Hierarchical Self-Tolerance to T Cell Determinants within the Ubiquitous Nuclear Self-Antigen La (SS-B) Permits Induction of Systemic Autoimmunity in Normal Mice

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Summary

Systemic autoimmune diseases are frequently associated with clustering of high titer autoantibody responses towards nuclear self-antigens. Little is known, however, about the extent of immune tolerance to the target nuclear antigens or the events leading to the complex autoantibody responses that are characteristic of systemic autoimmunity. To address these issues, we have examined the mouse immune response to La autoantigen (mLa) and the homologous human La antigen (hLa), which are components of the La(SS-B)/Ro(SS-A) ribonucleoprotein (RNP) complex targeted in systemic lupus erythematosus and primary Sjögren's syndrome. The findings reveal the presence of hierarchical T cell tolerance involving multiple autodeterminants within the La autoantigen expressed by normal H-2^k and H-2^a mice. At one end of this spectrum, there was no detectable T or B cell autoimmunity observed in mice that were immunized with the immunodominant $mLa_{287-301}$ determinant, which differed by a single residue in its core sequence from the homologous but highly immunogenic human $La_{288-302}$ determinant. Interestingly, the $mLa_{287-301}$ peptide acted as an altered peptide ligand that specifically antagonized the activation of an $hLa_{288-302}$-specific T cell hybridoma. In contrast to the tolerogenic $mLa_{287-301}$ determinant, a range of autoimmune potential was identified among poorly tolerizing, subdominant self-peptides present within mouse La autoantigen. Notably, immunization of normal mice with the autologous subdominant La_{25-44} and $La_{106-129}$ determinants resulted in limited or no detectable autoantibody response. In contrast, immunization with the subdominant mouse La_{13-30} determinant induced a proliferative T cell response associated with the appearance of specific autoantibodies recognizing multiple intrastructural (La) and intermolecular components (Ro) of the murine La/Ro RNP. The findings suggest how diversified autoimmunity might follow initiation of immunity to simple peptide mimics of poorly tolerogenic determinants that are present within ubiquitous self-antigens.

A key feature of many systemic autoimmune diseases is the presence of high titer autoantibodies that recognize ubiquitously expressed nuclear or cytoplasmic self-antigens. These autoantibodies are relatively disease specific and often recognize multiple components of discrete subcellular particles (1). For instance, autoantibodies that recognize nucleosomes, double-stranded DNA, and small nuclear ribonucleoproteins (snRNPs)[1] are characteristic of SLE, whereas antibodies that recognize components of the Ro (SS-A)/La (SS-B) RNP are most commonly associated with primary Sjögren's syndrome. Clustering of autoantibody responses probably reflects the physical association of the various target structures within distinct subcellular particles (1–3). This notion is supported by experiments that show spreading of autoimmunity towards the different components of the U1sn RNP (1, 4, 5) and the Ro/La RNP (6) after initiation of immunity to a single component of either complex.

We have previously shown that molecular spreading of immunity to the Ro/La RNP complex can be induced in normal mice by immunization with a single 107–amino acid (aa) fragment of the autologous La polypeptide (6). These observations suggest that immune tolerance to nuclear self-antigens may be absent or incomplete, suggesting a degree of immune ignorance even in normal individuals.

[1]*Abbreviations used in this paper:* aa, amino acid; GST, glutathione S-transferase; 6xHis, 6x-histidine; HEL, hen egg lysozyme; hLa, human La antigen; LIA, I-A^k–transfected L cells; mLa, mouse La antigen; PPD, purified protein derivative; SI, stimulation index; SnRNP, small nuclear ribonucleoproteins.

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Under these circumstances, spreading of autoimmunity may be naturally triggered by the initiation of T helper immunity. Incomplete self-tolerance to sequestered self-antigen is described in some models of tissue-specific autoimmunity where nontolerogenic cryptic peptides are believed to play an important role in driving autoreactive T cells and antibody diversification (7–10). There is also some evidence that nontolerogenic determinants exist in ubiquitously distributed self-components (11–16) including nuclear/cytoplasmic antigens targeted in systemic autoimmunity (1, 4, 17, 18). However, the precise extent to which the helper T cell compartment is tolerized to nuclear/cytoplasmic antigens is not known, and the general nature of the self-determinants recognized by T helper cells in systemic autoimmunity is unclear.

We now show that healthy normal mice are tolerant to immunodominant T helper epitopes that are present within the La polypeptide of the La/Ro RNP. Nonetheless, T cells from the same mice can react to subdominant or cryptic determinants of La protein after autoimmunization. Moreover, our data demonstrate that experimental autoimmunity involving multiple intrastructural and intermolecular components of the Ro/La RNP complex can be induced in normal mice that have been immunized with a single subdominant or cryptic peptide derived from the La protein. These observations reveal how autoimmunity to a simple peptide determinant can lead to complex autoantibody patterns through intra- and intermolecular spreading. In addition, the findings suggest how limited molecular mimicry of a single antigenic region of a ubiquitous autoantigen might initiate spreading of systemic autoimmune responses through activation of poorly tolerized autoreactive T cells.

Materials and Methods

Protein Antigens. Recombinant mouse La (mLa), human La (hLa), human Ro 60 (hRo 60), mouse Ro 52 (mRo 52), and hen egg lysozyme (HEL) were produced in bacteria as 6-histidine (6xHis) fusion proteins expressed in *Escherichia coli* from the vector pQE (QIAGEN, Inc., Chatsworth, CA). Alternatively, these proteins were expressed as glutathione *S*-transferase (GST) fusion proteins from the pGEX-2T bacterial plasmid vector (Amrad, Melbourne, Australia). 6xHis-hLa was also expressed in insect cells infected with recombinant baculovirus, and was kindly supplied by Dr. Neil Cook (Elias, Freiburg, Germany). Subfragments of mLa and hLa were expressed as GST fusion proteins as described previously (19, 20). Bacterial recombinant proteins were purified by either metal chelate affinity chromatography (Ni-NTA affinity chromatography) (21) or glutathione affinity chromatography (22). Recombinant proteins were passed through an endotoxin column (Pierce, Chemical Co., Rockford, IL) to remove bacterial endotoxins potentially contaminating the preparations.

Native and recombinant La antigen is known to be sensitive to proteolysis (23, 24), and mass spectrometric analysis of purified recombinant 6xHis-La antigens indicated that the majority of bacterial recombinant La proteins possessed a molecular mass of ~43 kD instead of the predicted 48 kD. This discrepancy in molecular mass is presumed to reflect bacterial proteolysis associated with protease-sensitive PEST regions of La (23–26). When 6xHis-hLa was produced in the baculovirus expression system, the resultant La protein was full length, as judged by its mol mass of ~48 kD after SDS-PAGE. Accordingly, baculovirus recombinant La protein was used in several immunizations to complete the T cell epitope mapping of the murine immune response to hLa.

Peptides. For initial screening of peptide specificity of lymph node T cells, a set of 155 overlapping 15-mer peptides of hLa was synthesized by the multipin synthesis system (Chiron Mimotopes, Clayton, Australia; 27) using optimized F-moc–based chemistry (28, 29) so that peptides could be cleaved from the pins. Peptides were dissolved in DMSO at ~1 mM concentration. Peptides spanned the complete length of the La molecule sequentially shifting by 2 or 3 amino acids, giving a 13–12-aa overlap of adjacent peptides. The peptides hLa 288-302, mLa 287-301, 13-30, 25-44, 106-129, and HEL 46-61 were synthesized on an automated peptide synthesizer (model 431A; Applied Biosystems, Inc., Foster City, CA) using both t-Boc–and F-moc–based chemistries, and were subsequently purified by reverse phase HPLC. The peptides were analyzed and their masses were confirmed by electrospray ionisation mass spectrometry using a triple quadrupole mass spectrometer (AP III; Perkin Elmer-Sciex, Ontario, Canada). The mLa amino acid sequence contains a single amino acid deletion at the position corresponding to hLa residue 217 and a 16-aa insertion after position 332, which changes the numbering of some mLa peptides relative to their hLa equivalent (30).

Immunization. 6–8-wk-old female A/J (H-2^a) and CBA/CaH (H-2^k) mice were purchased from the Animal Resource Centre (Perth, Australia) and maintained in the animal house at Flinders Medical Centre (Adelaide, Australia).

For in vitro T cell proliferation assays mice were immunized subcutaneously in the tail base and in one hind footpad with 20 μM La peptides or 100 μg either 6xHis-hLa, 6xHis-mLa fusion proteins, or baculovirus hLa protein in each site. The antigens were emulsified 1:1 in CFA containing *Mycobacterium tuberculosis* strain H37Ra (Difco Laboratories, Detroit, MI). For analysis of T cell reactivity, popliteal and inguinal lymph nodes were removed and recovered cells were pooled 9–10 d after the initial challenge.

For assessment of antibody responses, mice were immunized subcutaneously in the tail base with 20 μM La peptides or 100 μg 6xHis-La fusion proteins emulsified 1:1 in CFA. The mice were boosted twice at 10-d intervals with 10 μM peptides or 50 μg fusion proteins emulsified 1:1 in IFA, and 3–4 d after, the last boost animals were bled.

Lymph Node Proliferation Assays. 9–10 d after immunization, unfractionated popliteal and inguinal lymph node cells were pooled and processed as a single-cell suspension and cultured at 5×10^5 cells/well in flat-bottom 96 well-plates in HL-1 serum-free medium (Hycore Biomedical Inc., Irvine, CA) supplemented with 2 mM glutamine and 5×10^{-5} M 2-ME and 5 μM of a hLa peptide. For epitope screening, pairs of consecutive peptides were added to the same wells and tested in triplicate. T cell proliferation was measured by the addition of 0.5 μCi of [^{3}H]thymidine (ICN, Costa Mesa, Irvine, CA) for the last 18 h of 96-h cultures, and thymidine incorporation was measured using a multidetector direct beta counter system (Matrix 9600; Packard Instrument Co., Meriden, CT). The stimulation index (SI) was calculated as the ratio of cpm measured in the presence vs. absence of specific antigen.

In some experiments, before setting up the proliferation assay, lymph node T cells were purified by passing the cell suspensions through a nylon wool column (31). The T-enriched lymph node cells (2×10^5/well) were then cultured with irradiated syngeneic

spleen cells (2,000 rads; 4×10^5/well) in triplicate assays in the presence of either protein antigens, peptides, purified protein derivative (PPD; Commonwealth Serum Laboratories, Melbourne, Australia), Con A or medium alone. These assays were performed as for unfractionated LN cells, except that cells were cultured in complete DMEM medium (DMEM medium supplemented with 10% FCS, 2 mM glutamine, 1 mM Hepes, 5×10^{-5} M 2-ME, 1 mM pyruvate, and 1 mM nonessential amino acids).

Generation of T Cell Hybridomas. The I-A^k–restricted, hLa-specific T hybridoma 11B1 was generated by immunizing A/J mice with 6xHis-hLa (100 μg) emulsified in CFA and fusing the activated T blasts (32) from the draining lymph node cells to the TCR-negative, HGPRT-negative AKR thymoma line BW5147 (32, 33).

For preparation of T cell blasts, the immune LN cells (4×10^6/ml per well) were restimulated for 4 d in 24-well plates (Costar, Cambridge, MA) containing 6xHis-hLa (100 μg/ml) in 2 ml of complete RPMI 1640 medium (supplemented with 10% FCS, 2 mM glutamine, 1 mM Hepes, and 5×10^{-5} M 2-ME). Viable cells enriched for T blasts were isolated on Ficoll-Hypaque gradients, and 5×10^7 cells were fused with BW5147 (10^7 cells) in 42% polyethylene glycol (mol wt = 1,500; BDH Chemicals, Poole, England) and 15% DMSO. Cells (1 ml) were then plated in 24-well plates (10^5 cells/well) with A/J splenic feeder cells (2×10^5 cells/well) in a total volume of 2 ml complete RPMI 1640. HAT medium (complete RPMI containing 1.1×10^{-4} M hypoxanthine, 1.6×10^{-5} M thymidine, and 4×10^{-7} M aminopterin) and the medium (complete RPMI-HAT) was changed every 3 d. Hybrid growth was first observed at ~10 d after fusion. After ~3 wks, cells were transferred to new plates and gradually adapted to medium containing HT (complete RPMI plus 1.1×10^{-4} M hypoxanthine and 1.6×10^{-5} M thymidine). Hybrids were screened for specificity and then cloned by limiting dilution. The representative T cell hybridoma 11B1 was studied in further detail.

T Cell Hybridoma Antigen Presentation Assays. T hybridomas (10^5/well) and either A/J spleen cells (2×10^5/well) or I-A^k–transfected L cells (LIA; 5×10^4/well) were cocultured for 24 h in flat-bottom 96-well plates (Greiner Labortechnik, Frickenhausen, Germany) in the presence or absence of graded amounts of recombinant protein or peptide antigens. Cells were cultured in complete RPMI 1640. Recognition of antigen by T hybridomas results in their activation and production of IL-2. After 24 h the culture supernatants were harvested, freeze-thawed, and then added (25% vol/vol) to the IL-2–dependent cell line CTLL (34; 3.5×10^4/ml, 200 μl/well, final). Quantitation of IL-2 production was measured by [^{3}H]thymidine incorporation (0.5 μCi/well) in triplicate samples.

T Cell Antagonism Aassays. For assays of T cell antagonism, LIA (10^5/well) or spleen cells from A/J mice (2×10^5/well) were incubated with a nonsaturating concentration of $hLa_{288-302}$ peptide (100 nM) or 6xHis-hLa protein (4 μM) for 2 h at 37°C. The free antigen was removed and APC were washed once before further culture with graded amounts of either $mLa_{287-301}$ peptide or 6xHis-mLa protein. After 2 h, cells were washed once before the addition of the hLa-specific T cell hybridoma 11B1 (10^5 cells/well). The supernatants were harvested 24 h later and were assayed in triplicate for IL-2 content by [^{3}H]thymidine incorporation of CTLL.

Western Blotting. Recombinant antigens and control proteins were separated through 12.5% SDS-PAGE and electrotransferred to Hybond C extra nitrocellulose membranes (Amersham, Buckinghamshire, UK). The membranes were blocked with 3% skim milk in PBS (wt/vol) for 1 h and then incubated for 1 h with the relevant sera (1:250 or 1:500 dilution). After 3 × 10 min washes in 3% skim milk in PBS, the membranes were incubated for another 1 h with a 1:1,000 dilution of horseradish peroxidase–conjugated goat anti–mouse IgG (Sigma Immunochemicals, St. Louis, MO). After a further three washes in buffer, the bound antibodies were detected by enhanced chemiluminescence (Amersham, International, Buckinghamshire, UK).

Antibody Estimations by ELISA. Recombinant protein was coated on to Maxisorp microtiter plates (Nunc, Roskilde, Denmark) by incubating 200 μl of protein at 5 μg/ml in 0.03 M carbonate buffer at pH 9.6 overnight at 4°C. The plates were blocked for 1 h at 37°C and incubated with mouse serum diluted in PBS buffer containing 0.2% BSA and 0.05% Tween 20 for 2 h at 37°C. After washing in 0.05% Tween 20 in PBS, the plates were incubated with an alkaline phosphatase–conjugated goat anti–mouse IgG (Pierce) for another 2 h. Bound antibodies were detected by hydrolysis of the *p*-nitrophenyl phosphate substrate, and the developed color was measured at OD 405 nm.

Results

Immunization with Recombinant La Protein Induces Autoantibodies and Proliferative T Cell Responses to both hLa and mLa Antigen in Normal Mice. We have previously shown that immunization of normal healthy mice with recombinant mLa or hLa protein induced isotype-switched autoantibody production, suggesting T helper dependence of the immune response (data not shown and reference 6). The autoimmune response towards La is also associated with the development of autoantibodies to the Ro 60-kD antigen occurring 14–20 d after immunization (6). Immunization with either mLa or hLa induced anti-La autoantibodies, indicating that T helper determinants were present in La proteins from both species. Moreover, the hLa and mLa amino acid sequences are 77% identical (30), suggesting that shared T epitopes might exist in these homologous proteins. However, the magnitude of the induced autoantibody response was consistently greater in mice that were challenged with hLa compared to those challenged with mLa (6), implying the presence of additional T helper determinants (xenogeneic epitopes) in hLa and providing enhanced T help for the B cell response. To confirm that immunity to the La proteins was associated with specific T cell responses, normal mice were immunized with recombinant hLa protein, and 9 d later, responding lymph node T cells were examined for their specificity in vitro. Lymph node T cells from immunized mice specifically responded to 6xHis-hLa and 6xHis-mLa, but not to 6xHis-DHFR or recombinant GST protein (Fig. 1 *A*). The observation that T cells responded to both mLa and hLa confirmed the presence of shared or cross-reactive T cell epitopes present within these antigens (Fig. 1 *A*). Multiple T cell epitopes were apparently present in hLa, since La subfragments containing GST-hLaA (hLa amino acids 1–107) and GST-hLaC (hLa amino acids 111–243) also stimulated hLa-primed lymph node T cells. (Fig. 1 *A*). The presence of T cell autoepitopes in mLa was further verified by immunizing mice with 6xHis-mLa and examining the response of lymph node T cells to either 6xHis-

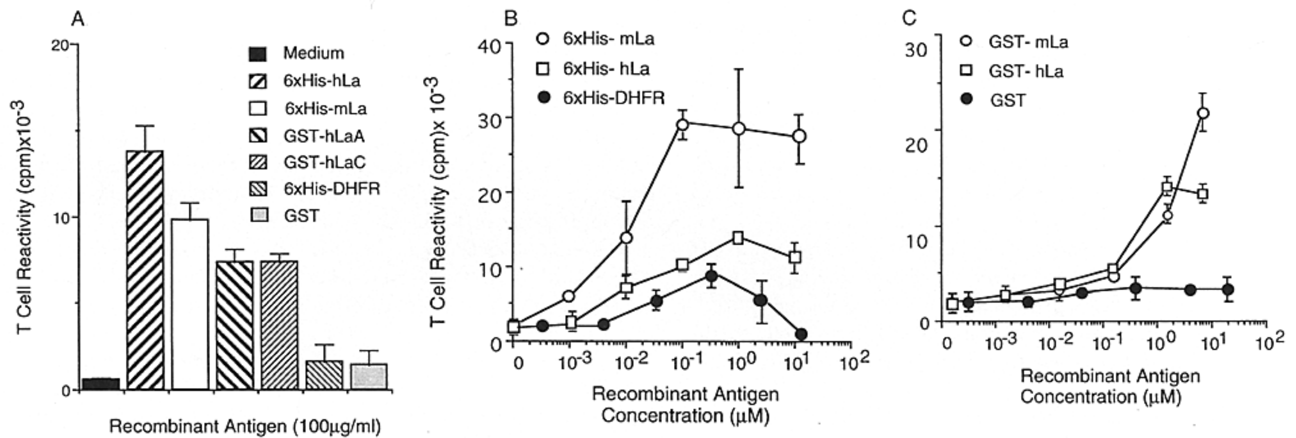

Figure 1. Proliferative response of murine lymph node T cells after immunization with recombinant hLa or mLa antigen. (A) A/J mice were immunized subcutaneously in the hind footpad and at the tailbase with 100 μg of 6xHis-hLa in CFA. The draining LN cells were pooled 9 d later, and the T-enriched population was cocultured (2×10^5/well) in triplicate with irradiated (2,000 rads) syngeneic spleen cells (4×10^5/well) in the absence (medium) or presence of the indicated recombinant antigens at a concentration of 100 μg/ml. After a 72-h coculture, the wells were pulsed for 18 h with [^{3}H]thymidine and then harvested for measurement of incorporated radioactivity. (*B* and *C*) Draining LN cells from A/J mice immunized with 100 μg of 6xHis-mLa in CFA were pooled, and the T-enriched cells were cocultured with irradiated syngeneic spleen cells in the absence or presence of graded amounts of either (*B*) 6xHis-mLa, 6xHis-hLa, or 6xHis-DHFR (mouse dihydrofolate reductase), or (*C*) GST-mLa, GST-hLa, or GST. Data are plotted as the mean +SD of triplicate cultures. Controls not shown included 100 μg/ml PPD (16,835 ± 40 cpm) and 1 μg/ml Con A (32,919 ± 29 cpm) in *A*, and 100 μg/ml PPD (25,400 ± 190 cpm) and 1 μg/ml Con A (30,190 ± 110 cpm) in (*B* and *C*).

mLa, 6xHis-hLa, GST-mLa, or GST-hLa proteins 9 d later (Fig.1, *B* and *C*). The results confirmed the presence of autoreactive T cells that responded in a dose-dependent manner to the 6xHis and GST forms of recombinant mLa protein (Fig. 1, *B* and *C*). Notably, in mice immunized with mLa, the in vitro T cell response upon subsequent challenge with mLa was sometimes (Fig. 1, *B* and *C*) but not always (data not shown) greater than the response observed after rechallenge with hLa. This observation suggested that mLa may also contain unique autodeterminants not found in hLa and which might contribute to the experimental autoimmunity induced by immunizing with mLa. It was also noted that responding T cells reacted to the GST-La fusion proteins with a dose dependence different from that observed for the 6xHis-La proteins implying that the covalent presence of the GST domain (mol mass ~26 KD) may alter the efficiency with which GST-La proteins are processed.

The Specificity of the Murine T Cell Response to hLa Includes Xeno- and Autodeterminants. In numerous experiments, we noticed that the T cell response to hLa immunogen was

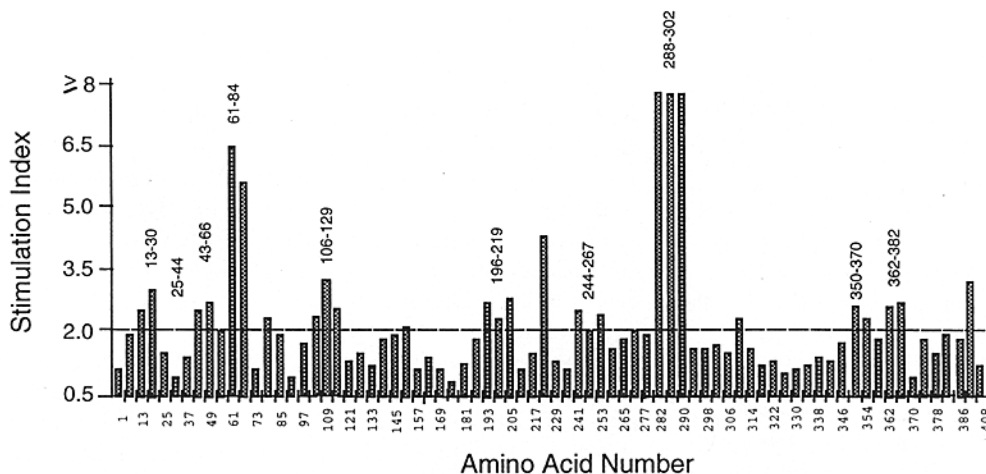

Figure 2. Peptide mapping of the murine T cell response to recombinant hLa. LN cells from A/J mice immunized once in the footpad with 6xHis-hLa antigen (100 μg; six experiments) or baculovirus hLa (100 μg; two experiments) in CFA were pooled 10 d later and tested for their in vitro reactivity against a panel of 15-mer peptides spanning the complete hLa molecule. The result of one experiment after challenge with baculovirus hLa is shown here. Peptides corresponding to residues 1–294 contained a 3-aa shift or 12-aa overlap (i.e., 1–15, 4–18, etc.). Peptides corresponding to residues 282–408 contained a 2-aa shift or 13 aa overlap (i.e., 282–296, 284–298, etc.). For epitope screening, peptides were pooled in pairs with the position of the amino terminus of the first peptide of every second pair shown on the x-axis (i.e., 1 corresponds to 1–15 and 4–18; 13 corresponds to 13–27 and 16–30, etc.). Triplicate LN cell cultures (5×10^5/well) were cultured in HL-1 medium (Hycore) containing 5 μM (final) of each of the relevant peptides. Proliferative data are shown as the SI. The average SI of control non-La peptides was ≤1. An SI ≥2 was considered positive if a clustered response (≥2 adjacent peptide pairs) occurred in one or more experiment. The amino acid residues of positive responses obtained in all experiments are shown above the relevant peptides. The dashed line corresponds to an SI of 2

Table 1. *H-2ᵃ–restricted T Cell Determinants within hLa*

Comparison of hLa peptide sequence and homologous mLa sequence*	Nature of determinant‡	Predicted amino acid residues	Positive§ experiments
h LEAKIC**HQIEYYFGD**FNL	AUTO	13-30	6/8
m ------------------			—
h FGDFNL**PRDKFLKEQIKL**DE	AUTO	25–44	3/8
m --------------------			
h DEGWVP**LEIMIKFNRLNR**LTTDFN	XENO	43-66	3/8
m --------T---------------			
h LTTDFN**VIVEALSKSKAE**LMEISE	XENO	61-84	6/8
m ---------Q-------K---V-A			
h NDVKN**RSVYIKGFPTD**ATLDDIKE	AUTO	106-129	4/8
m ------------------------			
h RKQNKV**EAKLRAKQEQEA**KQKLEE	XENO	196-219	1/8
m ----------N----H-GRH-PGM			
h TCREDL**HILFSNHGEIKW**IDFVRG	XENO	244-267	1/8
m -------F----------V--A--			
h D**ANNGNLQLRNK**EVT	XENO	288-302	8/8
m N------L----K--			
h SGKGKV**QFQGKKTKF**ASDDEH	XENO	350-370	1/2
m RRFKGK**GKGNRPGYA**GAPKGR			
h TKFASDD**EHDEHDEN**GATGPV	XENO	362-382	1/2
m GYAGAP**KGRGQFHG**RRTRFDD			

*The predicted minimum peptide epitopes are shown in bold type. A single amino acid deletion in the mLa sequence corresponding to hLa residue 217 and a 16–residue insertion in mLa after position 332 alters the numbering between equivalent mLa and hLa peptides after these positions (30).
‡AUTO, determinant of hLa where the same sequence is present in mLa; XENO, determinant sequences that differ between hLa and mLa. The $hLa_{61\text{-}84}$ and $hLa_{288\text{-}302}$ peptides were defined as immunodominant, based on the magnitude (SI generally ⩾6) and frequency of their reactivity. Although $hLa_{13\text{-}30}$ was reactive in six to eight experiments, the magnitude of these responses was consistently marginal (SI ~2), so this determinant was defined as subdominant.
§Positive proliferative T cell responses are defined as an SI ⩾2 present in two or more adjacent pairs of peptides and occurring in one or more experiment

consistently higher, and that it occurred at lower concentrations of hLa protein than the analogous responses that occur after immunization with mLa (data not shown). This observation was consistent with the differential pattern of autoantibody production after immunization with the two forms of La protein (hLa >mLa; 6). These findings suggested that in addition to determinants shared between hLa and mLa, xenogeneic T cell determinants present in hLa antigen might render this protein more immunogenic in mice than autologous mLa. Moreover, we reasoned that immunodominant regions of La may be easily revealed in hLa, but may be potentially tolerogenic and therefore invisible in mLa (12). To test this hypothesis, we mapped the mouse T cell response to hLa antigen. Lymph node T cells were isolated 9 d after immunization with 6xHis-hLa and tested for reactivity with a panel of 155 overlapping peptides spanning the complete hLa molecule. Peptides were pooled as pairs of 15 mers overlapping by two or three residues with the next peptide. A summary of these experiments is shown in Table 1, and the actual data from one such experiment is given in Fig. 2. Two determinants, $hLa_{288\text{-}302}$ (core amino acids 289–299) and $hLa_{61\text{-}84}$ (core amino acids 67–78), dominated the T cell response to the hLa antigen. Maximal proliferation was always observed to the $hLa_{288\text{-}302}$ peptide, which was stimulatory in eight out of eight experiments, while $hLa_{61\text{-}84}$ stimulated strongly in six out of eight experiments. The core amino acid sequence of both epitopes revealed that they were xenogeneic determinants differing from the homologous mLa determinants by a single residue (hLa295Q→mLa294L) in the case of $hLa_{289\text{-}299}$ (mouse homologue = $mLa_{288\text{-}298}$) and two residues (hLa70E→mLa70Q and hLa78E→mLa78K) in the case of $La_{67\text{-}78}$ (mouse homologue = $mLa_{67\text{-}78}$; Table 1). At least five other xenogeneic hLa determinants were identified as defined by clustering of the relevant T cell response to involve two or more pairs of overlapping peptides with a SI ⩾2 in one or more experiment. However, a feature of the responses to these additional xenogeneic determinants

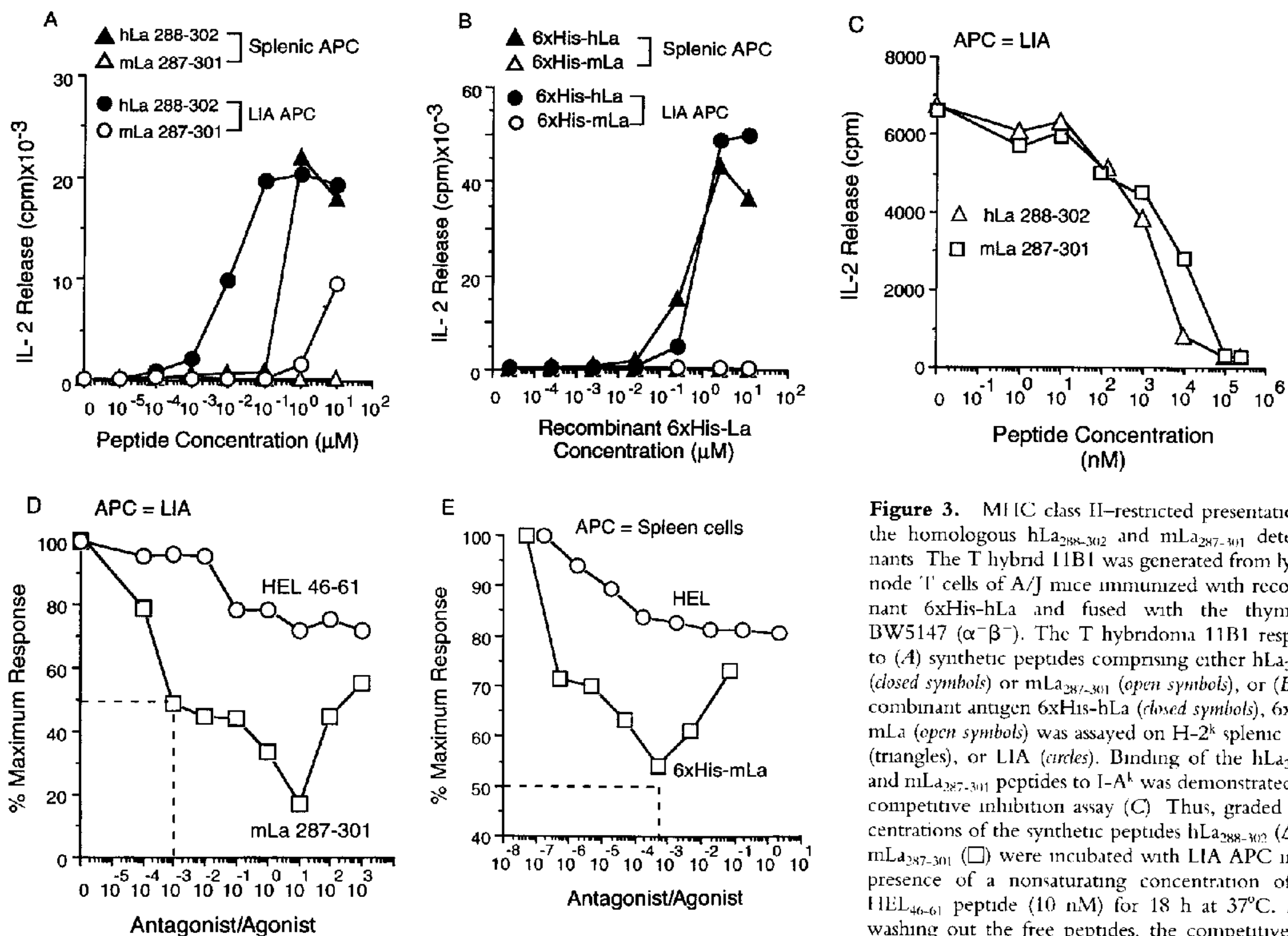

Figure 3. MHC class II–restricted presentation of the homologous $hLa_{288-302}$ and $mLa_{287-301}$ determinants The T hybrid 11B1 was generated from lymph node T cells of A/J mice immunized with recombinant 6xHis-hLa and fused with the thymoma, BW5147 ($\alpha^-\beta^-$). The T hybridoma 11B1 response to (*A*) synthetic peptides comprising either $hLa_{288-302}$ (*closed symbols*) or $mLa_{287-301}$ (*open symbols*), or (*B*) recombinant antigen 6xHis-hLa (*closed symbols*), 6xHis-mLa (*open symbols*) was assayed on H-2^k splenic APC (triangles), or LIA (*circles*). Binding of the $hLa_{288-302}$ and $mLa_{287-301}$ peptides to I-A^k was demonstrated in a competitive inhibition assay (*C*) Thus, graded concentrations of the synthetic peptides $hLa_{288-302}$ (△) or $mLa_{287-301}$ (□) were incubated with LIA APC in the presence of a nonsaturating concentration of the HEL_{46-61} peptide (10 nM) for 18 h at 37°C. After washing out the free peptides, the competitive displacement of HEL_{46-61} was assayed by measuring inhibition of IL-2 production by the I-A^k–restricted, HEL_{46-61}-specific T hybridoma 3A9. Each point represents the mean value of triplicate assays, and the experiment was repeated three times Functional presentation of $mLa_{287-301}$ was revealed by demonstrating specific antagonism of the 11B1 response to $hLa_{288-302}$ by the $mLa_{287-301}$ determinant after preloading APC with a nonsaturating amount of (*D*) synthetic $hLa_{288-302}$ peptide (100 nM) for 2 h before the addition of graded amounts of either $mLa_{287-301}$ (□) or the irrelevant I-A^k–binding peptide HEL_{46-61} (○). (*E*) APC were preloaded with a fixed, nonsaturating amount of 6xHis-hLa protein (4 µM) for 2 h before the addition of graded amounts of 6xHis-mLa protein (□) or control protein HEL (○) APC were then incubated for another 2 h and subsequent activation of 11B1 was measured as IL-2 production The maximum responses (100%) of 11B1 to the fixed amount of either $hLa_{288-302}$ (added to LIA APC) or 6xHis-hLa protein (added to splenic APC) were 5,500 and 6,200 cpm, respectively Each point represents the mean value of triplicate assays, and the entire assay was repeated three times. The dashed lines in *D* and *E* represent the estimated IC_{50} values for $mLa_{287-301}$ and 6xHis-mLa in their antagonism of the 11B1 T hybridoma response to the $hLa_{288-302}$ determinant

was the generally low level of T cell stimulation (SI = 2–6) and the failure to stimulate T cells in every experiment, consistent with the determinants being either subdominant or facultatively cryptic (35).

In addition to these xenoresponses, significant T cell reactivity was identified towards three hLa peptide determinants containing identical amino acid sequences to the homologous peptide regions of mLa. These "autoepitopes" corresponded to peptides in common with hLa and mLa at amino acid residues 13–30 (reactivity in six out of eight experiments), 25–44 (reactivity in three out of eight experiments), and 106–129 (reactivity in four out of eight experiments). In the experiment shown in Fig. 2, the La_{25-44} determinant did not stimulate hLa-primed T cells. Indeed, each autodeterminant behaved as cryptic or subdominant epitopes in that they produced only low levels of T cell proliferation, and in some experiments, they were nonstimulatory. Taken together, these findings confirmed the suspicion that hLa and mLa protein contained shared, notionally autologous T cell epitopes in addition to xenogeneic hLa determinants recognized by the murine immune system.

Efficient I-A^k-restricted Presentation of the $hLa_{288-302}$ and $mLa_{287-301}$ Determinants. The dominant hLa determinant $hLa_{288-302}$ contained a single species polymorphism within the core residues $La_{289-299}$ when compared with the homologous sequence $La_{288-298}$ present in mLa (note that mLa contains a single amino acid deletion at the position corresponding to hLa amino acid 217; see Table 1). This observation suggested that the immunodominance of $hLa_{288-302}$ depended on its xenogeneic sequence, and that the equivalent determinant in mLa might be tolerogenic in normal mice. Therefore, we examined the clonal specificity of T cells responding to the dominant $hLa_{288-302}$ determinant to eval-

uate whether these T cells cross-reacted with the equivalent mLa peptide (non self-tolerant) or whether they were hLa specific (putatively self-tolerant). After immunization with hLa protein, CD4-positive T cell hybrid clones were generated from the responding lymph node cells and analyzed for their peptide specificity. One such hybridoma clone (11B1) gave a strong in vitro response to the $hLa_{288-302}$ peptide, and the pattern of reactivity with four overlapping peptides spanning this region identified the minimal epitope as $hLa_{289-299}$ (data not shown). This finding verified the presence of mouse T cells recognizing $hLa_{288-302}$ in the bulk T cell responses shown in Fig. 2.

Notably, 11B1 did not respond to the autologous $mLa_{287-301}$ (or $mLa_{288-298}$) peptide, except at high peptide concentrations using the $I-A^k$–transfected L cell APC, (LIA; Fig. 3 *A* and data not shown). The T hybridoma 11B1 also secreted IL-2 in response to intact hLa, but not to mLa protein (Fig. 3 *B*). The species specificity of 11B1 for $hLa_{288-302}$ was observed with splenic APC and LIA (Fig. 3, *A* and *B*). The nonresponsiveness of 11B1 to $mLa_{287-301}$ was not the result of defective presentation or crypticity of $mLa_{287-301}$ for several reasons. First, $mLa_{287-301}$ was able to bind $I-A^k$ with equivalent efficiency to $hLa_{288-302}$ based on the comparable ability of these two peptides to compete with the $I-A^k$–restricted recognition of a HEL_{46-61} peptide (Fig. 3 *C*). Second, the $mLa_{287-301}$ peptide behaved as an altered peptide ligand (36, 37) for the T hybridoma 11B1. Altered peptide ligands characteristically contain a single amino acid substitution from the parent peptide and can block T cell responses even when present in concentrations too low to be explained by competitive displacement of the parent (agonist) peptide (36, 37). In the experiment shown in Fig. 3 *D*, APC were preloaded with a fixed concentration of $hLa_{288-302}$ (100 nM), washed free of agonist peptide, and then incubated with increasing concentrations of $mLa_{287-301}$ before assaying antigen presentation to 11B1. Specific antagonism of the 11B1 response occurred at a 50% inhibitory antagonist ($mLa_{287-301}$)/agonist ($hLa_{288-302}$) ratio of $\sim 10^{-3}$ (Fig. 3 *D*). When the $I-A^k$–restricted determinant HEL_{46-61} was titered into the Ag presentation assay under the same conditions, there was no evidence of specific T hybridoma antagonism, and only modest competitive inhibition of the 11B1 response was observed at a higher concentration of HEL_{46-61} (Fig. 3 *D*). Antagonism of the 11B1 response was also observed using the minimal determinants $mLa_{288-298}$ and $hLa_{289-299}$ as antagonist and agonist peptides (data not shown), indicating that the 11B1 response was dependent on position 294L→295Q (mLa→hLa). This observation confirms that the antagonist properties of $mLa_{288-298}$ arise from this single amino acid substitution, creating an altered peptide ligand for the hybridoma 11B1. At high concentrations of $mLa_{287-301}$, some agonist activity was observed when using the L cell transfectants (Fig. 3 *A*), which express higher levels of $I-A^k$ than splenic APC (data not shown).

The 11B1 response to intact hLa was also antagonized in the presence of very low ratios of intact mLa/hLa antigen (50% inhibitory antagonist/agonist ratio of $0.5–1 \times 10^{-3}$), indicating efficient processing of the La autoantigen resulting in presentation of peptides containing the $mLa_{288-298}$ determinant (Fig. 3 *E*). Under the same conditions where APC were prepulsed with a fixed stimulatory amount of intact 6xHis-hLa, titration of increasing amounts of HEL into the Ag presentation assay resulted in only modest competitive inhibition of the 11B1 response (Fig. 3 *E*). Notably, HEL contains mutiple $I-A^k$–restricted determinants that are capable of competitive displacement of $hLa_{288-302}$ (38) emphasizing the potent antagonist properties of the naturally presented $mLa_{288-298}$ determinant derived from mLa protein.

Cellular mLa is probably not presented through an endogenous pathway because its nuclear localization and low abundance render it inaccessible to the endosomal class II antigen presentation pathway (39, 40). Moreover, a human cell line expressing $I-A^k$ did not constitutively present the

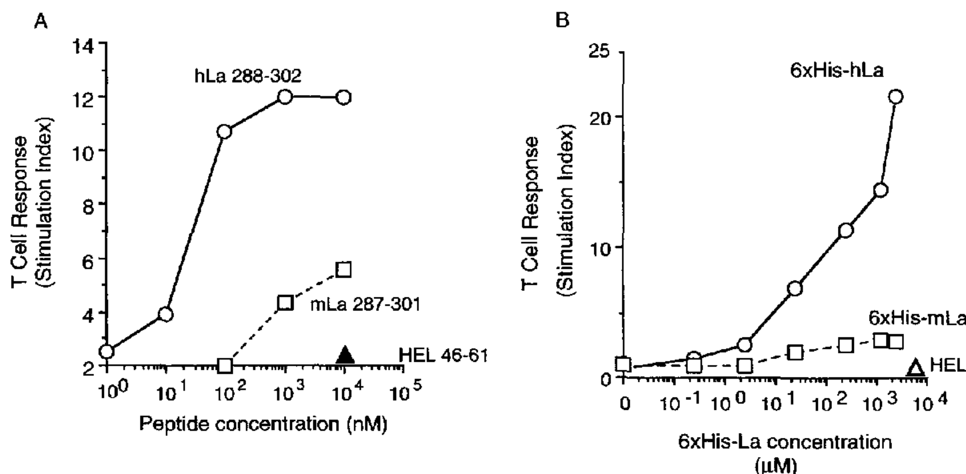

Figure 4. Immune tolerance to the $mLa_{287-301}$ determinant in normal mice. A/J mice (12 per group) were immunized subcutaneously in the hind footpad and at the tailbase with 20 µM of $hLa_{288-302}$ (○) or $mLa_{287-301}$ peptide (□) in CFA. The proliferation of T-enriched LN cells from hLa- or mLa-primed A/J mice was assayed 9 d later by coculture with irradiated syngeneic spleen cells (2,000 rads) in the presence of graded amounts of either (*A*) $hLa_{288-302}$ peptide (○) or $mLa_{287-301}$ peptide (□), or (*B*) recombinant 6xHis-hLa antigen (○) or recombinant 6xHis-mLa antigen (□). There was no T cell reactivity to HEL_{46-61} peptide (▲) or HEL protein (△; SI = 1). The SI shown represents the mean value of triplicate assays. The whole experiment was carried out twice.

endogenous $hLa_{288-302}$ determinant, but could present exogenous native and recombinant hLa antigen (data not shown). These observations are likely to explain why 11B1 responses to hLa are not constitutively antagonized by endogenous mLa antigen present in murine APC.

As in the case of $mLa_{287-301}$ peptide (Fig. 3, *A* and *D*), at high concentrations the intact mLa antigen (Fig. 3 *E*) showed some agonist activity by paradoxically stimulating the 11B1 hybridoma incubated with APC preloaded with a fixed amount of $hLa_{288-302}$ peptide or hLa protein antigen. Agonism induced by high concentrations of antagonist determinants is acknowledged in many systems where altered peptide ligands are recognized by the responding T cell (36, 37). Taken together, these data confirmed that $mLa_{287-301}$ was processed and presented by APC loaded with intact mLa antigen, and was readily recognized by specific T cells, even at low concentrations of exogenous antigen, consistent with this peptide being an immunodominant mLa determinant.

T Cell Tolerance to the Dominant $mLa_{287-301}$ Determinant in Normal Mice. The presentation of the $mLa_{287-301}$ determinant after antigen processing and the immunodominance of the homologous $hLa_{288-302}$ determinant suggested that the $mLa_{287-301}$ region might be highly tolerogenic in normal mice. To test whether this was the case, groups of normal H-2^a mice (I-A^k) were immunized with either $hLa_{288-302}$ or $mLa_{287-301}$ in Freund's adjuvant, and draining lymph node T cells were tested for proliferative reactivity 9 d later. T cells from mice primed with the $hLa_{288-302}$ determinant responded vigorously to this peptide (50% max response at ~20 nM; Fig. 4 *A*), and the response was recalled on the intact hLa antigen (Fig. 4 *B*). In contrast, T cells from mice primed with $mLa_{287-301}$ proliferated poorly to the $mLa_{287-301}$ peptide, even at high concentrations (50% max response at ~1 mM; Fig. 4 *A*), and failed to respond at all to the intact mLa antigen (Fig. 4 *B*).

As shown in Fig. 3, the lack of T cell reactivity to $mLa_{287-301}$ was not caused by a failure to bind I-A^k or by a lack of presentation of this determinant from intact antigen. However, it was possible that the $mLa_{287-301}$ peptide might have induced autoreactive T helper cells that proliferated poorly despite providing functional cytokine support for specific autoantibody responses. Therefore, we also tested the sera of animals immunized with $mLa_{287-301}$ peptide for the development of anti-La antibodies. Neither the $mLa_{287-301}$ nor the $hLa_{288-302}$ peptides provoked an autoantibody response under conditions where immunization with intact 6xHis-mLa induced a significant anti-La response (Fig. 5 *A*). Notably, immunization with $hLa_{288-302}$ induces a vigorous proliferative T cell response (Fig. 4, *A* and *B*) without inducing autoreactivity to endogenous murine La (Figs. 3 and 5 *A*), again reflecting the lack of functional T cell cross-reactivity between $mLa_{287-301}$ and $hLa_{288-302}$ in the murine T cell responses. Presumably, the lack of autoantibody production despite efficient T immunity to $hLa_{288-302}$ reflects the absence of B epitopes in this peptide and the failure of reactive T helper cells to recognize the mouse homologue of this determinant. Therefore, we concluded that the T cell repertoire in normal mice was specifically tolerant of the $mLa_{287-301}$ determinant, presumably because of its efficient presentation to the T cell compartment relative to other determinants. Hence, we inferred that the induction of autoimmunity to mouse La protein (Fig. 5 *A* and reference 6) was likely to depend on T helper cell responses that recognize nontolerogenic subdominant mLa determinants such as the mLa_{13-30}, mLa_{25-44}, and $mLa_{106-129}$ peptides rather than immunodominant determinants within mLa.

Induction of T Cell Immunity and a Diversified Anti–La/Ro RNP Autoantibody Response by a Nontolerogenic Subdominant Determinant of mLa. To test whether the subdominant La determinants La_{13-30}, La_{25-44} and $La_{106-129}$ could initiate anti-La autoantibodies, we immunized groups of normal mice with one or other of these peptides in Freund's adjuvant and boosted the response two to three times during a 4–5 wk period. Sera were then tested by ELISA for the presence of autoantibodies recognizing recombinant La protein. There was no detectable anti-La response after immunization with the $mLa_{106-129}$ determinant; however, both the mLa_{25-44} and mLa_{13-30} peptides induced a significant anti-La autoantibody response in the majority of immunized animals (Fig. 5 *B*). The pattern of responses to the three subdominant peptides suggested a hierarchy of autoimmune potential among self-peptides (Fig. 5 B), perhaps reflecting their intrinsic tolerogenicity or the nature of T cell responses associated with individual peptides.

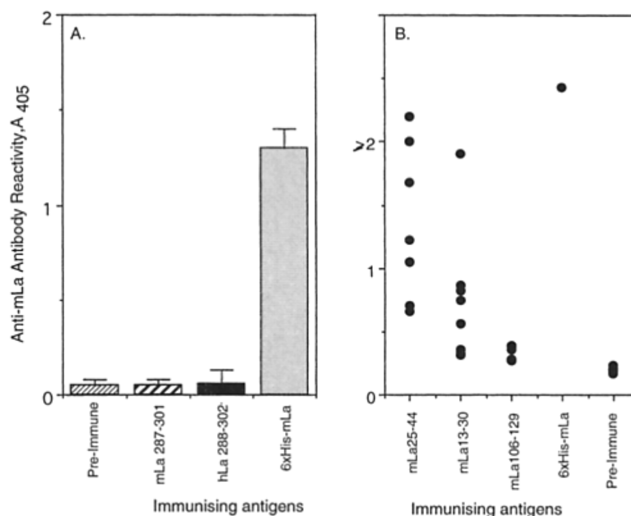

Figure 5. Hierarchical immunogenicity of murine La determinants in normal mice. Groups of six to eight A/J mice were immunized with (*A*) 20 μM of either $mLa_{287-301}$ peptide (▨), $hLa_{288-302}$ peptide (■), or 100 μg of 6xHis-mLa protein antigen (□), or (*B*) mLa_{25-44}, mLa_{13-30}, $mLa_{106-129}$, or 6xHis-mLa in CFA, and then boosted twice at 10-d intervals with 10 μM of the corresponding peptide or 50 μg of protein antigen in IFA. 3–4 d after the last boost, individual sera were pooled and tested at 1/100 (*A*), or were tested individually at 1/500 (*B*) for anti-mLa antibody reactivity by ELISA using 6xHis-mLa–coated microtiter plates. The reactivity of preimmune serum also is shown. The value shown for the 6xHis-mLa track in (*B*) is derived from a 1/500 dilution of pooled sera from six immune mice different to those described above in *A*

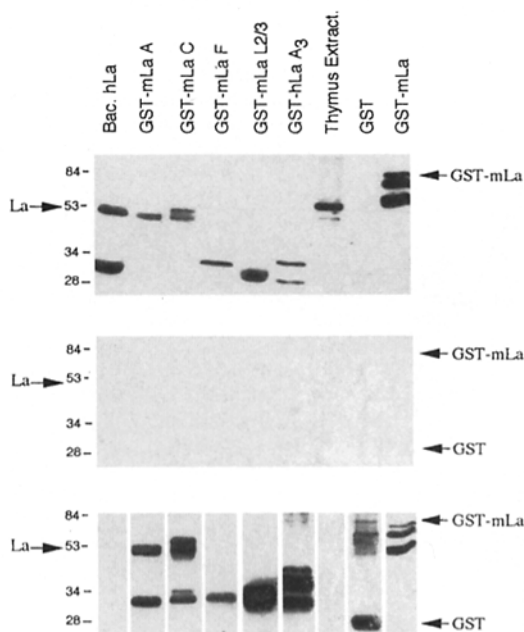

Figure 6. Initiation and intramolecular diversification of anti-La autoimmunity after immunization with the subdominant mLa_{13-30} peptide. Pooled sera (diluted 1/500) from A/J mice immunized and boosted twice with either mLa_{13-30} (*upper panel*) or $mLa_{287-301}$ peptides (*middle panel*) were used to immunoblot baculovirus 6xHis-hLa (*Bac hLa*), the GST-mLa subfragments; GST-LaA (aa 1–107 predicted mol mass ~41 kD); GST-LaC (aa 111–242 predicted mol mass ~43 kD); GST-LaF (aa 243–345 predicted mol mass ~33 kD), GST-LaL2/3 (aa 346–416 predicted mol mass ~31-33 kD; 19, 20), GST-hLaA$_3$ (aa 46–107 predicted mol mass ~30-33 kD) (41); rabbit thymus extract (containing several nuclear and cytoplasmic antigens, i.e., Sm antigens and La, Pel-Freez Biologicals, Rogers, AR); GST protein (predicted mol mass ~26 kD); and recombinant GST-mLa (predicted mol wt ~74 kD; 20) separated on a 12.5% SDS-PAGE before electrotransfer. The same proteins were immunoblotted with a rabbit anti-GST antiserum at 1:3,000 (*lower panel*). Bound antibodies were detected by ECL (Amersham) using goat anti-mIgG (Sigma) as a second antibody. Molecular masses are shown in kilodaltons. Bands corresponding to lower molecular weight degradation products are seen in some lanes.

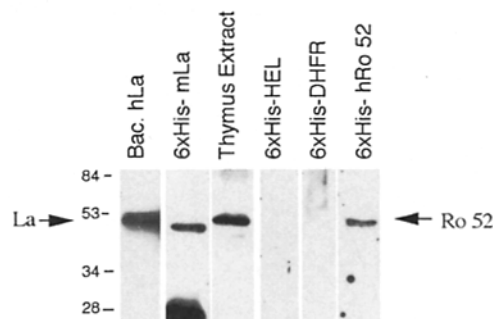

Figure 7. Intermolecular diversification of the autoantibody response to involve Ro autoantigens after immunization with the subdominant determinant mLa_{13-30}. Pooled immune serum (diluted 1/250) from A/J mice immunized with mLa_{13-30} peptide was reacted with baculovirus 6xHis-hLa (*Bac hLa*), 6xHis-mLa, rabbit thymus extract, 6xHis-HEL, 6xHis-DHFR, and 6xHis-hRo52 (Amrad, Melbourne, Australia), separated by SDS-PAGE and electrotransferred to nitrocellulose membrane. Bound antibodies were detected by ECL (Amersham) using goat anti-mIgG (Sigma) as second antibody. Molecular masses are shown in kilodaltons.

Intra- and intermolecular spreading of autoimmunity towards the different components of autologous subcellular particles has previously been described when immunity to a single component of the particle is triggered experimentally (1, 4, 6). To determine whether similar autoantibody spreading could be initiated by immunity to a single subdominant La peptide, we further examined the specificity of the autoantibody response to mLa_{13-30} and mLa_{25-44} by Western blot analysis. In A/J mice immunized with mLa_{25-44}, the autoantibody response was restricted to the La A subfragment containing the mLa_{25-44} peptide of La autoantigen (data not shown). By contrast, pooled sera from mice immunized and boosted with mLa_{13-30} specifically reacted with recombinant and mammalian sources of intact La antigen in immunoblots (Fig. 6, *upper panel*). Moreover, immune sera reacted with multiple regions of the mLa polypeptide, as shown by immunoblots of La subfragments spanning four nonoverlapping regions of the mLa molecule, LaA (aa 1–107), LaC (aa 111–243), LaF (aa 244–345), and LaL2/3 (aa 346–416; Fig. 6). In some lanes (e.g., *Bac.hLa*, Fig. 6, lane 1), immune mouse sera (*upper panel*) and hyperimmune rabbit anti-GST serum (Fig. 6, *lower panel*) reacted with multiple bands representing proteolytic degradation products of the recombinant antigens. Absorption of pooled immune sera with one subfragment of La (e.g., the LaC) specifically removed immunoblot reactivity to that fragment without affecting binding to the other subfragments (data not shown). By contrast, absorption with recombinant GST protein had no significant effect on the reactivity of the pooled serum with La subfragments (data not shown). This finding confirms that reactivity of the immune sera with different regions of the La polypeptide was not caused by cross-reactive antibodies. In addition, immunoblot reactivity of the immune sera was not observed with the control proteins 6xHis-DHFR, 6xHis-HEL, or with other nuclear proteins such as Sm polypeptides known to be present in the rabbit thymus extract (Figs. 6 and 7). A component of the anti-La response that develops after immunization with mLa_{13-30} is probably directed towards the La_{13-30} peptide itself since this peptide absorbs some of the antibodies directed towards the LaA subfragment that contains these residues (data not shown).

To determine whether intermolecular spreading of autoimmunity also follows immunization with mLa_{13-30}, pooled immune serum was examined for immunoblot reactivity with the Ro (SS-A) autoantigens (Fig. 7). The Ro 60-kD antigen is known to be associated with the La polypeptide in a snRNP particle (42), and autoantibodies to both Ro 60-kD and Ro 52-kD antigens are strongly associated with anti-La antibodies in systemic autoimmunity (43). Pooled sera from mice immunized with the La_{13-30} peptide reacted specifically with recombinant Ro 52-kD antigen (Fig. 7);

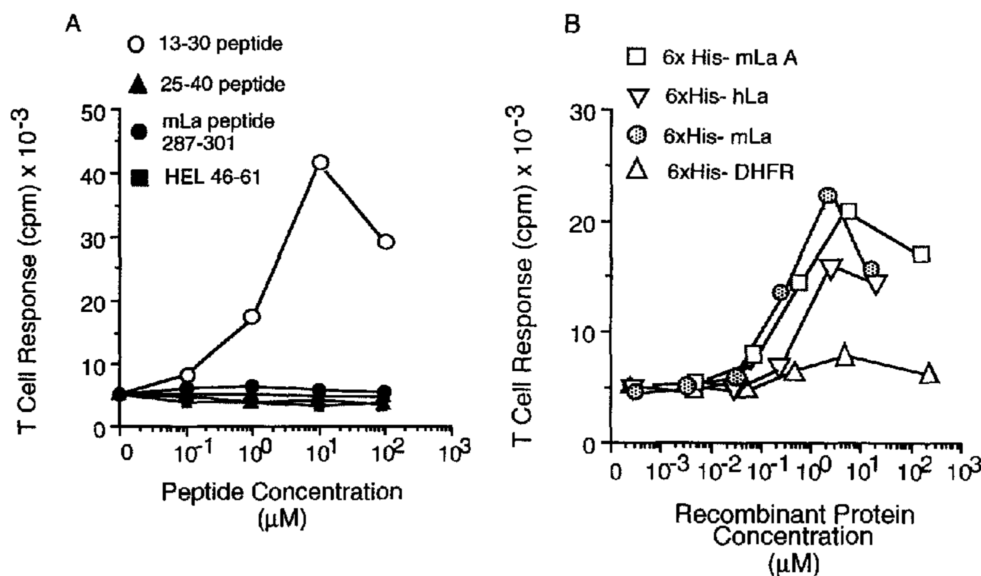

Figure 8. Immunization with the subdominant peptide mLa_{13-30} induces a proliferative T cell response that is recalled on intact mLa and hLa antigens. CBA mice were immunized in the hind footpad and subcutaneously at the tailbase with 20 μM mLa_{13-30} peptide in CFA. 9 d later, T-enriched draining LN cells were tested for their specificity by assaying the proliferative response to irradiated syngeneic spleen cells (2,000 rad) loaded either with (*A*) the peptides mLa_{13-30}, mLa_{25-44}, $mLa_{287-301}$, or HEL_{46-61}, or with (*B*) recombinant 6xHis-mLa, 6xHis-hLa, 6xHis-mLaA (1–107 aa), or recombinant 6xHis-DHFR as control antigens. Each point represents the mean value of triplicate assays.

however, reactivity with recombinant Ro 60-kD antigen was equivocal and not observed in all experiments (data not shown). The titer of anti–Ro 52-kD antibodies was significantly lower than that of the anti-La response (1/500 for anti–Ro 52 and ≤1/3,000 for anti-La antibodies). Nonetheless, this reactivity was specific, since binding to control recombinant proteins (6xHis-DHFR and 6xHis-HEL) was not observed under the same conditions, (Fig. 7). We conclude that immunization with the subdominant mLa_{13-30} peptide is sufficient to induce intramolecular spreading of autoimmunity to La autoantigen, as well as a low level of intermolecular antibody spreading of the autoimmune response.

The Autoantibody Response to the mLa_{13-30} Determinant Is Associated with an Autoreactive T Cell Response. Triggering of autoimmunity by immunization with subdominant peptides such as the mLa_{13-30} peptide was presumed to occur through the activation of nontolerized T helper cells that recognize this determinant. Therefore, we evaluated whether immunization with mLa_{13-30} peptide induced specific autoreactive T cells. CBA/CaH mice were immunized with mLa_{13-30} peptide in adjuvant, and draining lymph node T cells were examined for antigen reactivity 9 d later. Immune T cells proliferated specifically in response to mLa_{13-30}, but not control peptides mLa_{25-44}, $mLa_{287-301}$, or HEL_{46-61} (Fig. 8 *A*). These T cell responses were recalled in the presence of recombinant intact mLa, hLa, and mLaA subfragment (residues 1–107) but not the control protein 6xHis-DHFR (Fig. 8 *B*). Similar results were obtained after immunization of A/J mice (data not shown). Hence, the spreading autoimmunity that follows immunization of normal $H-2^k$ mice with the subdominant mLa_{13-30} peptide is associated with specific T helper responses recalled on intact mLa antigen.

Discussion

Although La is ubiquitously expressed in the nucleus and cytoplasm of diverse cell types, there is little data about the extent of immune tolerance that develops to this or many other intracellular sequestered self-antigens in normal individuals. Indeed, establishing whether the immune system is actively tolerant to a given determinant within a self-antigen is experimentally difficult because of the many ways in which nonresponsiveness may occur and the need to examine the host immune response in the presence and absence of expression of the self antigen. In the experiments reported here, we have immunized mice with a surrogate self-antigen, viz., the human La antigen that is 77% identical to mouse La (30). This approach has allowed identification of an immunodominant determinant of human La ($hLa_{288-302}$) and a homologous but tolerogenic epitope derived from endogenous mLa ($mLa_{287-301}$). The evidence that this determinant is tolerogenic in mice is based on the lack of either T cell responses or autoantibodies after immunization of normal mice with the $mLa_{287-301}$ peptide. The mLa determinant was efficiently presented after antigen presentation, as demonstrated by inhibition binding studies to $I-A^k$ and specific antagonism of the immunodominant $hLa_{288-302}$ homologue by both intact mLa and the $mLa_{287-301}$ peptide that possessed properties of an altered peptide ligand for $hLa_{288-302}$ recognition. Sercarz has cautioned that despite antigen presentation and immunodominance of a particular determinant in one species, comparable immunodominance cannot be assumed for homologous determinants across species (15). Nonetheless, in this case, the $hLa_{288-302}$ and $mLa_{287-301}$ determinants do appear to be equivalent in their antigen presentation and behave as immunodominant peptides in $H-2^k$ and $H-2^a$ mice.

The tolerogenicity of $mLa_{287-301}$ indicates that endogenous La polypeptides are indeed processed and presented to host T cells, presumably by an exogenous pathway involving uptake and catabolism of La/Ro RNP particles. This putative turnover and antigen presentation of endogenous RNPs clearly does not lead to autoimmunity under normal conditions. However, once autoreactive T cells have been primed towards poorly tolerogenic determinants within the La/Ro RNP, antigen presentation after the natural uptake of these RNPs appears to be potentially autoimmunogenic. In our experimental model, the poorly tolerogenic determinants are presumably subdominant or might behave as facultatively cryptic regions of the La molecule that are only presented under special circumstances. Regardless, humoral immunity to mLa is easily triggered experimentally and is associated with T cell responses to subdominant determinants. Although our assays have measured proliferative T cell responses, these responses are associated with functional T helper cells revealed by the isotype switching of associated autoantibody responses.

The autoreactive T cell response induced by the mLa_{13-30} subdominant peptide was recalled on intact La antigen, even though T cell responses to this and other subdominant mLa peptides were not evident in every experiment involving immunization with intact La protein. This pattern of autoreactivity is distinct from the autoimmune T cell responses that recognize cryptic peptides of the snRNP D protein described by Bockenstedt et al. (4). In that study, priming of T cells with peptides derived from snRNPs resulted in only a limited autoantibody production, and responding T cells were not stimulated by APC pulsed with intact snRNPs in vitro. Coimmunization and boosting of normal mice with a mixture of native snRNPs and cryptic peptides, however, induced spreading of the anti-snRNP antibody response. The diversification of the autoimmune response towards snRNPs (4) presumably occurs through selective uptake of the exogenous snRNPs by different autoreactive B cells that then present processed snRNP antigen to T cells that recognize newly revealed cryptic peptides. In the experimental autoimmunity we have described here, the diversification of the autoimmune response occurred without the need for any challenge with exogenous intact La/Ro RNPs suggesting that the autoimmunization driving the spreading of the response was caused by endogenous turnover of intracellular RNPs.

We propose that under normal conditions, subdominant self-determinants are continuously being presented to T cells by APC, including self-reactive B cells (44), but this presentation is qualitatively (45) and quantitatively (46) insufficient to prime naive self-reactive T cells. In particular, the necessary costimulatory signals are likely to be absent under conditions of constitutive antigen presentation, and the density of subdominant determinants presented on the surface of the APC probably falls below the threshold for activation of naive T cells.

It seems likely that experimentally induced autoimmunity to the La/Ro RNP occurs because immunization overcomes the priming threshold by using larger amounts of antigen than is normally encountered in vivo and by using adjuvants to activate APC and induce the qualitative costimulatory APC functions that are necessary for T cell priming (45, 47–52). In other words, experimental induction of autoimmunity exposes the extent of immune self-ignorance.

Experimental autoimmunity to autologous recombinant La (6) and Ro 52-kD antigen (53) can be induced by immunization with recombinant autologous La protein (6), whereas immunization with autologous native snRNPs (1, 4), cytochrome *c* (17, 18, 54), histone preparations (6, 55), nDNA (56), and P53 antigen (57) all fail to induce high titer autoantibody responses in experimental animals. These differences in the immunogenicity of intracellular self-antigens might reflect degrees of host immune tolerance based on antigen abundance, turnover, adjuvanticity, intracellular trafficking, or structural complexity affecting antigen processing. Alternatively, the use of recombinant intact La antigen may facilitate its processing compared with native La antigen. Notably, the recombinant La was highly soluble and retained the capacity to bind poly U RNA (30), ATP, patient autoantibodies, and mouse anti-La mAbs, suggesting that some native structure is maintained by the bacterial La antigen. Nonetheless, it remains possible that challenge with native La/Ro RNP antigens may result in a hierarchy of peptide determinants different from that observed with recombinant proteins. However, this does not alter the evidence that immunization with peptide determinants nominally defined as subdominant resulted in a natural spreading of autoimmunity to involve multiple components of the La/Ro RNP.

We propose that the initial diversification of the autoimmune response is driven by selective constitutive uptake of endogenous "native" La/Ro RNP complexes by autoreactive B cells expressing specific mIg (44) leading to presentation of the same subdominant determinants after antigen processing. Thus, T cells specific for one subdominant determinant might provide helper signals to distinct autoreactive B cells with differing specificities within the RNP complex. It is also possible that once the autoimmune response is initiated, natural presentation of multiple subdominant (or cryptic) determinants will lead to spreading of the T cell response (7). Spreading of T cell autoimmunity may depend on priming by specific autoreactive B cells (54), but could also involve enhanced antigen presentation through FcR uptake of autoantibody–autoantigen complexes. The relative contribution of these potential mechanisms in explaining diversification of the autoimmune response needs clarification. If systemic autoimmunity involving ubiquitous self-antigens is initiated by T cell responses to one or two subdominant self peptides, then selective antigen presentation might explain HLA class II allele association with certain antinuclear autoantibodies (58). Maintenance of autoimmunity may require chronic exposure to an antigenic trigger as well as a genetic predisposition affecting functions such as MHC restriction of non-

tolerogenic determinants (59), defective immune clearance, or exaggerated cytokine production (60). Notwithstanding these considerations, the mechanism by which particular sets of nuclear autoantigens are selected in different systemic autoimmune disorders remains mysterious. Our observation here, which has shown that complex patterns of autoantibody production can be triggered by immunity to a single peptide determinant, suggests how limited molecular mimicry by exogenous agents might initiate spreading of the autoimmune response (61).

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