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

By Pakathip Reynolds,* Tom P. Gordon,* Anthony W. Purcell,* David C. Jackson,† and James McCluskey*

From the *Centre for Transfusion Medicine & Immunology, Flinders Medical Centre, Bedford Park, South Australia 5042, Australia; and †Department of Microbiology, University of Melbourne, Royal Parade, Parkville, Victoria 3052, Australia

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-2k and H-2a 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 mLa28v 301 determinant, which differed by a single residue in its core sequence from the homologous but highly immunogenic human La288-302 determinant. Interestingly, the mLa287_301 peptide acted as an altered peptide ligand that specifically antagonized the activation of an hLa288_302-specific T cell hybridoma. In contrast to the tolerogenic mLa287_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 La25_44 and La106_120 determinants resulted in limited or no detectable autoantibody response. In contrast, immunization with the subdominant mouse La13_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) 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.

Abbreviations used in this paper: aa, amino acid; GST, glutathione S-transferase; HEL, hen egg lysozyme; hLa, human La antigen; LIA, I-Ak-transfected L cells; mLa, mouse La antigen; PPD, purified protein derivative; SI, stimulation index; SnRNP, small nuclear ribonucleoproteins.
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 tolerant 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 autoimmune body 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. Neel Cook (Ehas, 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 protected 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 multiplex synthesis system (Chiron Miniotopes, 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 ionization 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–2I) and CBA/CaH (H–2b) 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 (Difeo 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 × 10^6 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 [3H]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 T helper-restricted, hLa-specific T hybridoma 11B1 was generated by immunizing A/J mice with 6xHis-hLa (100 µg) emulsified in CFA and fusng the activated T blasts (32) from the draining lymph node cells to the TCR-negative, HGPR.T-negative AKR, thymoma line BW5147 (32). 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 × 10^5 cells were fused with BW5147 (10^4 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 spleen feeder cells (2 × 10^6 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^{-5} M amphotericin) 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 HAT (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 × 10^6/well) or A/J-transfected L cells (LIA; 5 × 10^5/well) were cocultured for 24 h in flat-bottom 96-well plates (Greiner Laborzotechnik, 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 × 10^5/ml, 200 µl/well, final). Quantitation of IL-2 production was measured by [3H]thymidine incorporation (0.5 µCi/well) in triplicate samples.

T Cell Antigenicity Assays. For assays of T cell antigenicity, LIA (10^5/well) or spleen cells from A/J mice (2 × 10^5/well) were incubated with a nonsaturating concentration of hLa_20-30 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 mLae_20-30 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 [3H]thymidine incorporation of CTLL.

Western Blotting. Recombinant antigens and control proteins were separated through 12.3% 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 phosphate–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 Antigens 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-
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 X Q)
Table 1. H-2- restricted T Cell Determinants within hLa

| hLa peptide sequence and homologous mLa sequence* | Nature of determinant† | Predicted amino acid residues | Positive‡ experiments |
|--------------------------------------------------|------------------------|------------------------------|----------------------|
| LEAKICHQIEYYFGDFNL                               | AUTO                   | 13-30                        | 6/8                  |
| FGDFNLPRDKFLKEQILDE                               | AUTO                   | 25-44                        | 3/8                  |
| DEGWVPLEIMIKFNRLLTRLDFN                           | XENO                   | 43-66                        | 3/8                  |
| LTTDFNVIVEALSKSAELMEISE                          | XENO                   | 61-84                        | 6/8                  |
| NDVMEKSVYIKGEPITDAFLDDEKE                        | AUTO                   | 106-129                      | 4/8                  |
| ROKVACEKLRAQQEAMQKLLE                            | XENO                   | 196-219                      | 1/8                  |
| TCREDLHIFFSHGEIKWDFVGR                           | XENO                   | 244-267                      | 1/8                  |
| DANNIGNLQRNKWVT                                  | XENO                   | 288-302                      | 8/8                  |
| SGKGVQFQKXPFASDEH                                | XENO                   | 350-370                      | 1/2                  |
| RKKRCGKGNRPGYAGAPKGR                              |                       |                              |                      |
| TKFASDEDEHDEHEATGFPV                             | XENO                   | 362-382                      | 1/2                  |
| GYAGAPKGRQFQHGRRTFDD                              |                       |                              |                      |

*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 hLa13-3, hLa106-129 and hLaXeno_30a peptides were defined as immunodominant, based on the magnitude (SI generally $\geq 6$) and frequency of their reactivity. Although hLa13-3 was reactive in six to eight experiments, the magnitude of these responses was consistently marginal (SI $\sim 2$), so this determinant was defined as subdominant.

‡Positive proliferative T cell responses are defined as an SI $\geq 2$ present in two or more adjacent pairs of peptides and occurring in one or more experiment.

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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 mLα. These “autoepitopes” corresponded to peptides in common with hLa and mLα 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 mLα235-44 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 non-stimulatory. Taken together, these findings confirmed the suspicion that hLa and mLα protein contained shared, notionally autologous T cell epitopes in addition to xenogeneic hLa determinants recognized by the murine immune system.

Efficient I-A\(^{b}\)-restricted Presentation of the hLa288,302 and mLα287,301 Determinants. The dominant hLa determinant hLa288,302 contained a single species polymorphism within the core residues Lα289-295 when compared with the homologous sequence Lα288-295 present in mLα (note that mLα 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 hLa288,302 depended on its xenogeneic sequence, and that the equivalent determinant in mLα might be tolerogenic in normal mice. Therefore, we examined the clonal specificity of T cells responding to the dominant hLa288,302 determinant to eval-

**Figure 3.** MHC class II-restricted presentation of the homologous hLa288,302 and mLα287,301 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 (or β°C). The T hybridoma 11B1 response to (A) synthetic peptides comprising either hLa288,302 (closed symbol) or mLα287,301 (open symbol), or (B) recombinant antigen 6xHis-hLa (closed symbol), 6xHis-mLα (open symbol) was assessed on H-2\(^{k}\) spleenic APC (trangles), or LIA (squares). Binding of the hLa288,302 and mLα287,301 peptides to I-A\(^{k}\) was demonstrated in a competitive inhibition assay (C). Thus, graded concentrations of the synthetic peptides hLa288,302 (D) or mLα287,301 (E) were incubated with LIA APC in the presence of a nonsaturating concentration of the 6xHis-hLa peptide (10 nM) for 18 h at 37°C. After washing out the free peptides, the competitive displacement of HEL46,61 was assayed by measuring inhibition of IL-2 production by the I-A\(^{k}\)-restricted, HEL46,61-specific T hybridoma 3A9. Each point represents the mean value of triplicate assays, and the experiment was repeated three times.
ulate whether these T cells cross-reacted with the equivalent mLα 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 hLa288–302 peptide, and the pattern of reactivity with four overlapping peptides spanning this region identified the minimal epitope as hLa289–299 (data not shown). This finding verified the presence of mouse T cells recognizing hLa288–302 in the bulk T cell responses shown in Fig. 2.

Notably, 11B1 did not respond to the autologous mLα287–301 (or mLα288–298) peptide, except at high peptide concentrations using the I-Ak-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 mLα protein (Fig. 3 B). The species specificity of 11B1 for hLa288–302 was observed with splenic APC and LIA (Fig. 3 A and B). The nonresponsiveness of 11B1 to mLα287–301 was not the result of defective presentation or crypticity of mLα287–301 for several reasons. First, mLα287–301 was able to bind I-Ak with equivalent efficiency to hLa289–299 based on the comparable ability of these two peptides to compete with the I-Ak-restricted recognition of a HEL46–61 peptide (Fig. 3 C). Second, the mLα287–301 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 (agonistic) peptide (36, 37). In the experiment shown in Fig. 3 D, APC were preloaded with a fixed concentration of hLa288–302 (100 nM), washed free of agonist peptide, and then incubated with increasing concentrations of mLα287–301 before assaying antigen presentation to 11B1. Specific antagonism of the 11B1 response occurred at a 50% inhibitory antagonist (mLα287–301)/agonist (hLa288–302) ratio of ~10−5 (Fig. 3 D). When the I-Ak-restricted determinant HEL46–61 was titrated 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 HEL46–61 (Fig. 3 D). Antagonism of the 11B1 response was also observed using the minimal determinants mLα288–298 and hLa289–299 as antagonist and agonist peptides (data not shown), indicating that the 11B1 response was dependent on position 294L→295Q (mLα→hLa). This observation confirms that the antagonist properties of mLα289–298 arise from this single amino acid substitution, creating an altered peptide ligand for the hybridoma 11B1. At high concentrations of mLα287–301, some agonist activity was observed when using the L cell transfectants (Fig. 3 A), which express higher levels of I-Ak than splenic APC (data not shown).

The 11B1 response to intact hLa was also antagonized in the presence of very low ratios of intact mLα/hLa antigen (50% inhibitory antagonist/agonist ratio of 0.5–1 × 10−3), indicating efficient processing of the La autoantigen resulting in presentation of peptides containing the mLα288–298 determinant (Fig. 3 D). Under the same conditions where APC were preincubated 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 multiple I-Ak-restricted determinants that are capable of competitive displacement of hLa288–302 (38) emphasizing the potent antagonist properties of the naturally presented mLα288–298 determinant derived from mLα protein.

Cellular mLα 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-Ak did not constitutively present the

Figure 4. Immune tolerance to the mLα287–301 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 hLa288–302 (O) or mLα287–301 peptide (□) in CFA. The proliferation of T-enriched LN cells from hLa- or mLα-prime A/J mice was assayed 9 d later by coculture with irradiated syngeneic spleen cells (2,000 rad) in the presence of graded amounts of either (A) hLa288–302 peptide (O) or mLα287–301 peptide (□), or (B) recombinant 6xHis-hLa antigen (□) or recombinant 6xHis-mLα antigen (□). There was no T cell reactivity to HEL46–61 peptide (△) or HEL protein (○; SI = 1). The SI shown represents the mean value of triplicate assays. The whole experiment was carried out twice.
endogenous hLa288-302 determinant, but could present exogenous native and recombinant La 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 mLa287-301 peptide (Fig. 3, A and D), at high concentrations the intact mLa antigen (Fig. 3 B) showed some agonist activity by paradoxically stimulating the 11B1 hybridoma incubated with APC preloaded with a fixed amount of hLa288-302 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 mLa287-301 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 mLa287-301 Determinant in Normal Mice.** The presentation of the mLa287-301 determinant after antigen processing and the immunodominance of the homologous hLa298-301 determinant suggested that the mLa287-301 region might be highly tolerogenic in normal mice. To test whether this was the case, groups of normal H-2d mice (I-Ak) were immunized with either hLa288-302 or mLa287-301; 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 hLa288-302 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 mLa287-301 proliferated poorly to the mLa287-301 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 mLa287-301 was not caused by a failure to bind mLa or by a lack of presentation of this determinant from intact antigen. However, it was possible that the mLa287-301 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 mLa287-301 peptide for the development of anti-La antibodies. Neither the mLa287-301 nor the hLa288-302 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 hLa288-302 induces a vigorous proliferative T cell response (Fig. 4, A and B) without inducing autoactivity to endogenous murine La (Figs. 3 and 5 A), again reflecting the lack of functional T cell cross-reactivity between mLa287-301 and hLa288-302 in the murine T cell responses. Presumably, the lack of autoantibody production despite efficient T immunity to hLa288-302 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 mLa287-301 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 mLa13-30, mLa25-44, and mLa106-129 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 La13-30, La25-44 and La106-129 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 mLa206-129 determinant; however, both the mLa25-44 and mLa13-30 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.
with either mLal ~ 3~,
predicted tool mass ~33 kD), GST-LaL2/3 (aa 346-416 pre&cted 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 cellular autoantigens, i.e., Sm antigens and La, Pel-Freez Biologicals, Rogers, AR); GST protein (predicted mol mass ~26 kD); and recombinant DHFR, and 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-mlgG (Sigma) as second antibody. Molecular masses are shown in kilodaltons.

Figure 6. Initiation and intramolecular diversification of anti-La autoantibody after immunization with the subdominant mLal3_30 peptide. Pooled sera (diluted 1/500) from A/J mice immunized and boosted twice with either mLal3_30, (upper panel) or mLAl413-50 peptides (middle panel) were used to immunoblot baculovirus 6xHis-mLa (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-hLaA3 (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 antisera at 1:3,000 (lower panel). Bound antibodies were detected by ECL (Amersham) using goat anti-mlgG (Sigma) as second antibody. Molecular masses are shown in kilodaltons. Bands corresponding to lower molecular weight degradation products are seen in some lanes.

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 mLal413-50 and mLAl25-44 by Western blot analysis. In A/J mice immunized with mLAl413-50, the autoantibody response was restricted to the La A subfragment containing the mLAl25-44 peptide of La autoantigen (data not shown). By contrast, pooled sera from mice immunized and boosted with mLAl3_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 mLAl3_30 is probably directed towards the LaA413-50 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 mLAl3_30, pooled immune sera was examined for immunoblot reactivity to 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 LaA3_30 peptide reacted specifically with recombinant Ro 52-kD antigen (Fig. 7).
Associated with an Autoreactive T Cell Response.

specific T helper responses recalled on intact mLa antigen. mice with the subdominant mLa 13_3 peptide is associated with was equivocal and not observed in all experiments (data not shown). Hence, the spreading autoreactivity that follows immunization of normal H-2 k mice was equivalent in their antigen presentation and behaved as 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 (hLa288-302) and a homologous but tolerogenic epitope derived from endogenous mLa (mLa287-301). The evidence that this determinant is tolerogenic in mice is based on the lack of either T cell responses or autoantibody production after immunization of normal mice with the mLa287-301 peptide. The mLa determinant was efficiently presented after immunization with the subdominant mLa13_30 peptide in H-2 k mice. Therefore, we evaluated whether immunization with mLa13_30 peptide induced specific autoreactive T cells. CBA/CaI-I mice were immunized with recombinant intact mLa, hLa, and mLaA subfragment 8 A). These T cell responses were recalled in the presence of antigen reactivity 9 d later. Immune T cells proliferated specifically in response to mLa13_30, but not control peptides mLa25_44, mLa287_301, or HEL46_61 (Fig. 8 A). These T cell responses were recalled in the presence of 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).

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 nonspecificity 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 (hLa288-302) and a homologous but tolerogenic epitope derived from endogenous mLa (mLa287-301). 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 mLa287-301 peptide. The mLa determinant was efficiently presented after antigen presentation, as demonstrated by inhibition binding studies to 1-A k and specific antagonism of the immunodominant hLa298-302 homologue by both intact mLa and the mLa287-301 peptide that possessed properties of an altered peptide ligand for hLa298-302 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 hLa298-302 and mLa297-301 determinants do appear to be equivalent in their antigen presentation and behave as immunodominant peptides in H-2 k and H-2 a mice.

Figure 8. Immunization with the subdominant peptide mLa13_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 mLa13_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 mLa13_30, mLa25_44, mLa287_301, or HEL46_61, or with (B) recombinant 6xHis-mLa, 6xHis-hLa, 6xHis-mLa (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 mLa13_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 mLa13_30 Determinant Is Associated with an Autoreactive T Cell Response. Triggering of autoimmunity by immunization with subdominant peptides such as the mLa13_30 peptide was presumed to occur through the activation of nontolerized T helper cells that recognize this determinant. Therefore, we evaluated whether immunization with mLa13_30 peptide induced specific autoreactive T cells. CBA/CaH mice were immunized with mLa13_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 mLa13_30, but not control peptides mLa25_44, mLa287_301, or HEL46_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 mLa13_30 peptide is associated with specific T helper responses recalled on intact mLa antigen.
The tolerogenicity of mLae87_3m indicates that endogenous La poly- 
peptides 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 deter-
minants 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 experiment-
ally and is associated with T cell responses to subdominant 
determinants. Although our assays have measured prolifer-
ative 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 mLae13_30 
subdominant peptide was recalled on intact La antigen, 
even though T cell responses to this and other subdomi-
nant mLa peptides were not evident in every experiment 
involving immunization with intact La protein. This pat-
tern of autoreactivity is distinct from the autoimmune T cell 
responses that recognize cryptic peptides of the snRNPs 
described by Bockenstedt et al. (4). In that study, 
priming of T cells with peptides derived from snRNPs re-
sulted in only a limited autoantibody production, and re-
sponding 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 au-
toreactive B cells that then present processed snRNP anti-
gens to T cells that recognize newly revealed cryptic pep-
tides. In the experimental autoimmunity we have described 
here, the diversification of the autoimmune response oc-
curred without the need for any challenge with exogenous 
intact La/Ro RNPs suggesting that the autoimmune 
immunization driving the spreading of the response was caused by endog-
ogenous 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 pre-
sentation is qualitatively (45) and quantitatively (46) insuffi-
cient 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 sur-
face of the APC probably falls below the threshold for acti-
vation of naive T cells.

It seems likely that experimentally induced autoimmu-
nity 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 
adjutants to activate APC and induce the qualitative 
costimulatory APC functions that are necessary for T cell 
priming (45, 47-52). In other words, experimental induc-
tion 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 im-
munization 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 ti-
ter autoantibody responses in experimental animals. These 
differences in the immunogenicity of intracellular self-anti-
gens might reflect degrees of host immune tolerance based 
on antigen abundance, turnover, adjuvanticity, intracellular 
trafficking, or structural complexity affecting antigen pro-
cessing. Alternatively, the use of recombinant intact La an-
tigen 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, suggest-
ing 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 those observed with 
recombinant proteins. However, this does not alter the ev-
edence that immunization with peptide determinants nom-
inally defined as subdominant resulted in a natural spread-
ing of autoimmunity to involve multiple components of the 
La/Ro RNP.

We propose that the initial diversification of the autoim-
mune response is driven by selective constitutive uptake of 
endogenous "native" La/Ro RNP complexes by autoreac-
tive B cells expressing specific mlgs (44) leading to presenta-
tion of the same subdominant determinants after antigen 
processing. Thus, T cells specific for one subdominant de-
terminant might provide helper signals to distinct autoreac-
tive B cells with differing specificities within the RNP 
complex. It is also possible that once the autoimmune re-
sponse is initiated, natural presentation of multiple sub-
dominant (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 com-
plexes. The relative contribution of these potential mecha-
nisms in explaining diversification of the autoimmune 
response needs clarification. If systemic autoimmunity 
involving ubiquitous self-antigens is initiated by T cell re-
sponses to one or two subdominant self peptides, then se-
lective antigen presentation might explain HLA class II al-
lele association with certain antinuclear autoantibodies (58).

Maintenance of autoimmunity may require chronic expo-
sure to an antigenic trigger as well as a genetic predisposi-
tion 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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Address correspondence to Prof. James McCluskey, Centre for Transfusion Medicine & Immunology, Flinders Medical Centre, Bedford Park, South Australia 5042, Australia.

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Author/s:
Reynolds, P; Gordon, TP; Purcell, AW; Jackson, DC; McCluskey, J

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