How Well Can a T-Cell Epitope Replace Its Parent Carrier Protein? A Dose–Response Study

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Received December 11, 2002; accepted December 16, 2002

Purpose. This work examines the effectiveness of synthetic peptide immunogens derived from immunodominant T-cell epitopes as replacements for their intact parent protein in vaccines.

Methods. Fluorescein was conjugated to hen egg lysozyme (FL-HEL, positive control) and three synthetic peptide immunogens: (a) murine B10.A (H-2^a) immunodominant T-cell epitope of HEL [FL-(T-cell epitope)]; (b) multiple antigenic peptide (MAP) multimer of this epitope {[FL-(T epitope)]$_n$-MAP, n = 2–4}; and (c) negative control MAP with T-cell epitope residues replaced with glycine [(FL-Gly$_{18}$)$_4$-MAP]. The dose response of each immunogen was examined over a 300-fold range in B10.A mice. The immune response was monitored using antifluorescein ELISA assays.

Results. FL-(T epitope)'s immune response correlated positively with dose, with maximum response comparable to that of [FL-(T epitope)]$_n$-MAP, or FL-HEL. This trend was consistent across 1°, 2°, and 3° responses, although interanimal variability was higher in the latter two because of an all-or-none response in mice immunized with this peptide. [FL-(T epitope)]$_n$-MAP's immune response was consistently high and nearly dose independent, a trend observed across 1°, 2°, and 3° responses. FL-HEL's immune response correlated negatively to dose in the 1° response but was nearly dose independent in the 2° and 3° responses. The magnitude of these latter responses was comparable to that observed for [FL-(T epitope)]$_n$-MAP. (FL-Gly$_{18}$)$_4$-MAP did not elicit an immune response except at the highest dose. This trend was consistent across 1°, 2°, and 3° responses.

Conclusions. The monomeric epitope was 300-fold less potent than its parent carrier protein, but increasing immunogen valency using MAP technology compensated totally for reduced potency. (FL-Gly$_{18}$)$_4$-MAP's lack of response at all but the highest dose strongly suggests that a specific immunodominant T-cell epitope sequence for HEL is necessary for successful peptide mimicry of HEL. This work also demonstrates the importance of quality assessment of commercial MAP core resins.

KEY WORDS: T-cell epitope; multiple antigenic peptide; MAP; chicken hen egg lysozyme; HEL; dose reponse; fluorescein hapten.

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ABBREVIATIONS: CV, coefficient of variation; DCC, *N,N*'-dicyclohexylcarbodiimide; ELISA, enzyme-linked immunosorbent assay; ESI-MS, electrospray ionization mass spectrometry; FAB-MS, fast atom bombardment mass spectrometry; FL, fluorescein; FL-KLH, fluorescein-labeled keyhole limpet hemocyanin; HEL, chicken hen egg lysozyme; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry; MAP, multiple antigenic peptide; MHC, major histocompatibility complex; MS, mass spectrometry; TLC, thin-layer chromatography; UV/VIS, ultraviolet/visible absorption spectroscopy.

INTRODUCTION

Peptides have been investigated as immunogens in vaccines since the 1980s, but initially only as haptens conjugated to a carrier protein. However, peptide haptens conjugated to carrier proteins can be difficult to characterize because of variable degrees of labeling and variable batch consistency. Also, there is the possibility of eliciting an immune response to extraneous epitopes peculiar to the carrier protein, with the potential for allergies or other immunologic side effects. Consequently, it is advantageous to dispense completely with carrier proteins for human and livestock animal vaccines and to use instead well-defined, immunologically relevant peptides. In order to be effective (involving a T-cell-dependent response), a peptide vaccine formulation must include one or more T-cell epitopes that match the MHC haplotypes encountered in the population. Several different pharmaceutical approaches [liposomes (1–3), iscoms (1,3), microspheres (2), numerous adjuvants (4)] have appeared in the literature with the goal of improving the efficacy of peptide (and other) vaccines.

In 1988 Tam introduced the multiple antigenic peptide (MAP) consisting of a branched oligolysine core to which are attached the peptide epitopes of interest (5). This gives an advantage over the carrier protein approach in that it is a chemically well-defined immunogen as well as having other possible synthetic and safety issues. There have now been over 150 papers involving MAPs, some using peptides as simultaneous T-cell and B-cell epitopes (6,7), some using a more cassette approach with distinct T- and B-cell epitopes (8–10), and some comparing MAPs with the traditional carrier protein approach (11,12). Nevertheless, to the best of our knowledge, no studies have specifically addressed how well a T-cell epitope can replace the carrier protein from which it is derived, from the pragmatic point of view of an *in vivo* immune response.

The simplest way to address this question would be to compare animal cohorts immunized with a hapten coupled either to a carrier protein or a T-cell epitope derived from such a carrier protein. Fluorescein (FL) was chosen for this purpose because of its ease of detection and its widespread use as a hapten (13). Considerable effort has gone into mapping many T-cell epitopes for a variety of proteins; one model protein used for immunologic studies is chicken hen egg lysozyme (HEL) (14). HEL's immunodominant T-cell epitope in B10.A (H-2^a) mice (along with flanking residues) consists of residues 49–66 (15). In synthetic peptides based on this sequence, cysteine 64 was replaced with serine to prevent dimerization, giving a sequence of GSTDYGILQINSRWWSND (referred to as "T epitope"). Because peptides are generally believed to be poorly immunogenic unless coupled to a carrier protein or incorporated into a MAP (or other special delivery system), we also included a MAP version of the peptide in our comparison as well as a negative control MAP consisting of the same number of amino acids as in the experimental MAP, but with the T-epitope sequence replaced by glycine residues. Four different immunogens were prepared: FL-HEL, FL-(T epitope), [FL-(T epitope)]$_n$-MAP (n = 2–4, see Methods), and [FL-(Gly)$_{18}$]$_4$-MAP. Because the FL-HEL species is not a chemically well-defined species,

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there was some variability in the degree of labeling of the HEL with the FL (about 2–4 FL per HEL).

We examined the primary, secondary, and tertiary responses over what initially seemed a wide enough concentration range to see the interesting region of the dose–response curve. This range was chosen based on the typical recommendations for protein immunogens, about 25 to 100 μg per mouse (16). Consequently, in the present study we spanned 1 to 300 μg per mouse for the FL-HEL case, as a positive control. This was converted to a molar basis and then the same epitope molar concentrations were used for the other immunogens. Because the primary and secondary immunization results showed that this range did not extend far enough into the low concentration region of the dose–response curves for the FL-HEL and for the [FL-(T epitope)$_n$]-MAP, the doses for all cohorts were reduced 100-fold for the tertiary immunization. The assay for measuring total antibody as a function of dose was a sandwich ELISA.

MATERIALS AND METHODS

Preparation of FL-HEL

HEL was purchased from Sigma, dialyzed against water, lyophilized, and stored frozen until use. Then it was dissolved in formamide and reacted with fluorescein NHS ester, dialyzed, and lyophilized. There was still some undialyzed FL remaining, which was removed by dissolving the FL-HEL in formamide, diluting it with dimethylformamide, and then precipitating this out with diethyl ether. TLC showed that the removal of free FL was quantitative. Degree of labeling was assessed by MS and UV/VIS and found to be approximately 3.

Preparation of Fluoresceinated Peptide Immunogens

A professional peptide synthesis facility located at the University of Utah synthesized the sequence GSTDYGILQINSRWWSND by batch method solid-phase peptide synthesis using standard Fmoc chemistry both as the linear peptide and as a MAP construct using the tetrameric MAP resin supplied by Advanced ChemTech. This facility also prepared a control tetrameric MAP consisting of the same number of residues but with every residue replaced by glycine. The control MAP was synthesized using the tetrameric MAP resin commercially available from Novabiochem; different suppliers were used for the two MAP constructs simply because of availability at the time. All of these synthetic peptides were fluoresceinated with 5- (and 6-) carboxyfluorescein using DCC as coupling reagent while still attached to the resin.

Purity and Structure Assessment of Fluoresceinated Peptide Immunogens

Purity was assayed by HPLC and MS. The latter worked very well for the linear peptide and produced a mass within 1.0 Da of the expected value. However, it had been the experience of the peptide synthesis facility, and also reported in the literature (17), that MAPs sometimes failed to give clean mass spectra. Nevertheless, both the experimental and control MAPs were analyzed by MS. MALDI-, ESI-, and FAB-MS failed to give interpretable and self-consistent spectra for the (FL-Gly$_{18}$)$_4$-MAP, and MALDI-MS for the experimental MAP formulation showed mostly dimeric MAP. Although this latter result was unexpected, its validity was questionable for the reasons mentioned above. Thus, we turned to amino acid sequencing to validate the experimental MAP and amino acid analysis to validate the control MAP (amino acid sequencing was not thought to be productive for the control MAP because of its branched Gly$_{18}$ sequence).

Amino acid sequencing of a small portion of experimental MAP that was left unfluoresceinated was found to be in agreement with the expected sequence. Comparison of the ratios of Gly:FL, Lys:FL, and Gly:Lys from amino acid analysis and UV/VIS was done for the control MAP. (Note that although the MAP resins from both vendors were sold as a β-Ala plus three Lys, so that taking Lys:β-Ala would seem to be the most accurate measure, this turned out to be problematic because the different vendors—especially Novabiochem—used an excess of β-Ala but capped it, resulting in an excess of β-Ala, which rendered this ratio noninformative.) The expected ratio of Gly:Lys was 24:1, and the experimental value by amino acid analysis performed at the University of Utah's Amino Acid Analysis Facility was in agreement with the expected, within experimental uncertainty. Furthermore, the ratio of Gly:FL (expected, 18:1) was also acceptable within experimental error. Based on these results, the purity of the fluoresceinated peptide immunogens was deemed adequate for immunization.

Immunization Protocol

Male B10.A mice (Jackson Laboratories, ME) 4–6 weeks of age were immunized subcutaneously in two injections of 100 μL each, with the dose comprised of a 1:1 emulsion of the desired dilution of the appropriate immunogen and Freund's complete adjuvant for the primary immunization or Freund's incomplete adjuvant for the secondary immunization. The animals were bled 2 weeks after they were immunized for both injections by tail vein or artery bleeding. The blood was allowed to clot in Eppendorf tubes, centrifuged, and stored frozen. The primary, secondary, and tertiary immunizations were 8 weeks apart. The research adhered to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985).

ELISA Protocol

ELISA test strips were purchased from Nunc and coated with a preparation of FL-KLH [prepared as described by Kranz and Voss (18)], diluted with a high-salt coating buffer (1.2 M NaCl, 50 mM $NaPO_4$, pH 7.5). All steps were done at room temperature. After coating of the wells for 60–90 min, the plates were washed with tap deionized water and tap dried (turned upside down on paper towels sharply), then postcoated (blocked) with casein in ELISA wash buffer (150 mM NaCl, 10 mM Tris, 1 mM EDTA, 0.01–0.05% Tween 20) adjusted to pH 11 with NaOH. After postcoating for ~30 min. The plates were washed again; then samples were applied and serially diluted. The plates were then washed, and goat anti-(mouse Fab) (Sigma) was used in a 1:10,000 dilution in ELISA wash buffer, and the plates were washed again. Substrate (Blue-Phos, KPL) was used but diluted 1:1 with water, and the absorbances were read in a Titertech plate reader 63 min after application of the substrate.

Further Analysis of the MAP Resins Using Test Peptides

Our structure assessment of the experimental and control MAPs did not yield any information about the distribution of the number of branches per MAP, so we decided to have a test peptide synthesized at the same peptide facility using the same resins as were used for the control and experimental MAPs. The sequence FLRG was made into a MAP using both the Novabiochem resin used for the $(FL\text{-}Gly_{18})_4$-MAP and the Advanced ChemTech resin used for the $[FL\text{-}(T\ epitope)]_4$-MAP, and then analyzed by MALDI-MS. The MAP with the former resin was >95% tetramer, whereas the latter resin yielded mostly dimer with only one lysine as being the core; both are supposed to have a β-Ala residue that is used for anchoring the growing peptide to the resin during synthesis. These results with the test peptide would strongly support the conclusion that the control MAP was predominantly tetrameric, whereas the experimental MAP was predominantly dimeric. For this reason we have chosen to designate the experimental MAP as "$[FL\text{-}(T\ epitope)]_n$-MAP."

RESULTS

Primary Response

Twenty cohorts of five mice (less one that died) were immunized subcutaneously utilizing the four different immunogens, each at five different concentrations. An additional control cohort of five mice was injected with adjuvant but no immunogen. The vaccine dose range spanned from 65.0 pmol to 19.5 nmol (equivalent to 1–300 μg for the FL-HEL case). Concentrations were based on the fluorescein absorption with the assumption that for every mole of fluorescein there was a mole of T-cell epitope, except in the case of FL-HEL, in which the assumption was that for every 3 moles of fluorescein there was 1 mole of HEL, based on the degree of labeling seen in the MALDI-MS. The mice were bled 2 weeks after immunization, and antifluorescein antibody responses were measured by ELISA. Serum components such as complement can inhibit the formation of the immune complex in the microtiter well (19–21), but such effects generally disappear on dilution of serum components. Thus, a full titration was performed on each mouse serum to determine whether such serum effects were present (none were observed, with one minor exception). Intraassay precision was determined by performing one titration in quadruplicate, yielding a CV value of 6.5%. Figure 1 shows the response at the 1.95 nmol dose for the FL-HEL, in order to more clearly show the differences in titrations from animal to animal. These results indicate that the assay variability is much less than the mouse-to-mouse variability.

The results from Fig. 1 and other similar titrations from the primary immunization are summarized in Fig. 2. In the absence of serum effects, titrations generally decreased in response with increasing dilution, and so the best signal was with the highest concentration. The average of this highest-concentration signal is plotted in Fig. 2, with the vertical error bars (shown either up or down for the sake of clarity) being the standard deviations for the different mice in each cohort. The negative control $(FL\text{-}Gly_{18})_4$-MAP gave no antibody response except at the highest dosage. The dose–response curve of the FL-(T epitope) immunogen appeared sigmoidal in shape (increasing linearly at immunogen concentrations above 10 μM), although it is unclear whether the curve would saturate at higher immunogen concentrations. This immunogen also exhibited moderately high variability in the responding animals. Increasing the valency of the immunogen, i.e., switching to the experimental $[FL\text{-}(T\ epitope)]_n$-MAP, resulted in dramatically increased immunogenicity at the lowest doses, but there was little change over the concentration range examined. Hence, it seems that increasing the valency of the peptide vaccine resulted in a strong shift in the dose–

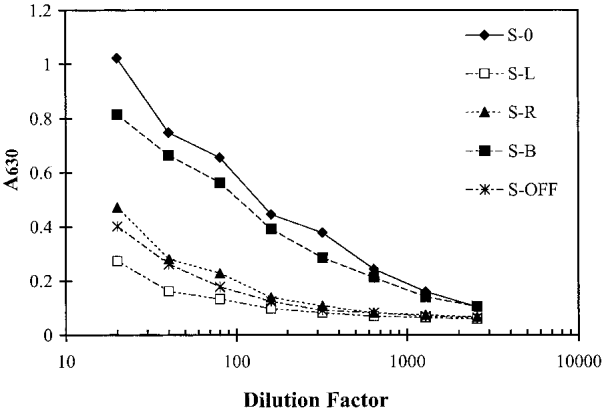

Fig. 1. Primary response for 19.5 μM FL-HEL cohort. The five curves are titrations (twofold serial dilutions) for each mouse in this cohort (designated as S). Samples were applied to polystyrene ELISA strips coated with fluorescein-KLH and blocked with casein. Goat anti-(mouse Fc) was used as the tracer antibody. The polystyrene strips were washed, and then substrate was added. Color (A_{630}) was read after 63 min from the time at which the substrate was added.

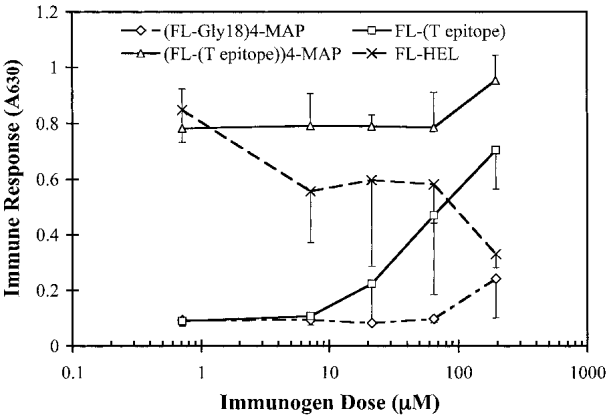

Fig. 2. Primary dose–response functions for control and experimental immunogens in B10.A mice measured by ELISA with FL-KLH as coating Ag. This summarizes the primary immune response for all cohorts except for the adjuvant-only (infinite Ag dilution) cohort because of the log scale. The adjuvant-only cohort was indistinguishable in response relative to the cohorts for the lowest concentration of $(FL\text{-}Gly_{18})_4$-MAP or of FL-(T epitope). At each immunogen concentration, 100 μl of immunogen was mixed with 100 μl of Freund's complete adjuvant and injected subcutaneously. ELISA assays were performed as described in Fig. 1. Immune response is reported as the A_{630} values for data with a dilution factor of 20. Every datum shown represents the average of five mice per cohort, except for the second to lowest dose for the $(FL\text{-}Gly_{18})_4$-MAP negative control, which had four mice (one died). The *error bars* are the sample standard deviations, shown either up or down for clarity.

response curve to the left. The experimental MAP gave the strongest overall primary antibody response among the four immunogens. Finally, the FL-HEL positive control actually showed a decreasing antibody response with increasing dose, although with fairly wide variation among animals.

Secondary Response

The immunization protocol was the same as for the primary response, except for the use of Freund's incomplete adjuvant. There was some evidence of increased titer levels in the FL-(T epitope) response and, surprisingly, in the (FL-$Gly_{18})_4$-MAP response, but not in the other two. It is important to note, however, that because titers were measured rather than affinities, higher titer could reflect increased antibody production by responding clones and/or increased antibody affinity. FL-(T epitope) at low doses exhibited a strong all-or-none response, as shown in Fig. 3. Similarly, the negative control MAP showed one responder at a lower dose than previously. The newly responding mouse in the intermediate dosage cohort was of the IgM isotype, whereas the other responding mice were primarily of the IgG isotypes (data not shown). At the highest dose, the response for $(FL\text{-}Gly_{18})_4$-MAP was amplified in the secondary immunization over that in the primary, as shown in Fig. 4. Increases in titer were not observed for either the FL-HEL or the $[FL\text{-(T epitope)}]_n$-MAP immunogens. Neither the $[FL\text{-(T epitope)}]_n$-MAP nor the FL-HEL positive control showed a strong dose dependency in the concentration range examined (Fig. 4).

Tertiary Response

Mice were bled before the tertiary (3°) immunization in order to examine carryover from the secondary (2°) response. Because of the saturating behavior observed in the 2° response for the experimental MAP and the positive control, all four immunogens were diluted 100-fold before 3° injections. Results from the carryover and tertiary bleedings are shown in Figs. 5 and 6, respectively. Comparison of these figures shows that there is little difference between the carryover and tertiary responses, making it difficult to say how much of the 3° response is caused by the 3° immunization vs. carryover

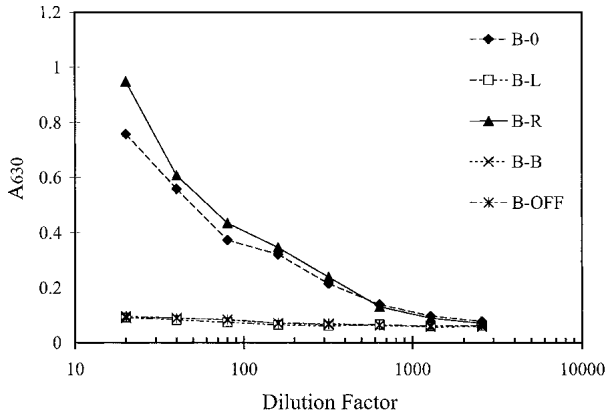

Fig. 3. Secondary response for 0.65 μM FL-(T epitope) cohort. Two mice showed strong secondary responses, but three showed no response in this low-dosage cohort. The next higher dosage was similar. The ELISA conditions were as in Fig. 1.

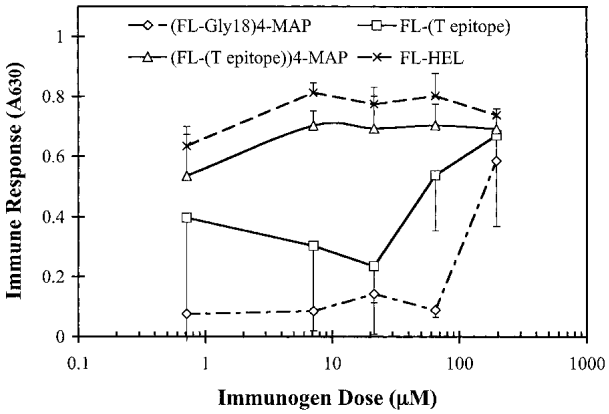

Fig. 4. Secondary dose–response functions for control and experimental immunogens in B10.A mice using ELISA with FL-KLH as coating Ag. Methods are the same as in Fig. 2, except that Freund's incomplete adjuvant was used instead of Freund's complete adjuvant. Again, the infinite-dilution (adjuvant-only) cohort (not shown) gave no response. The very wide error bars for the FL-(T epitope) result from the strong all-or-none response shown in detail in Fig. 3.

from the 2° immunization. Also, there is a small (one out of five mice) response for the middle dose of the $(FL\text{-}Gly_{18})_4$-MAP in Fig. 4, which disappears in Fig. 5 because the mouse died in the interim. The most striking observation is that the response for FL-(T epitope) is higher at 56 days than at 14 days, and the others are still about as high.

DISCUSSION

Comparing the monomeric and multimeric MAP versions of the fluoresceinated peptide clearly shows a dramatic increase in the effectiveness of the T epitope on incorporation into the MAP. This is consistent with earlier studies comparing the valency of different monomeric or multiple antigenic peptides (8) but also extends these results across a much wider concentration range.

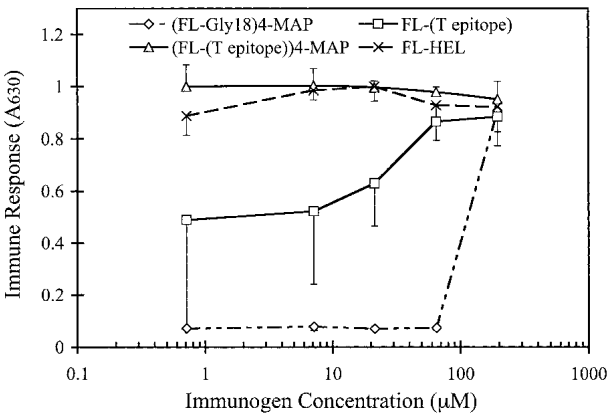

Fig. 5. Secondary dose–response functions 56 days postimmunization for control and experimental immunogens in B10.A mice using ELISA with FL-KLH as coating Ag. This shows the carryover from 2° immunization, 56 days postimmunization. Methods are the same as in Fig. 4. The middle dose for the FL-(T epitope) cohort, the middle and second-highest control MAP dose cohorts, and the second-highest and highest doses of the $[FL\text{-(T epitope)}]_4$-MAP contained four mice each instead of the usual five.

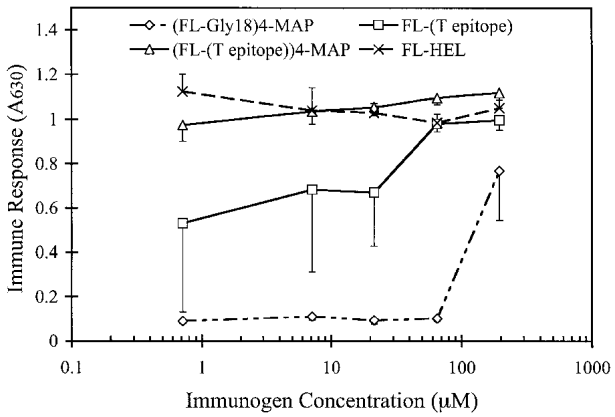

Fig. 6. Tertiary dose–response functions for control and experimental immunogens in B10.A mice using ELISA with FL-KLH as coating Ag. Results of 3° immunization. Methods are the same as in Fig. 4, except that immunogens were diluted 100-fold before injection. No additional mice died since the data in Fig. 5 were recorded.

MALDI-MS analysis of both the $[FL\text{-}(T\ epitope)]_n$-MAP and test peptide $[(FLRG)_n$-MAP] synthesized using the nominally tetrameric Advanced ChemTech MAP core resin indicated that the vast majority (80–85%) of the $[FL\text{-}(T\ epitope)]_n$-MAP formulation was dimeric. Although this does raise some questions about the relative potencies of two- and four-branched MAPs (discussed below), it actually strengthens the central conclusion, namely, that T-cell epitopes incorporated into MAPs are far more potent than monovalent peptides on an epitope molar basis except at extremely high (midnanomolar) doses, where they become equivalent. This begs the question of whether a more prodigious immune response would have been observed if $[FL\text{-}(T\ epitope)]_n$-MAP were purely tetrameric. Because the immune response with the present dimer/tetramer mixture was comparable to that of FL-HEL and relatively dose independent, it is doubtful whether increasing the tetramer fraction would improve the response over the dosage range examined. Still, it is possible that pure $[FL\text{-}(T\ epitope)]_4$-MAP would be more potent (exhibit higher titer) than the present dimer/tetramer mixture.

As for the original question of how well a T-cell epitope can replace the carrier protein from which it is derived, a comparison between the monomeric peptide and the carrier protein shows that the immunodominant peptide from HEL for B10.A mice is at least 300 times less potent at stimulating a T-cell-dependent response than is the intact carrier protein. It is worth noting that FL-HEL is also monomeric in terms of the immunodominant T-cell epitope but is roughly trimeric in terms of the hapten fluorescein. However, when the T-cell epitope is incorporated into a MAP, there is little difference between the MAP and the carrier protein, with the MAP possibly being slightly more potent for the primary response.

B cells can differentiate into antibody-secreting plasma cells by one of three known mechanisms: (a) direct stimulation by a T-helper cell specific for the same antigen (albeit different epitopes) as is the B cell, (b) activation by a type 1 T-cell-independent (TI-1) response (which actually does have some T-cell dependence), or (c) activation by a type 2 T-cell-independent (TI-2) response (which is truly T-cell independent) (22). TI-1 responses involve polyclonal B-cell activators, but TI-2 responses rely on receptor cross-linking and therefore require that the antigen be multimeric with respect to the epitope recognized by the reactive B-cell clone. In the present case this would mean that the valency of the FL-HEL with respect to FL, and likewise of the $[FL\text{-}(T\ epitope)]_n$-MAP, is a confounding variable; i.e., the response seen could be caused by the B-cell receptor cross-linking or by specific assistance by helper T cells. However, $(FL\text{-}Gly_{18})_4$-MAP effectively controls for B-cell receptor cross-linking. Hence, the response seen in the case of the FL-HEL was caused by the immunodominant and subdominant epitopes present in HEL rather than by direct cross-linking of the B-cell receptors, at least at the lower concentrations used. Likewise, the response evoked by the $[FL\text{-}(T\ epitope)]_n$-MAP preparation involves T-cell help. Because cross-linking is concentration dependent, it is reasonable to assume that the anti-FL response observed at the highest concentration of the control MAP results from B-cell receptor cross-linking of FL-specific clones, and that this response should not exist at lower concentrations. We surmise that $(FL\text{-}Gly_{18})_4$-MAP is acting as a type 2 T-cell-independent (TI-2) immunogen (22, p. 210).

Although this work was originally intended as a dose–response study over a fairly broad concentration range, the optimal concentrations were not known *a priori,* and a significant dynamic range was observed only for the FL-(T epitope) species (Fig. 2). The $[FL\text{-}(T\ epitope)]_n$-MAP response has reached a plateau throughout its dose–response curve. However, unlike most other saturation phenomena in biology, T-cell stimulation with respect to increasing antigen dose does not simply reach a stable plateau but rather exhibits a maximum. It is known that at high antigen doses T-cell anergy or deletion becomes operative (23,24). It is somewhat surprising that this development of tolerance is seen over a concentration range typically used for protein antigens, but HEL is a low-molecular-weight protein, and so a typical dose of 50 to 100 μg of HEL corresponds to a greater number of molecules than 50 to 100 μg of a higher-molecular-weight protein.

Increases in titer were not observed in 2° or 3° responses for either FL-HEL or $[FL\text{-}(T\ epitope)]_n$-MAP, presumably because the doses of these immunogens were already in the saturation region of the dose–response curve, so there is little, if any, competition for antigen and hence very limited selective pressure on high-affinity clones in the germinal centers (25). Surprisingly, titer increases were observed in the negative control MAP. Thymus-independent antigens are believed to show little if any memory (22), so one might conclude that this negative control vaccine is acting in a T-cell-dependent fashion. Although possible, it is difficult to reconcile with current understanding of how peptides are presented in the MHC and recognized by the T-cell receptor. It is more likely that this negative control immunogen is in fact a TI-2 immunogen, but that class switching and increased titer levels are nevertheless being seen.

Finally, it should be noted that commercially available, off-the-shelf MAP resins do not always yield the desired product, and it is advisable to first double-check the vendor's quality control. The MAPs used in this paper were synthesized using the same level of care as are ordinary peptides routinely synthesized at the University of Utah's Peptide Facility, but one vendor's resin gave mostly the expected four-branched MAP, whereas the other's gave mostly two-branched MAP.

ACKNOWLEDGMENTS

We thank Dr. Robert Schackmann and Scott Endicott of the University of Utah DNA/Peptide Facility for preparing the synthetic immunogens and for helpful discussions, Vajira Nannayakkara of the University of Utah's Pharmacy Mass Spectrometry Facility for doing the MALDI-MS and most of the ESI-MS, Prof. Elliot Rachlin of the University of Utah's Chemistry Department Mass Spectrometry Facility, and Pamela Crain for helpful discussions in the interpretation of some of the mass spectra. We would also like to thank Prof. Dennis Winge of the University of Utah's Amino Acid Analysis Facility for doing the amino acid analyses described herein, and Parke Byron for doing supplemental amino acid analyses. We would also like to especially thank Prof. Lorise Gahring for helpful discussions with her. This work was supported by grants from the University of Utah Research Committee and the University of Utah Funding Seed Grant Incentive Program.

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