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Induction of Autoimmunity by Multivalent Immunodominant and Subdominant T Cell Determinants of La (SS-B)

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We investigated the consequences of altering the form and valence of defined autodeterminants on the initiation and spreading of experimentally induced La/Ro autoimmunity. Anti-La and Ro (SS-A) Ab responses were monitored following immunization of healthy mice with defined immunodominant and subdominant T cell determinants of the La (SS-B) autoantigen synthesized as either monomeric or multiple antigenic (MAP) peptides. Abs to mouse La (mLa) developed faster and were of higher titer in mice immunized with the subdominant mLa25–44 MAP compared with mice immunized with the 25–44 monomer. Rapid intermolecular spreading of the autoimmune response to 60-kDa Ro was observed in AKR/J mice immunized with mLa25–44 MAP, but not in mice immunized repeatedly with monomeric peptide. A/J mice immunized and boosted with the known tolerogenic mLa287–301 determinant delivered as monomeric peptide failed to develop Abs to either intact mLa or mLa287–301 peptide. However, immunization with the multivalent mLa287–301 peptide led to the rapid production of high titer mLa autoantibodies associated with a proliferative T cell response to the mLa287–301 peptide. The data suggested that the enhanced immunogenicity of MAPs was not due to augmented Ag presentation or T cell stimulation. However, MAP-, but not monomer peptide-, containing immune complexes were potent substrates for Ab-dependent fixation of complement. These results demonstrate that the form of Ag responsible for inducing autoimmunity can profoundly influence the nature and magnitude of the immune response. Thus, molecular mimicry of tolerogenic and nontolerogenic self determinants might trigger autoimmunity under conditions of altered valence. The Journal of Immunology, 1999, 162: 3079–3087.

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pearding of autoimmunity to involve multiple components of the La/Ro ribonucleoprotein (RNP) complex (1–3) and spliceosomal proteins (4–6) has been reported following immunization with a single component of the RNP complex. For the La (SS-B) autoantigen there is a hierarchy of self tolerance to T cell determinants, with recent identification of several subdominant determinants and an immunodominant I-Ak-restricted determinant in A/J mice (7). Ironically, those determinants that are subdominant in their overall immunogenicity are poorly tolerogenic as self determinants, so that autoreactive T cells recognizing these epitopes are present in normal individuals. By contrast, immunodominant determinants efficiently tolerize the host T cell compartment when they are present as self determinants so that it is difficult to identify T cells recognizing these epitopes in normal individuals. Nonetheless, there is still conflicting evidence as to what type of T cell determinant drives autoimmunity. On the one hand, cryptic or subdominant determinants that are poorly presented may drive autoimmunity (7–10). On the other hand, immunodominant determinants, which are thought to be highly tolerogenic, have also been implicated (11). The relative roles of these determinants and the nature of the autoimmunity they engender might depend upon the form and immunogenicity of the Ag responsible for initiating autoimmune responses. For example, a highly immunogenic initiating stimulus might evoke T cell autoreactivity driven by both subdominant and dominant determinants, whereas a moderately potent immunogen might rely upon subdominant peptide determinants to elicit self-reactive T cells.

In experiments involving immunization with monomeric cryptic small nuclear RNP D peptides, additional administration of exogenous native murine small nuclear RNPs was required for a diversified B cell response (6), yet immunization with multimeric Sm B/B′ peptides was sufficient to induce autoantibodies binding multiple spliceosomal proteins in rabbits and mice (4–5). The relative immunogenicities of multivalent vs monovalent determinants have not been studied in models of autoimmunity initiated by immunization with short peptides. To examine these issues, we have altered the structural valence of defined self-peptides from the La autoantigen to enhance their initial immunogenicity (12).

Our findings reveal enhanced immunogenicity of multimeric I-Ak-restricted autodeterminants from the La autoantigen (7) and an enhanced potential to initiate spreading of the humoral autoimmune response to include 60-kDa Ro (R60). A subdominant La autoepitope became strongly immunogenic when delivered as a multivalent peptide, so that the autoimmune response diversified to involve the R60 component of the La/Ro RNP under conditions...
where the same monomeric determinant induced only limited au-
toimmunity. Moreover, mice were unresponsive to an immuno-
dominant self-La determinant in monomeric form, as predicted, but autoimmunity was induced rapidly following immunization 
with the same determinant in a multimeric form.

Materials and Methods

Peptides and protein Ags

Peptides were synthesized by the Molecular Biology Core Laboratory 
of the University of Oklahoma Health Sciences Center. The subdominant I-A\(^{k}\)-restricted mouse mLa\(^{25–44}\) peptide (FGDFNLPRDKFLKEQIKLDE) and immunodominant I-A\(^{k}\)-restricted mLa\(^{287–301}\) peptide (NANNGLQQRNKEVT) (7) were prepared as monomeric peptides and as eight copies of linear peptide synthesized onto a branched lysine backbone (Perkin-Elmer, Applied Biosystems Division, Foster City, CA) (12) to produce multiple 
antigenic peptides (MAPs). Monomeric and MAP forms of the immuno-
dominant hen egg lysozyme peptide (HRLa\(^{6–6}g\)) (13) were synthesized as I-A\(^{k}\)-restricted control peptides. All peptides were purified by reverse 
phase HPLC and verified by amino acid analysis. All experiments reported 
here were conducted using the same lots of purified peptides.

Recombinant soluble mLa (14) was produced in bacteria as an in-frame 
six-histidine fusion protein in the pQE vector (Qiagen, Chatsworth, CA) 
and was purified by Ni\(^{2+}\) affinity chromatography. Dihydrofolate reductase 
(DHFR) was similarly produced as a six-histidine fusion protein for use 
as a negative control. Recombinant human Ro60 (hRo60) was purified under 
denaturing conditions as previously described (15), and native bovine La 
and bovine Ro60 were purified as previously described (16, 17).

Immunizations

Six- to 8-week-old female A/J (H-2\(^{a}\)), I-A\(^{k}\)- and AKR/J (H-2\(^{b}\)) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained 
in the Oklahoma Medical Research Foundation animal facility. Athymic 
BALB/c-nu mice were purchased from the Animal Resources Center 
(Perth, Australia) and maintained in the animal facility at the Department of 
Microbiology and Immunology, University of Melbourne. For Ab stud-
ies, groups of six or seven mice from each strain were immunized s.c. with 
either monomeric (47.5 \(\mu\)g) or MAP (50 \(\mu\)g) mLa peptides emulsified 1:1 
in CFA, resulting in the injection of equal moles of specific epitopes. Mice 
were boosted three times with 47.5 \(\mu\)g (monomeric) or 50 \(\mu\)g (MAP) of 
the immunizing peptides emulsified 1:1 inIFA on day 10 and thereafter at 
14-day intervals. Tail bleeds were performed 3 days after each boost, and 
mice were exsanguinated on day 56. Control mice (two or three per group) 
were similarly immunized and boosted with MAP or monomeric HEL\(^{46–61}\) 
peptides.

ELISAs

Antipeptide responses were monitored by solid phase assay as previously 
described (2, 18). ELISA plates were coated with 50 \(\mu\)l of peptide (5 
\(\mu\)g/ml in 0.05 M carbonate buffer, pH 9.6) overnight, blocked with 0.1% 
gelatin in PBS, and incubated with sera from immunized mice diluted in 
PBS. Washing with 0.1% Tween-20 in PBS, bound Abs were detected with 
an alkaline phosphatase-labeled anti-mouse IgG (Sigma, St. Louis, MO) followed 
by addition of the substrate p-nitrophenylphosphate. Absorbance was measured at 405 nm. Similarly, reactivity with mLa, human Ro60, and the control protein DHFR were measured by incubating 
mouse serum with ELISA plates coated with 1 \(\mu\)g/ml of recombinant six-
histidine-mLa, hRo60, or six-histidine-DHFR, respectively.

Immunoblots

One or two micrograms of protein per lane were electrophoresed in 10% 
SDS-PAGE gels, then electroblotted to nitrocellulose membranes. Strips 
corresponding to specific lanes were prepared and blocked overnight at 4°C 
in 3% nonfat milk/TBST (TBST is 0.01 M Tris, 0.16 M NaCl, and 0.05% 
Tween-20). All other incubations were performed at room temperature. 
Following brief washes in TBST, strips were agitated for 2 h in 1/100 
dilutions of pooled mouse sera or in 1/50,000 dilutions of human 
reference antisera. Strips were washed five times for 5 min each time in 
TBST, then incubated for 2 h in either goat anti-mouse or anti-human IgG 
conjugated to alkaline phosphatase (The Jackson Laboratory). Following 
additional TBST washes as described above, strips were developed in 
5-bromo-4-chloro-3-indolyl-phosphate/4-nitro blue tetrazolium chloride 
solution (Kirkgaard and Perry Laboratories, Gaithersburg, MD). Develop-
ment was stopped with 0.02 M EDTA. With some blots, nonspecific 
binding was blocked by preincubating sera for 2 h at room temperature 
with Escherichia coli whole cell lysate. Briefly, the bacterial pellet from a 10-ml overnight culture of E. coli was lysed on ice in 1 ml of 0.25 mg/ml 
lysozyme in 0.1 M NaCl, 0.05 M Tris-HCl (pH 8.0), and 0.01 M EDTA, 
then sonicated for 10 s. For preincubations, bacterial cell lysate comprised 
one-fifth the volume of serum dilutions.

T cell hybridoma presentation assays

The I-A\(^{k}\)-restricted T cell hybridoma 11B1 (10^5 cells/well) and I-A\(^{k}\)-trans-
fected L cells (LIA; 5 × 10^5 cells/well) were cocultured for 24 h in the presence 
of graded amounts of various peptides. Culture supernatants were then 
assayed for IL-2 production by adding them to the IL-2-dependent cell 
line CTLL and measuring [3H]thymidine incorporation (0.5 \(\mu\)Ci/well) in 
triplicate samples as described previously (7). I-A\(^{k}\)-restricted HEL\(^{46–61}\) 
MAP or monomeric peptides were used as control peptides. In parallel Ab 
presentation experiments, LIA were fixed with 0.1% paraformaldehyde 
before coculture for 24 h with 11B1 and peptides, and IL-2 production 
was quantitated as described above.

T cell proliferation assays

Ten-week-old A/J mice (purchased from the Animal Resources Center 
and maintained in the animal facility at the Department of Microbiology 
and Immunology, University of Melbourne) were immunized s.c. in each hind 
foot with a total of 50 \(\mu\)g of mLa\(^{287–301}\) MAP or 47.5 \(\mu\)g of mLa\(^{287–301}\) 
monomeric peptide emulsified 1/1 in CFA (Sigma, Castle Hill, Australia). 
Control mice were immunized with saline and CFA alone. Seven days 
following immunization, the draining inguinal and popliteal lymph nodes 
were harvested, and T cells were purified on nylon wool columns (19). 
Irradiated (2000 rad) spleen cells from normal A/J mice, treated with Tris-
buffered ammonium chloride buffer to remove RBC (20), were used as 
a source of APC. Microcultures were established in 96-well flat-bottom mi-
croplate wells containing 3 × 10^5 purified T cells, 3 × 10^4 APC, and 
several concentrations of peptide Ags in a final volume of 250 \(\mu\)l of T cell 
culture medium (RPMI 1640 supplemented with 10% FCS, 2 mM t-
glutamine, 2 mM sodium pyruvate, 100 \(\mu\)M 2-ME, 100 IU/ml penicillin, 100 
\(\mu\)g/ml streptomycin, and 30 \(\mu\)g/ml gentamicin). Plates were incubated 
for 4 days at 37°C in 5% CO_2, with 1 \(\mu\)Ci/well of [3H]thymidine present 
during the final 18 h of culture. Cells were harvested onto glass-fiber filters 
(Micro 96 Harvester, Skatron Instruments, Lier, Norway), and radioactivity 
was determined using a gas phase Matrix 9600 Direct Beta Counter (Can-
berra-Packard, Victoria, Australia).

Binding of C3d to peptide immune complexes by ELISA

ELISA plates (Nunc, Roskilde, Denmark) were coated with increasing 
concentrations of L_{25–44} monomer, L_{25–44} MAP, or HEL_{46–61} MAP 
(0.05–10 \(\mu\)g/ml in 0.03 M carbonate buffer, pH 9.6). Following blocking 
with 1% BSA in PBS, duplicate wells were incubated with heat-inactivated 
sera from L_{25–44}-MAP-immunized A/J mice (diluted 1/1000). Wells were 
incubated with 1% normal human serum as a source of complement, 
then bound C3d was detected with a sheep anti-human C3d Ab (Sielenus, 
Melbourne, Australia) incubated for 1 h at 37°C, followed by an alkaline 
phosphatase-labeled anti-sheep Ig (Sigma, St. Louis, MO) and addition of 
substrate para-nitrophenylphosphate. To control for peptide coating 
of plates, bound anti-L_{25–44} MAP Ab was detected with an alkaline phos-
phatase-labeled anti-mouse IgG followed by substrate.

Results

Induction of autoimmunity following immunization with a 
multivalent subdominant L_{25–44} T cell determinant

Groups of A/J (H-2\(^a\), I-A\(^a\)) or AKR/J (H-2\(^b\)) mice were immunized 
and boosted with the I-A\(^{k}\)-restricted L_{25–44} subdominant T cell 
determinant synthesized as either a monomeric or MAP peptide (7). 
Serial dilutions of pooled successive serum samples from each 
group were analyzed by ELISA for IgG Ab reactivity with the 
L_{25–44} peptide, recombinant mLa, hRo60, and a control recom-
binant protein (DHFR). Sera taken from individual mice on day 86 
were also examined for Ab reactivity to the same Ags.

Both MAP and monomer peptides induced anti-mL_{25–44} peptide 
Abs, suggesting the presence of a B as well as a T epitope on 
L_{25–44}. However, the kinetics of Ab production were slower for 
the monomeric than for the MAP groups. High titer IgG anti-
L_{25–44} peptide Abs were detected on day 13 in MAP-immu-
nized A/J and AKR/J mice, but not in monomer-immunized mice.
at the same time point (Fig. 1A). Furthermore, by day 27, high titer IgG autoantibodies to intact mLa (but not to the unrelated protein DHFR) were present in the MAP-immunized mice when low titer anti-mLa and anti-La25–44 Abs were being first detected in the monomeric groups. In A/J monomer-immunized mice, Abs to mLa were detected before those to La25–44 peptide, suggesting that although monomeric La25–44 is a defined T cell determinant, it may be a poor B epitope in this strain, with early B cell reactivity directed at other regions of the La molecule. Despite repeated immunizations, the responses to La25–44 and mLa remained approximately 10-fold lower in the monomeric immunized mice (Fig. 1A). No anti-La25–44 or anti-mLa Abs were detected in control mice immunized with either the I-AK-restricted HEL46–61 monomeric or MAP control peptides, nor were Abs to the MAP backbone detected in MAP-immunized mice (data not shown).

To determine whether immunization with a single La determinant could also initiate intermolecular epitope spreading to involve other components of the La/Ro RNP, pooled sera from serial bleeds were tested for reactivity with recombinant Ro60 by ELISA. In the AKR/J (but not A/J) MAP-immunized mice, significant anti-Ro titers were observed on day 44 and continued to rise until the final bleed on day 86 (Fig. 1A). The responses for individual mice reflected the pooled serum results, with anti-Ro60 Abs being detected in five of six AKR/J mice immunized with mLa25–44 MAP, while none of six animals immunized with monomeric peptide produced Abs to Ro60 (Fig. 1B; p < 0.005, by Mann-Whitney U test). Neither monomer- nor MAP-immunized A/J mice developed immune responses that spread to Ro60 despite high responder rates against both the La peptides and whole mLa protein.

Pooled sera from day 86 were tested for reactivity with recombinant and purified La and Ro60 Ags on immunoblot to confirm reactivities detected by ELISA (Fig. 2). In agreement with the ELISA data, immune sera from A/J and AKR/J mouse strains reacted with both recombinant mLa and purified bovine La for both monomeric and multimeric peptide immunogens. Bands corresponding to proteins of lower m.w. are presumed to represent degradation products and were detected more strongly with higher titer sera from the MAP-immunized mice and human anti-La control sera. Immune sera from La25–44 MAP-immunized AKR/J mice bound both recombinant Ro60 and purified bovine Ro60 on immunoblots, consistent with intermolecular epitope spreading to Ro60 in these animals, while pooled sera from mice immunized either with monomeric La25–44 or with HEL46–61 MAP did not bind either recombinant or purified Ro60 Ags (Fig. 2). Binding to Ro60 was also detected in five of six individual sera from 25 to 44 MAP-immunized AKR/J mice (data not shown). No reactivity was observed with recombinant mouse Ro52 (21) or baculoviral human Ro52 (22) on immunoblots (data not shown).

Thus, immunization with a multivalent subdominant La determinant rapidly led to the production of high titer IgG anti-mLa Abs. For one I-AK-positive inbred strain (AKR/J), but not the other (A/J), the initial response to the mLa25–44 MAP was followed by recruitment of autoantibodies to Ro60, suggesting a potential role for non-MHC class II genes in controlling intermolecular spreading initiated by this epitope.

**Tolerance to the dominant mLa287–301 determinant is apparently overcome by immunization with mLa287–301 MAP**

Previous studies have shown no detectable B cell and weak to undetectable T cell autoimmunity in A/J mice immunized with the putative immunodominant I-AK-restricted mLa287–301 determinant, although this epitope binds I-AK and is processed and presented from intact mLa protein by mouse cells (7). To determine whether a multivalent form of mLa287–301 might be able to break immune tolerance and initiate anti-La autoimmunity, groups of six A/J and AKR/J mice were immunized and boosted with monomeric or...
MAP mLa\textsubscript{287–301} peptides. Pooled serial and individual mouse sera were tested by ELISA for Abs recognizing the intact mLa Ag as well as for Abs recognizing the mLa\textsubscript{287–301} peptide. In agreement with our earlier findings (7), repeated immunization with the mLa\textsubscript{287–301} MAP did not provoke an Ab response directed to either the intact mLa protein or the mLa\textsubscript{287–301} peptide in either strain. In contrast, the mLa\textsubscript{287–301} MAP rapidly induced autoimmunity, with detection of high titer IgG anti-mLa 287–301 peptide Abs on day 13 followed by high titer IgG anti-mLa autoantibodies on day 27 in both A/J mice (Fig. 3A) and AKR/J mice (data not shown). The results for individual mice reflected the pooled serum data in both strains (Fig. 3B and data not shown). Intermolecular spreading to involve Ro60 was not observed in either A/J or AKR/J strains, and this may reflect a more restricted response to the tolerized mLa\textsubscript{287–301} epitope relative to the subdominant 25–44 determinant.

MAP-induced Ab responses are T cell dependent

Although we have used multivalent MAPs primarily as a device to increase the autoimmunogenicity of defined self T cell determinants, the basis of their immunogenicity remains unclear. Presumably, the induction of class-switched, high titer IgG autoantibodies in MAP-immunized mice required the presence of T cell help. However, the observation that immunization of mice with defined T cell determinants in the form of MAP constructs elicited Abs specific for these same determinants prompted us to examine whether the observed Ab responses were T cell dependent. To this end, cohorts of athymic nude mice (BALB/c-\textit{nu}) or control A/J mice were immunized and boosted with mLa\textsubscript{287–301} MAP or monomeric peptides, and their pooled serum samples were examined for Abs to mLa\textsubscript{287–301} or intact mLa as described above (Fig. 4). As expected, MAP-immunized, but not monomeric peptide-immunized, A/J mice developed high titer IgG Ab responses to mLa and Ro60.
the peptide of immunization. MAP- or monomer-immunized nude mice produced no detectable IgG Ab to intact mLa or mLa287–301 (Fig. 4), although B cells from normal BALB/c mice produce Ab in response to mLa287–301 MAP (data not shown). Despite secondary abnormalities in nude mice, such as an overabundance of NK cells, they remain a benchmark test for T cell dependence of Ab. These data indicate that the observed responses were indeed T cell dependent.

Presentation of MAPs by APC does not lead to enhanced T cell hybridoma stimulation

The T cell-dependent nature of the vigorous anti-mLa Ab responses produced in mice immunized with a highly tolerized T cell epitope suggested that the enhanced immunogenicity of MAPs might be explained by augmented T cell stimulation, perhaps simply due to an increased density of T cell determinants presented by APC. To test this possibility, we stimulated the I-Aκ-restricted hLa288–302-specific T hybridoma 11B1 (7) with unfixed I-Aκ-transfected L cells (LIA) as APC and graded amounts of either monomeric or MAP forms of the hLa288–302 peptide. Maximal stimulation of 11B1, as assessed by IL-2 release, required approximately 100 times more MAP than monomeric peptide, suggesting that a higher density of peptide on APC is not responsible for the high immunogenicity of MAPs (Fig. 5A).

To further characterize the nature of presentation of MAP T epitopes by APC, we next assessed whether there was an absolute requirement for internalization of MAPs by APC for the stimulation of 11B1. Interestingly, hLa288–302 MAP retained the ability to stimulate 11B1 in the presence of formaldehyde-fixed APC, although a decrease in stimulatory capacity of fixed relative to unfixed APC was observed for both mono- and multivalent hLa288–302 peptides (Fig. 5B). The presentation of intact hLa Ag by APC fixed under these conditions is abolished (data not shown). Equivalent results were obtained when fixed and unfixed irradiated spleen cells were used as APC (data not shown). Thus, MAPs do not demonstrate an absolute requirement for internalization by APC for T cell stimulation and appear capable of stimulating a T hybridoma following direct binding to I-Aκ molecules at the surface of APC. These data indicate that the enhanced immunogenicity of MAPs is unlikely to be due to a higher density of presented determinants than achieved by equivalent monomeric peptide.

T cells primed by mLα287–301 MAP are peptide specific

The data presented above, which apparently exclude a critical role for Ag presentation in mediating the enhanced immunogenicity of MAPs, exploits the largely costimulus-independent nature of T cell hybridoma stimulation and does not address any effects MAPs might have on costimulus-dependent T cell activation. Furthermore, the apparent ability of MAPs to overcome tolerance to mLα

FIGURE 4. Ab responses elicited by MAPs are T cell dependent. Pooled preimmune (day 0) and immune sera from nude mice (BALB/c- nu; squares) or control A/J mice (triangles) immunized with the MAP (filled symbols) or monomeric (open symbols) forms of mLα287–301 peptide were titrated by ELISA for reactivity to mLα287–301 peptide or recombinant mouse La Ag as described in Fig. 3.

FIGURE 5. Stimulation of an hLa288–302-specific T cell hybridoma (11B1) by MAP and monomeric peptides. A, The 11B1 response to monomeric (mono; open symbols) or MAP (filled symbols) hLa288–302 or control HEL46–61 peptides was assayed with nonfixed I-Aκ-transfected L cells (LIA) as APC. IL-2 concentrations present in the resulting supernatants were then assayed by the proliferation of the IL-2-dependent cell line CTLL (reported as counts per minute). Each point represents the mean value of triplicate assays. B, Similarly, the 11B1 response to MAP or monomeric hLa288–302 peptides was assayed after addition of graded concentrations of peptide to either nonfixed (open symbols) or paraformaldehyde-fixed (closed symbols) LIA APC.
following immunization with a tolerized T cell determinant might be explained by the generation of a novel T cell epitope formed by parts of the mLα287–301 peptide and the MAP lysine backbone. To address these issues, groups of AJ mice were immunized in the footpad with either mLα287–301 MAP or monomer peptide delivered in CFA, then T cells from draining lymph nodes were purified and assayed in vitro for proliferation in response to mLα287–301 MAP or monomer peptides presented by irradiated splenic APC. While no responses were measurable in groups of CFA/saline-immunized mice (data not shown), a weak, but specific and reproducible, recall response to mLα287–301 monomeric peptide was observed with T cells from animals immunized with mLα287–301 MAP (Fig. 6, left panel). However, this T cell reactivity to the 287–301 peptide was not significantly different from that of monomeric primed animals (Fig. 6). Importantly, mLα287–301 MAP-primed T cells do not recognize mLα287–301 MAP to a greater extent than mLα287–301 monomer peptide, strongly arguing against the creation of any neo-epitopes recognized by MAP-primed mice. Thus, the peptide-specific proliferative T cell response elicited by immunization with MAP and that produced by immunization with monomeric peptide are similar in magnitude. On the one hand, it is clear from Fig. 3 that priming and boosting by mLα25–44 monomer do not provide adequate T cell help for production of anti-mLa Abs. On the other hand, the data presented above suggest that the ability of mLα25–44 MAP to elicit high titer IgG anti-mLa Ab responses is not mediated by either an enhanced T cell response to specific peptide or the creation of any neo-epitopes comprised partly of lysines derived from the MAP backbone. Therefore, the weak, but specific, T cell response to mLα287–301 peptide detected in mLα287–301 MAP-immunized mice appears to provide sufficient help for the production of high titer IgG Ab recognizing mLα.

MAP immune complexes are potent substrates for the fixation of complement

The lack of detectable differences between MAP vs monomer peptide Ag presentation and T cell proliferation suggested the possibility that the action of MAPs in enhancing immunogenicity may not depend upon differential T cell stimulation. It has been established that the covalent attachment of C3-derived complement fragments on Ags or immune complexes can enhance T cell-dependent humoral immunity through interactions with complement receptor CR2 (CD21), which is present on both B cells and follicular dendritic cells (23–29). Therefore, we considered whether MAPs could differentially fix complement in either the absence or the presence of peptide-specific Ab. Microtiter plates were coated with varying concentrations of MAP or monomer peptides, incubated with 1% normal human serum as a source of complement, then assayed for the presence of bound C3d as an indication of complement fixation. Neither solid phase monomer nor MAP peptides bound C3d in the absence of Ab, implying that these peptides do not fix complement directly (data not shown). We next assayed whether MAP- or monomer-containing immune complexes could fix complement by incubating the plate-bound peptides with specific Ab before incubations with sources of complement and C3d detection reagents. While no bound C3d could be detected in wells containing monomer peptide, MAP-containing immune complexes were potent substrates for the fixation of C3d (Fig. 7, top panel). Microtiter plate coated MAP and monomer peptides bound comparable levels of specific IgG from MAP-immunized mice over a range of peptide-coating concentrations (Fig. 7, bottom panel).

Discussion

We have asked whether enhancing the immunogenicity of a stimulus initiating autoimmunity can influence the establishment of diversified autoantibody responses. This study has shown that immunization with highly immunogenic multimeric forms of short mLa peptides containing a subdominant (normally nontolerogenic) or dominant (normally tolerogenic) T cell determinant can induce autoimmunity in normal mice. For the subdominant mLα25–44 determinant, autoimmunity was evidenced by intermolecular spreading of the immune response to Ro60, without a requirement for any exogenous source of Ro60. Furthermore, immunization with the mLα25–44 MAP resulted in Abs to intact mLa that developed more rapidly and were higher in titer than those from mice immunized with equal moles of the specific 25–44 epitope in monomeric form.

IgG anti-Ro60 Abs were elicited only in AKR/J mice and not in the class II-identical A/J strain, suggesting that non-MHC class II genes are involved in the B cell epitope spreading initiated by the 25–44 subdominant T epitope. Indeed, non-MHC genes have been
We originally hypothesized that the enhanced immunogenicity of MAPs might be the result of augmented T cell stimulation, resulting in the delivery of increased T cell help to La-reactive B cells. Such enhanced stimulation of human T cell clones by T cell epitopes oligomerized in a linear format has been reported (34); however, our data do not support this explanation for the high immunogenicity of MAPs. The former study noted that spacing between epitopes was an important factor for enhancing oligomer immunogenicity, and our results suggest that geometry may also be critical. The lack of enhanced stimulation of a T cell hybridoma by specific MAP suggests that MAPs do not achieve a higher density of MHC class II-presented determinants relative to monomer peptides. Furthermore, when naive T cells were primed with either specific MAP or monomer peptides in vivo (a situation where any differential T cell activation dependent upon costimuli would be allowed to occur), no difference in specific recall to monomer peptides was observed. Additional experiments will be required to determine whether MAP and monomer peptides have the capacity to differentially stimulate cytokine production by T cells in a proliferation-independent manner. The observation that mLa287–301 MAP-primed T cells are no better stimulated to proliferate in the presence of specific MAP relative to monomer peptide argues against the generation of a neo-T epitope comprised partly of MAP lysine backbone as an explanation for these findings.

While subtle differences in mLa25–44 T cell stimulation cannot at present be ruled out as an explanation for the enhanced immunogenicity of MAPs, our inability to demonstrate such a phenomenon using both Ag presentation and T cell proliferation experiments suggested that the influence of MAPs may not depend solely upon T cells. It is entirely likely that MAPs containing both T and B cell epitopes might cross-link B cell surface Ig, delivering stimulatory signals that could account for our observations. For example, studies in both human and murine systems demonstrate synergy between membrane Ig receptor cross-linking by multivalent ligands and signaling through CD40, resulting in B cell proliferation and increased Ig production (35, 36). The increased IgG production observed in mice immunized with MAP vs monomeric forms of the mLa25–44 T determinant (which also contains a B epitope) could in part be the result of a similar phenomenon following membrane Ig cross-linking during cognate T cell help. It is also possible that surface Ig cross-linking may activate autoreactive B cells, resulting in expression of costimulatory molecules. The activated B cell might then become a competent APC, presenting MHC-peptide complexes to naive peptide-specific T cells (37–39). While MAPs appear capable of binding cell surface MHC class II directly, our experiments suggest that such binding does not result in either a high density of MHC-peptide complexes capable of TCR cross-linking and triggering of autoreactive T cells (presented here) or induction of the costimulatory molecules B7-1 or B7-2 on the surface of mouse splenic APC in vitro (data not shown).

The induction of high titer anti-mLa autoantibodies by the multimeric mLa287–301 peptide was unexpected, since we had no previous evidence that this peptide harbored a B cell epitope. We have shown previously that the mLa287–301 T cell determinant is efficiently presented by I-A× and is highly tolerogenic in A/J mice. These findings are based in part on poor T cell responses and the lack of specific Ab production following immunization of normal A/J mice with the linear mLa287–301 peptide (7). Even after repeated immunization of mice with the nontolerized immunodominant human La288–302 T epitope in monomeric form, which would be expected to provide more than adequate T cell help for anti-hLa288–302-reactive B cells (which would be expected to cross-react with mLa287–301-reactive B cells), anti-peptide Abs are not produced (data not shown). Nevertheless, immunization with mLa287–301 MAP reveals the presence of mLa287–301-specific B cells in the normal murine B cell repertoire. Perhaps the multimeric nature of MAPs allows the activation of B cells with a very
low affinity for La, which would not otherwise be stimulated. Alternatively, if normal mice possess a degree of tolerance to La in the B cell compartment, MAPs might overcome anergy or developmental arrest in such cells through enhanced B cell receptor signaling. This latter possibility may be less likely, since reversal of anergy or developmental arrest in tolerized B cells has only been shown to occur in the absence of specific Ag (40, 41). Unfortunately, testing of any hypotheses involving direct cross-linking of B cell surface Ig by MAPs would require mice bearing transgenic B cell receptors specific for a known peptide antigen, a reagent that is not yet available.

The finding that MAP-containing immune complexes are potent substrates for the fixation of complement could help explain a more potent Ab response in the case of immunization with the subdominant La25–44 T epitope, where both monomer and MAP forms of the immunogen induced peptide-specific Ab production. This could be due to enhanced activation and proliferation of Ag-specific B cells following binding to and signaling through CR2 (27, 29). Alternatively, MAP-containing immune complexes could be better retained on the surface of follicular dendritic cells through enhanced binding to CR2 (23, 42). These possibilities are currently under investigation.

There is increasing evidence from models of both tissue-specific and systemic autoimmunity that nontolerogenic cryptic or subdominant peptides can prime autoreactive T cells, which then drive T and B epitope spreading (6, 7, 9, 43–46). Genetic factors, the immunogen and the initial peptide immunogen, and an available source of endogenous antigenic complexes are all likely to contribute to the extent of antigen spread. The present study suggests that the immunogenicity of the self peptide may be of critical importance in the induction and diversification of an autoimmune response and that multivalent T cell determinants may induce immunity toward even highly tolerogenic determinants. While it is unlikely that multimeric peptides per se are responsible for the induction or perpetuation of natural autoimmunity, it is not implausible to suggest that multimeric peptide constructs such as MAPs may imitate encounters with nontolerogenic or tolerogenic determinants that repeat in structure. Potential sources of these multimeric stimuli may include viral proteins displayed on the cell surface, self proteins concentrated in apoptotic blebs, endogenous immune complexes, and proteins with repeated epitopes. Of interest, an epitope of the Sm B protein capable of initiating autoimmune disease in rabbits and mice is effectively repeated four times within the C-terminal 50 amino acids of Sm B (4–5). Regardless of the degree to which multiple antigenic peptides mimic natural autoimmune stimuli, however, MAPs are useful reagents for probing the extent of immune tolerance in the B and T cell compartments to nuclear autoantigens such as Ro and La.

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References


