule. The Poisson distribution fit the observed species fractions with excellent correlation (r = 0.997). Immunogen concentrations used in subsequent animal studies were based on $(Bep)_n$-HEL composition, rather than total HEL composition.

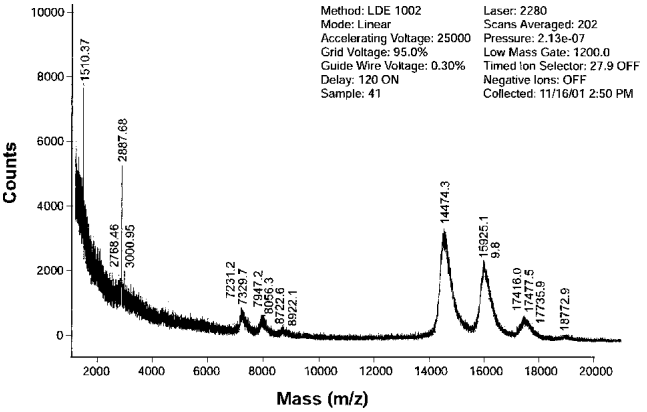

Fig. 1. MALDI-MS of Bep-HEL reaction mixture product. The observed mass of HEL is about 160 Da higher than its expected mass of 14,314 Da. This increase is most likely due to salts or other molecules complexed tightly with it. The conjugation reaction was carried out in a solution containing PBS (pH 7.4), plus formamide and DMSO (to dissolved the Bep peptide), making these molecules likely suspects for complexation. In fact, the molecular weights of phosphate, formamide, and water total 158 d. At pH 7.4, HEL has a net positive charge and may complex with a phosphate counter ion. The same holds true of formamide and water, both of which are effective at hydrogen bonding. Given the observed mass of 14,474.3 Da for HEL, the observed masses for Bep_1-HEL and Bep_2-HEL are consistent with their expected masses computed with the following equation, give or take a water molecule: $M_{expected} = n \cdot MW_{Bep} + MW_{HEL} + n \cdot (M_{DSG} - 2^* M_{HO\text{-}NHS})$ where n is the number of Bep peptides per conjugate molecule, *DSG* is the disuccinimidyl glutarate cross-linker, and *HO-NHS* is its *N*-hydroxysuccinimide leaving group. The observed mass of Bep_3-HEL is also consistent with this formula, but appears to contain an additional DSG group. The peaks around 7–8 kDa can be assigned to the double-ionized HEL, Bep_1-HEL, Bep_2-HEL, and Bep_3-HEL species. The degree of labeling is discussed in the text.

The HEL component in the immunogen did elicit anti-HEL antibodies (data not shown). However, this was not considered a problem because a previous study had shown that neither the idiotype (9-40 Fab) ELISA nor the idiotope (Bep-BSA) ELISA cross-reacted with such antibodies (34). Furthermore, underivatized HEL did not show any adjuvant properties in this same study when formulated in a noncovalent 50:50 mixture with the 9-40 Fab.

Specificity of Response

The specificity of the anti-idiotype antibody response was established in pilot studies with B10.A mice immunized with the Fab'-HEL conjugate. Fab fragments of four different monoclonal antibodies (9-40, 4-4-20, BDC1, and BV04-01) were used as ELISA plate coating antigens. 4-4-20 is a high-affinity anti-fluorescein antibody that is idiotypically cross-reactive with 9-40 and is clonotypically related to 9-40 (23,24). BDC1 is another anti-fluorescein antibody, developed in our laboratory, which is not idiotypically cross-reactive with 4-4-20 or 9-40. BV04-01 is an anti-ssDNA antibody (specific for oligothymidine) that has extensive (>90%) sequence homology with the 9-40 light chain and about 60% homology with the 9-40 heavy chain (36), but is not idiotypically cross-reactive with 9-40.

Results are shown in Figs. 2 and 3 for the primary and

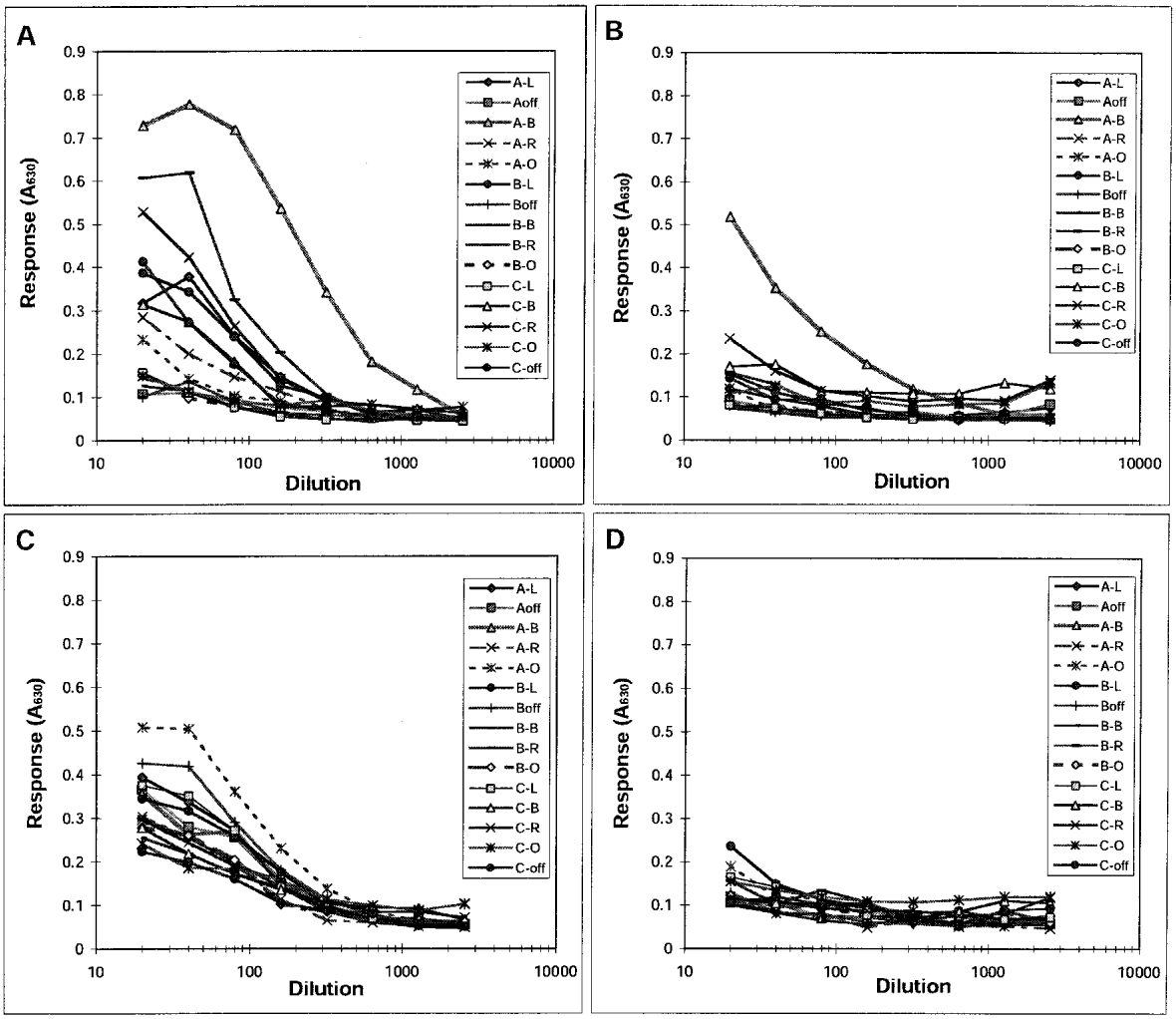

Fig. 2. Primary immune response (14-day post injection) for fifteen B10.A mice used in the idiotype specificity study. Keys for individual mice are in the box on the right. Panels A–D are the responses against the Fab fragments of 9-40, 4-4-20, BV04-01, and BDC1, respectively. See text for details.

secondary responses, respectively. An immune response to homologous antigen (9-40 Fab) was observed after both primary and secondary immunizations (Figs. 2A and 3A), whereas little or no cross-reactivity was observed to the non-idiotypically related BDC1 Fab (Figs. 2D and 3D). The percentage of mice responding to Fab'-HEL increased from 60% (9 of 15) in the primary response to 93.3% (14 of 15) in the secondary response (Figs. 2A and 3A). In the primary response, cross-reactivity to the idiotypically related 4-4-20 Fab was observed in only 20% (3 of 15) of the mice (Fig. 2B), but this figure increased to 93.3% (14 of 15 mice) in the secondary response (Fig. 3B). Nearly all mice exhibited some cross-reactivity to the BV04-01 Fab (which exhibited high sequence homology, but not idiotypic cross-reactivity to the 9-40 Fab) in the primary response, but this cross-reactivity disappeared in the secondary response (Figs. 2C and 3C).

Cross-reactivity with Parent Idiotype

Having established the specificity of the anti-idiotype response, we moved on to test the principal hypothesis of this study—namely, how well does the idiotope peptide immunogen (Bep-HEL) mimic its parent idiotype immunogen (Fab'-HEL)? Four different cohorts of B10.A mice were immunized with the following immunogens: Bep-HEL, Fab'-HEL, Bep+HEL, or Bep. Sera were collected after 14 and 44 days and each was assayed with two different ELISA test antigens—9-40 Fab, which tested for anti-idiotype activity, and Bep-BSA, which tested for anti-idiotope activity. For this cross-vaccination approach to be successful, sera collected from mice immunized with Bep-HEL (the idiotope mimic) should cross-react in the ELISA with the heterologous test antigen (9-40 Fab, which contains the native idiotype), in addition to reacting strongly to the homologous test antigen (Bep-BSA).

Figure 4 shows primary responses at 14 days (top panels) and 44 days (bottom panels) post immunization. Anti-idiotype and anti-idiotope responses are shown in the left panels (Figs. 4A and 4C) and right panels (Figs. 4B and 4D), respectively. Each data point represents the mean response for a cohort of ten animals, while error bars indicate the interanimal variability within each cohort. An anti-idiotype response was observed for the homologous immunogen (Fab'-HEL) after 14 days and persisted through 44 days, with a small increase in titer. An anti-idiotope response to the homologous immunogen (Bep-HEL) was only observed at

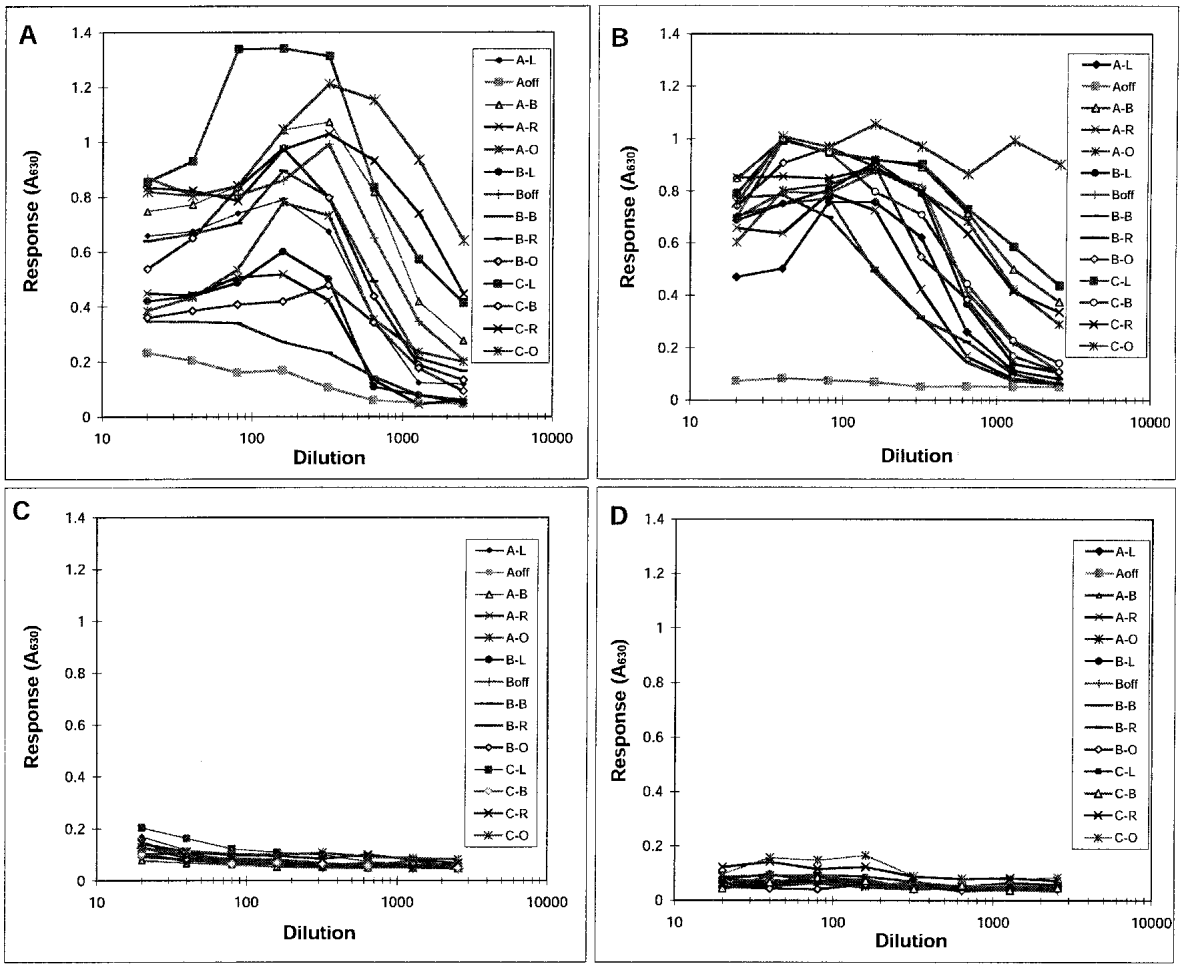

Fig. 3. Secondary immune response (14-day post injection) for fifteen B10.A mice used in the idiotype specificity study. Panels A–D are the responses against the Fab fragments of 9-40, 4-4-20, BV04-01, and BDC1, respectively. The ordinate scales in the 1° and 2° responses are not directly comparable due to a shorter ELISA development time used in the 2° response to avoid saturation of the microplate reader.

the 44-day time point. Interestingly, no heterologous responses (i.e., cross-reactivity) were observed in the primary response. Because BSA was used as a carrier protein for the idiotope peptide in ELISA assays, the anti-BSA response was also measured and found to be negative (data not shown). These results indicate that the kinetics of the anti-idiotope response is slower than the kinetics of the anti-idiotype response.

Data for the 14-day post immunization secondary response are shown in Fig. 5. In these plots, maximum anti-idiotype response (maximum A_{630} value of a given 9-40-Fab ELISA titration) is plotted against maximum anti-idiotope response (maximum A_{630} value of a given Bep-BSA ELISA titration) for each animal in each cohort. The horizontal and vertical lines denote 99% confidence limits for background absorbance values in the 9-40 Fab and Bep-BSA ELISAs, respectively. Maximum A_{630} values were scored positive for an anti-idiotype and/or anti-idiotope response if they were greater than the respective 99% confidence limit. It should be noted that the maximum A_{630} values for various ELISAs reported in Figs. 5–7 are not on directly comparable scales due to the different test antigens and/or color development times used in these assays.

All 10 mice in the Bep-HEL cohort developed an anti-idiotope response and 50% exhibited anti-idiotype cross-reactivity. One mouse from this cohort actually had a better anti-idiotype response than did any of the mice immunized with the intact idiotype (Fab'-HEL). Conversely, 100% of the mice in the Fab'-HEL cohort exhibited an anti-idiotype response, as well as a marginal, but statistically significant ($p = 0.015$) cross-reactive anti-idiotope response. Interestingly, all of the Bep+HEL and Bep control mice exhibited an anti-idiotope response, and two of seven mice in the Bep+HEL cohort exhibited a cross-reactive anti-idiotype response as well.

Another bleeding was taken prior to the tertiary immunization (at 39 days for control cohorts and 48 days for experimental cohorts). These data are shown in Fig. 6. Anti-idiotype cross-reactivity was observed in 77.8% (7/9) of the mice in the Bep-HEL cohort, although one mouse in this cohort no longer exhibited an anti-idiotope response. For mice in the Fab'-HEL cohort, both the intensity and consistency of the anti-idiotype response increased after 48 days, however the fraction of animals exhibiting a marginal cross-reactive anti-idiotope response dropped to 40%. Two of six mice in the Bep+HEL control cohort exhibited an anti-

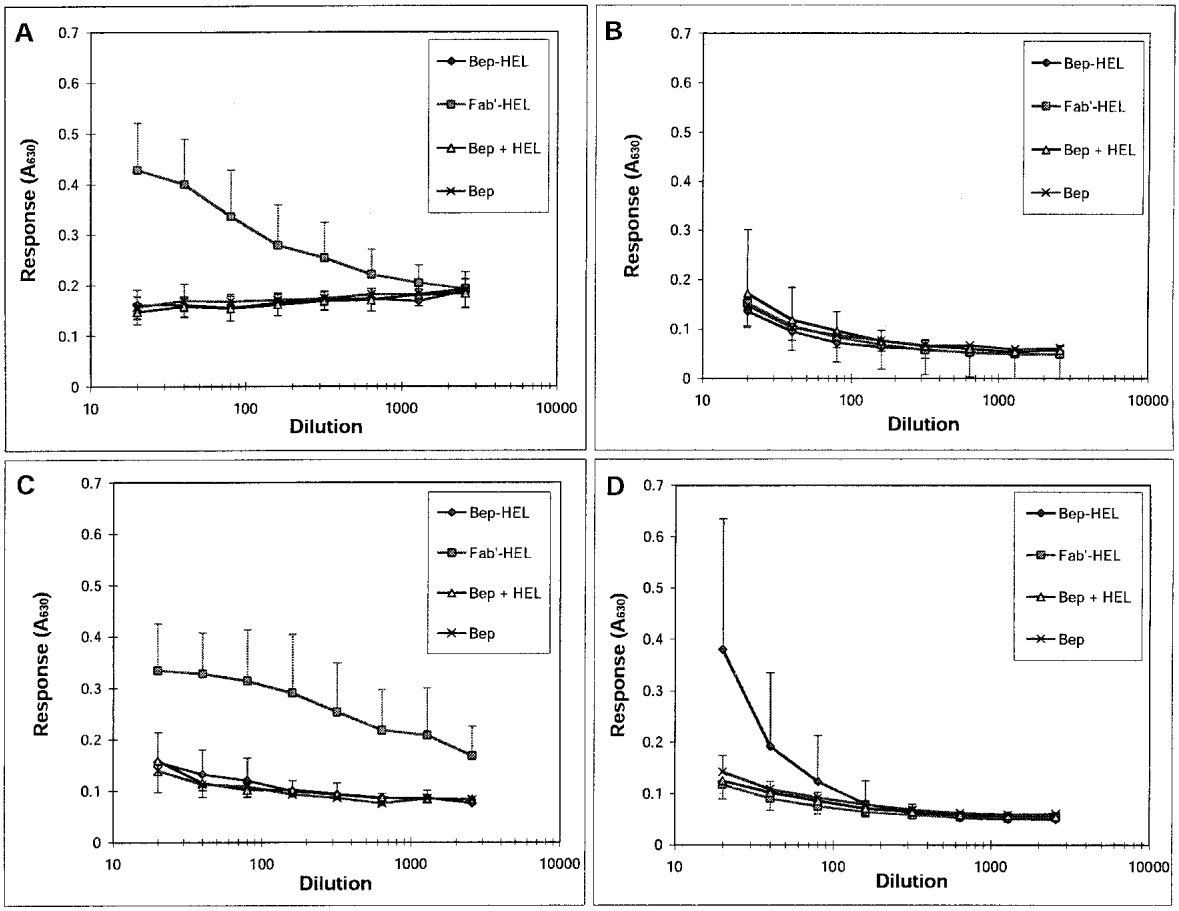

Fig. 4. Anti-idiotype and anti-idiotope responses at 14-days and 44-days post primary immunization. Panel A: anti-idiotype response 14 days post immunization. Panel B: anti-idiotope response 14 days post immunization. Panel C: anti-idiotype response 44 days post immunization. Panel D: anti-idiotope response 44 days post immunization. Each data point represents the mean response for a cohort of 10 animals, and error bars indicate the interanimal variability within each cohort. Only the upper or lower error bars are shown for clarity.

idiotope response; one of these also exhibited a marginal cross-reactive response. All mice in the Bep cohort exhibited an anti-idiotope response, although none exhibited any cross-reactivity.

The tertiary response is shown in Fig. 7. All eight surviving mice in the Bep-HEL cohort exhibited both anti-idiotope and cross-reactive anti-idiotype responses. Conversely, an anti-idiotype response was observed for all animals in the Fab'-HEL cohort, whereas a cross-reactive anti-idiotope response was observed in 55.6% (5/9) of the animals. Interestingly, all of the control animals exhibited an anti-idiotope response, and all but one exhibited cross-reactive anti-idiotype response. It is worth noting that all of the Bep control mice had higher anti-idiotype titers than those in the Bep+HEL cohort.

In summary, the results showed that (i) both the idiotype (Fab'-HEL) and idiotope (Bep-HEL) immunogens produced homologous, allogenic immune responses when covalently conjugated to a xenogenic carrier protein (HEL), (ii) the Bep-HEL idiotope vaccine produced cross-reactive antisera in the secondary and tertiary responses, (iii) the percentage of cross-reactive mice increased with increasing number of immunizations (14-day post primary, 0%; 14-day post secondary, 50%; 48-day post secondary, 77.8%; 16-day post tertiary, 100%), (iv) the underivatized Bep peptide in the control vaccines produced cross-reactive antisera in the tertiary response, (v) the Fab'-HEL idiotype vaccine elicited antisera that recognized the idiotope peptide mimic in some animals, although the response was variable across secondary and tertiary immunizations, and (vi) an anti-idiotype response was observed more consistently with the Fab'-HEL idiotype vaccine than with the Bep-HEL idiotope vaccine.

DISCUSSION

Mice in the idiotype specificity study responded well to the homologous idiotype and to a clonotypically related antibody (4–4-20) known to be idiotypically cross-reactive, but not to another anti-fluorescein antibody (BDC1) nor to an anti-DNA antibody (BV04-01) that shares the same framework regions as 9-40. It is significant that an anti-framework response was not seen in the secondary response, as these epitopes could also be considered part of an immunologically foreign molecule after coupling to HEL. This leads to speculation that the anti-framework reactive clones had been deleted or rendered anergic in the development of the mouse immune systems.

When conjugated to chicken hen egg lysozyme, the peptide mimic based on the 9-40 CDR-H3 produced a homolo-

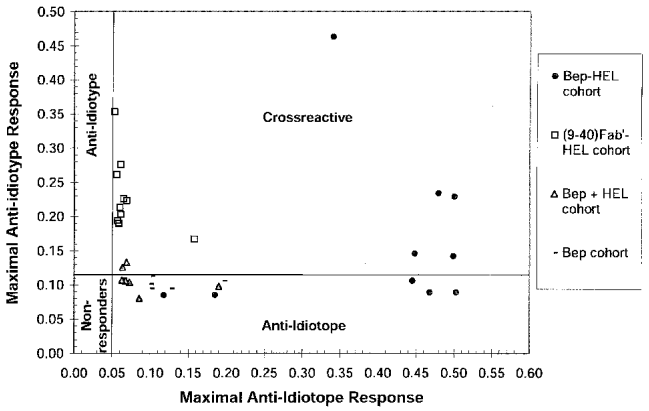

Fig. 5. Idiotype-idiotope cross-reactivity plot for 14-day post secondary response. Maximal anti-idiotope (maximum A_{630} value of a Bep-BSA ELISA titration curve for a given mouse) and anti-idiotype (maximum A_{630} value of a 9-40-Fab ELISA titration curve for a given mouse) responses are plotted on the abscissa and ordinate, respectively, for each mouse in each cohort. The vertical line intersecting the abscissa at 0.05 denotes the 99% confidence limit for a background response in the anti-idiotope (Bep-BSA) ELISA (determined using a one-tailed *t* test, n = 76). Likewise, the horizontal line intersecting the ordinate at 0.118 denotes the 99% confidence limit (one-tailed *t* test, n = 51) for a background response in the anti-idiotype (940 Fab') ELISA.

gous anti-idiotope response following all three (1°, 2°, 3°) immunizations. However, a cross-reactive anti-idiotype response was only observed following 2° and 3° injections. An important question is why the cross-reactive response takes so long to develop? One possibility is that only a small fraction of the B cell clones selected by the peptide mimic during the 1° response is capable of recognizing the conformation of the CDR-H3 loop as found in the parent 9-40 idiotype (e.g., perhaps only a few residues are exposed to bulk solvent, the rest being either juxtaposed to contiguous regions of the heavy chain or forming part of the antigen binding cavity). Thus, repeated stimulation (via 2° and 3° immunizations) of such

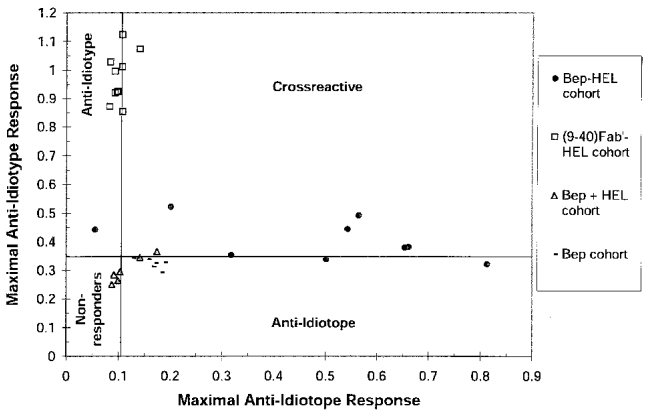

Fig. 6. Idiotype-Idiotope cross-reactivity plot for 48-day post secondary response. Data were plotted as described in Fig. 5. The vertical line intersecting the abscissa at 0.105 denotes the 99% confidence limit (one-tailed *t* test, n = 80) for a background response in the anti-idiotope (Bep-BSA) ELISA. Likewise, the horizontal line intersecting the ordinate at 0.35 denotes the 99% confidence limit (one-tailed *t* test, n = 63) for a background response in the anti-idiotype (940 Fab') ELISA.

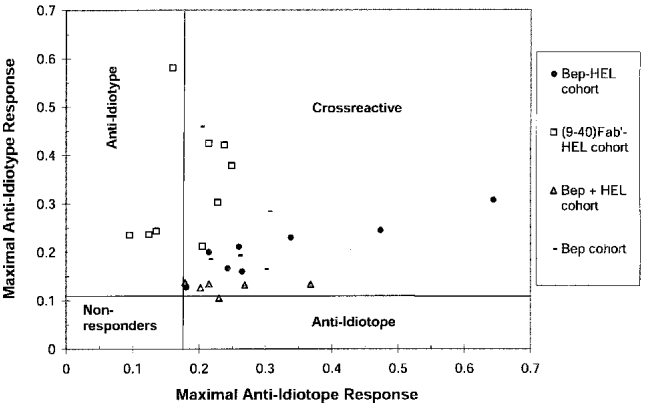

Fig. 7. Idiotype-Idiotope cross-reactivity plot for 14-day post tertiary response. Data were plotted as described in Fig. 5. The vertical line intersecting the abscissa at 0.174 denotes the 99% confidence limit (one-tailed *t* test, n = 69) for a background response in the anti-idiotope (Bep-BSA) ELISA. Likewise, the horizontal line intersecting the ordinate at 0.11 denotes the 99% confidence limit (one-tailed *t* test, n = 54) for a background response in the anti-idiotype (940 Fab') ELISA.

clones is necessary to achieve an observable cross-reactive anti-idiotype response.

The intensity of the cross-reactive anti-idiotype response for the Bep-HEL cohort appeared to decrease between 14 and 48 days post secondary, relative to the homologous anti-idiotype response in the Fab'-HEL cohort (although the number of marginally cross-reactive animals in the Bep-HEL cohort actually increased). This reduction in intensity may indicate the subsidence of specific cross-reactive B cell clones, while anti-idiotope clones persist to at least 48 days. Another study (37) investigating cross-reactivity between an idiotope mimic and an internal image idiotype mimic also found this same pattern of temporal cross-reactivity—initial idiotope-idiotype cross-reactivity that subsided into a more specific anti-idiotope response with time. These observations, taken together with those in the preceding paragraph, suggest the need for continual stimulation of the cross-reactive clones during the immune response—a postulate supported by the increase in both the intensity of the cross-reactive anti-idiotope response and the number of responding animals in the Bep-HEL cohort following 3° immunization.

A marginal cross-reactive anti-idiotope response was observed in the Fab-HEL cohort, but only after 2° and 3° immunizations. This response was less intense and more variable than the cross-reactive anti-idiotype response observed in the Bep-HEL cohort. These results are consistent with previous studies in which CDR-mimicking peptides produced an effective anti-idiotype response, but the native antibody produced little if any anti-peptide response (17,38). They also suggest that the CDR-H3 idiotope may not be completely accessible in the parent 9-40 idiotype. It should also be mentioned that our own studies are a more direct comparison of anti-idiotype and anti-idiotope responses because they were performed in the same species (mouse) as the immunizing 9-40 idiotype and the same carrier protein was used for both the idiotype and its peptide mimic.

The CDR-H3 loop was chosen primarily because it offers high specificity, being the product of V-D-J gene rearrangements and not exhibiting any canonical structures. However,

the 9-40 CDR-H3 is rather short, so another antibody with a longer, more solvent exposed CDR-H3 loop may have been a better choice for testing our principal hypothesis. This also begs the question of whether a longer CDR, such as CDR-H2 would give a stronger response. Several previous studies have demonstrated strong cross-reactive anti-idiotype responses for CDR-H2 mimicking peptides (12–14). Nevertheless, at least two other studies comparing cross-reactive anti-idiotype responses for synthetic peptide mimics of CDR-H1, CDR-H2, and CDR-H3 indicated that only the latter produced effective cross-reactivity (10,11). Thus, a peptide mimic for a given CDR may not be effective in all cases.

Are anti-idiotope peptide mimic vaccines a promising approach to targeting pathological B lymphocytes? They do offer the significant advantage of being readily prepared by solid phase synthesis using sequence information obtainable from biopsy. In addition, they cross-react with the parent idiotype and are expected to cause apoptosis of B cells expressing this idiotype. Nevertheless, the current study indicates that the parent 9-40 idiotype is a more effective immunogen than its peptide mimic, especially with 1° and 2° immunizations. An intriguing solution to this problem would be to construct an ensemble idiotope vaccine that would combine CDR-H1, -H2, & -H3 peptide mimics to better approximate the parent idiotype while maintaining the advantages of synthetic peptide antigens.

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