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Intra- and intermolecular spreading of autoimmunity involving the nuclear self-antigens La (SS-B) and Ro (SS-A)

(anti-nuclear antibodies/ribonucleoproteins/immune tolerance)

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ABSTRACT **We have tested the extent of immune self-tolerance to the ubiquitously expressed nuclear/cytoplasmic autoantigens La (SS-B) and Ro (SS-A) in healthy, nonautoimmune mice. Immunization of mice with recombinant mouse La resulted in a specific, isotype-switched autoantibody response, which was initially directed toward the La C subfragment (aa 111–242) but rapidly spread to involve the La A (aa 1–107) and La F (aa 243–345) regions of the La antigen. Intramolecular spreading of the anti-La antibody response was further demonstrated by the appearance of autoantibodies to multiple, nonoverlapping antigenic regions of La, after immunization of mice with the 107-aa La A subfragment. Moreover, immunization of mice with recombinant mouse or human La also elicited specific anti-60-kDa Ro IgG antibodies in all strains tested. Mice immunized with 60-kDa Ro produced a high titer anti-Ro antibody response, which was also associated with intermolecular spreading, resulting in the specific appearance of anti-La autoantibodies. These findings show that the development of autoantibodies to multiple components of the La/Ro ribonucleoprotein complex may follow initiation of immunity to a single component. In addition, the data reveal the incomplete nature of immune tolerance to La and Ro despite their endogenous expression in all nucleated cells. These observations are likely to account for the coexistence of anti-La/Ro antibodies in autoimmune disease and suggest a general explanation for the appearance of mixed autoantibody patterns in systemic autoimmune disorders.**

Antibodies to the La and Ro ribonucleoproteins (RNPs) are a prominent feature of Sjögren syndrome, yet little is known about how these autoantibodies arise, why they occur together, or indeed the extent of immunological tolerance to these ubiquitous, nuclear/cytoplasmic antigens in normal individuals (1–3). Considerable evidence suggests that the autoantibody response is antigen-driven and involves T-helper cells. For instance, B-cell epitope mapping of the La and Ro polypeptides indicates the autoimmune response is polyclonal (4), is class switched (5), and contains species-specific epitopes (6). These studies are consistent with molecular mimicry (7) or "altered self" (8) acting as an initiating event ultimately leading to antigen-driven autoimmunity. However, it is unclear how these mechanisms could lead to simultaneous targeting of the La and Ro autoantigens in Sjögren syndrome (2) or result in the specific subsets of anti-nuclear antibodies associated with other autoimmune disorders (1).

La is an ATP-dependent (9) transcription-termination factor for RNA polymerase III (10) that binds the 3′ uridine-rich region of polymerase III RNA transcripts (11). At least two Ro polypeptides have been identified: 52-kDa Ro and 60-kDa Ro (Ro60). The function of the Ro proteins is undefined; however, it is known that Ro60 associates with the Y RNAs (cytoplasmic small RNAs) (12) and, at least transiently, with the La molecule (13, 14). Thus it is possible that the occurrence of autoantibodies to both La and Ro in autoimmune disease is a consequence of their structural association intracellularly (2).

We have examined whether immunity to La and Ro60 autoantigens can be triggered by immunization with recombinant antigen in normal, healthy mice. The data indicate incomplete immune tolerance to the La and Ro autoantigens after immunization of normal mice and show that initiation of immunity to a single component of the La/Ro RNP complex is sufficient to trigger autoantibodies reactive with other components of this complex.

MATERIALS AND METHODS

Expression and Purification of Recombinant Protein. Recombinant mouse La (mLa), human La (hLa), human Ro60, and hen egg lysozyme (HEL) were produced in bacteria as six-histidine (6×His) fusion proteins expressed from the pQE expression vector (Qiagen, Chatsworth, CA) or as glutathione *S*-transferase (GST) fusion proteins (mLa, hLa, and mLa subfragments), expressed from the pGEX expression vector (Pharmacia) (6, 15). 6×His fusion proteins possess six histidine residues at the amino terminus, which facilitates purification of the proteins by Ni^+ affinity chromatography, whereas GST fusion proteins possess a 26-kDa amino-terminal domain derived from GST, which allows glutathione affinity purification. Mass spectrometry of the purified "full-length" recombinant 6×His-La antigen indicated a molecular mass of ≈41 kDa instead of the predicted 47 kDa, representing removal of ≈70 amino acids from the carboxyl terminus (A. W. Purcell, personal communication). This discrepancy in molecular mass is presumed to reflect bacterial proteolysis associated with protease-sensitive PEST regions within the carboxyl-terminal region of La (16). Recombinant Scl-70 was the generous gift of R. Eisenberg (University of North Carolina, Chapel Hill).

Animals and Immunization. Six- to 8-week-old C3H/HeJ ($H\text{-}2^k$), BALB/c ($H\text{-}2^d$), A/J ($H\text{-}2^a$), and C57BL/6 ($H\text{-}2^b$) mice were purchased from the Animal Resources Centre (Perth, Australia) and maintained in the animal house at Flinders Medical Centre (Adelaide, Australia). Groups of four to six animals were immunized subcutaneously with 100 μg of antigen in complete Freund's adjuvant (CFA) (GIBCO/BRL) and boosted twice with 50 μg of antigen in incomplete Freund's adjuvant (ICFA) (GIBCO/BRL) by the same route

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Abbreviations: 6×His, six histidine; CFA, complete Freund's adjuvant; GST, glutathione *S*-transferase; HEL, hen egg lysozyme; hLa, human La; ICFA, incomplete Freund's adjuvant; mLa, mouse La; RNP, ribonucleoprotein; Ro60, 60-kDa Ro.

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at ≈10-day intervals. Separate immunizations were carried out using the bacterially produced recombinant mLa, hLa, human Ro60, and HEL 6×His fusion proteins or with commercially prepared histones (Sigma). Animals were bled every 7–10 days after primary immunization. ELISAs were carried out as described (17).

Western Blotting. Recombinant proteins, purified histones (Sigma), or rabbit thymus extract were separated by SDS/PAGE and then electrotransferred to Hybond C extra nitrocellulose membranes (Amersham). Filters were blocked with 3% (wt/vol) skim milk powder (Bonlac Foods, Melbourne, Australia) in PBS and probed with the relevant sera diluted 1:500–1:1000 or with monoclonal antibodies as indicated. Reactive antibodies were detected by enhanced chemiluminescence (Amersham) using an anti-mouse IgG horseradish peroxidase-conjugated second reagent (Silenus, Melbourne, or Kirkegaard & Perry Laboratories).

RESULTS

Limited B-Cell Tolerance to La (SS-B) in Normal Mice. To determine whether it was possible to initiate autoimmunity to the La autoantigen, groups of four to six mice from four nonautoimmune strains were immunized with recombinant 6×His-mLa or 6×His-hLa emulsified in Freund's adjuvant. Nearly all animals immunized with mLa or hLa made a significant IgG anti-mLa antibody response (Fig. 1*A*), with C57BL/6 mice being low responders and C3H/HeJ, BALB/c, and A/J mice being high responders. Notably, immunization of mice with hLa elicited an ≈10-fold greater anti-La response than immunization with mLa. The specificity of the immune response was confirmed by immunoblotting of cell lysates from human and mouse cell lines, and all sera cross-reacted with hLa and mLa antigen regardless of the species of immunizing antigen (data not shown).

The specificity of anti-La antibodies was determined in serial serum samples by testing for immunoblot reactivity to a mixture of four, nonoverlapping subfragments of the mLa protein (La A, aa 1–107; La C, aa 111–242; La F, aa 243–345; and La L⅔, aa 346–415) (4). As shown in Fig. 1*B*, mice immunized with hLa initially responded to the La C subfragment with subsequent spreading of the immune response occurring within 14–21 days to involve the La A and La F subfragments. A similar evolution of antibody reactivity was observed in sera from mice immunized with mLa, except that the initial response to La C was detected 7 days later, and even after 35 days, sera from these animals reacted poorly with the La F fragment (aa 243–345).

Thus healthy, nonautoimmune mice challenged with recombinant La responded by making a polyclonal, isotype-switched autoantibody response, indicating incomplete immune tolerance to this autoantigen.

The Autoantibody Response Undergoes Intramolecular Spreading to Involve Multiple Determinants Despite Initial Challenge with a Single Subfragment of Autoantigen. The evolution of the anti-La autoantibody response in hLa-immunized mice involved the late appearance (35 days) of weak reactivity with the La L⅔ subfragment (aa 346–415) (data not shown), even though this region was truncated in the recombinant La protein used for immunization. This observation suggested that some of the anti-La autoantibody response may have been driven by endogenous mouse La. Therefore, we tested whether immunization of C3H and BALB/c mice with the 107-aa hLa A subfragment would lead to intramolecular spreading of immune reactivity to other regions of the La polypeptide. As shown in Fig. 1*C*, mice immunized with the 6×His-La A subfragment not only produced antibodies that bound GST-La A but also made lower titer autoantibodies reactive with the GST-fused mLa C (aa 111–242), mLa F (aa 243–345), and mLa L⅔ (aa 346–415) subfragments. This reactivity reflects intramolecular spreading of the antibody response, since antibodies to these disparate regions of the La molecule were not cross-reactive (data not shown).

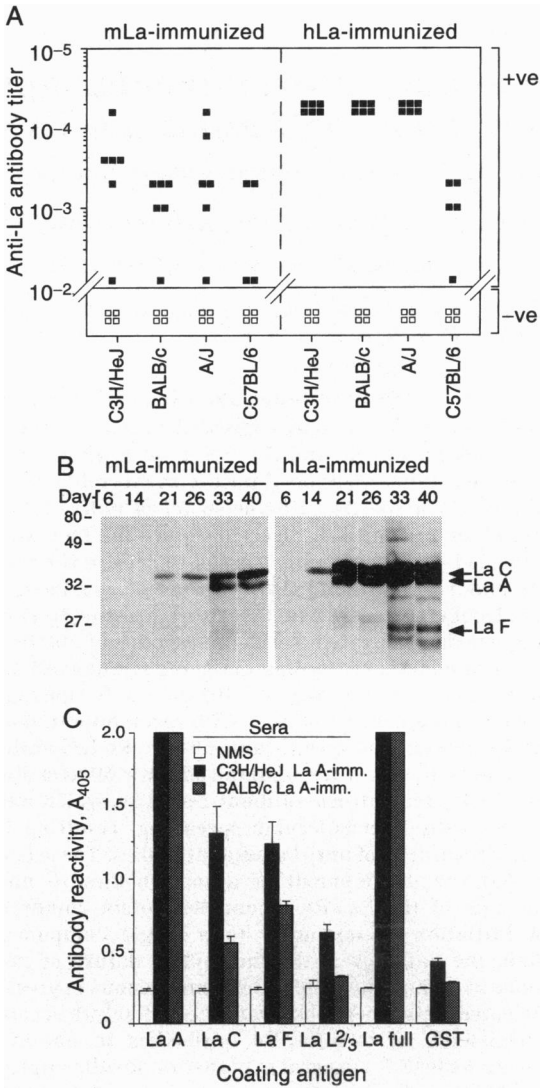

FIG. 1. Autoimmunity and intramolecular spreading of the autoantibody response in mice immunized with recombinant La autoantigens. (*A*) Mice from the indicated nonautoimmune strains were immunized with 100 μg of recombinant 6×His-mLa or 6×His-hLa in CFA and boosted twice at 10-day intervals with 50 μg of antigen in ICFA. Sera from individual mice were tested for reactivity with recombinant GST-mLa and GST-hLa by ELISA. The end-point reactivity of sera was determined using a positive cutoff value ≥ 3 SD above the mean value obtained from preimmunization sera. ■, Immune sera; □, preimmune sera; mLa-immunized, immunization and reactivity with mLa; hLa-immunized, immunization and reactivity with hLa. Positive (+ve) and negative (−ve) values are shown. (*B*) Evolution of autoantibody specificities during the immune response to La autoantigen. Serial serum samples (diluted 1:500) from mLa- and hLa-immunized C3H/HeJ mice were immunoblotted with a mixture of recombinant GST-mLa subfragments; La A (aa 1–107), La C (aa 112–242), La F (aa 243–345), and La L⅔ (aa 346–415) were present in each lane. Weak reactivity to the La L⅔ fragment was detectable but is not evident in the fluorograph shown. (*C*) Intramolecular spreading of the autoimmune response. C3H/HeJ and BALB/c mice were immunized (La A-imm.) with recombinant 6×His-hLa A (aa 1–107) as described above. Pooled sera from each group of six animals (diluted 1:250) were then allowed to react with the indicated GST-mLa subfragments, GST-mLa (La full), or GST alone, and reactivity was determined by ELISA. The reactivity of normal mouse serum (NMS) also is shown.

Intermolecular Spreading of Autoimmune Reactivity to Involve Other Components of the La/Ro RNP Particle. In

Sjögren's syndrome and systemic lupus erythematosus, anti-La antibodies are almost invariably associated with anti-Ro60 autoantibodies. To determine whether immunity to La was sufficient to induce spreading of the response to Ro60, sera from La-immunized mice were tested for reactivity to recombinant Ro60. Immune sera from each of the four mouse strains, immunized with either mLa or hLa, contained IgG anti-Ro60 antibodies (Fig. 2*A*). Immunization with hLa resulted in a stronger anti-Ro60 antibody response than immunization with mLa in all strains except for C57BL/6. Neither preimmune sera nor sera from mice immunized with Freund's adjuvant alone specifically reacted with La or Ro60 (Fig. 2*A*). To evaluate the kinetics of autoantibody spreading, C3H/HeJ and BALB/c mice were immunized with either mLa or hLa as described above, and serial serum samples were obtained. Pooled sera from the various time points after immunization were then tested for reactivity to recombinant La and Ro60 by ELISA (Fig. 2*B*). The results confirmed the findings in Fig. 2*A* and demonstrated that the appearance of anti-Ro60 antibodies was detectable within 10–15 days of the anti-La antibody response (Fig. 2*B*).

To test the reciprocity of intermolecular spreading of anti-La and anti-Ro60 immunity, mice were immunized with 6×His-human Ro60, and immune sera were then tested for anti-La antibodies reactive with Ro60 and GST-mLa. A high-titer anti-Ro60 antibody response was observed in both C3H/HeJ and BALB/c mice (Fig. 2*C*). Notably, immunization with recombinant Ro60 also elicited IgG anti-La antibodies in nearly all animals tested (Fig. 2*C*). The titer of the anti-La response was lower than the anti-Ro60 antibody response. These data demonstrate that immunization of nonautoimmune mice with either La or Ro60 antigen ultimately results in reciprocal intermolecular spreading of the autoantibody response to involve both autoantigens contained within the endogenous La/Ro RNP particle. Autoantibodies are class switched, suggesting a T helper-dependent mechanism of autoimmune spreading.

Autoantibody Spreading Is Specific and Not Due to Cross-Reactivity. Immune sera from mice immunized with recombinant La contained both anti-Ro60 and anti-La antibodies and did not react with commercially purified HEL, recombinant GST, or recombinant 6×His-HEL (Fig. 3*A* and data not shown). However, the mixed autoantibody reactivity may have been due to antibody cross-reactivity with La and Ro polypeptides. To exclude this possibility, antibodies reactive with immobilized La antigen were affinity-purified from the sera of mice previously immunized with recombinant La or Ro60 antigen (18) and retested for their capacity to bind recombinant La and Ro60 antigens in a Western blot. As shown in Fig. 3*B*, affinity-purified anti-La antibodies from both immunization groups specifically bound La but not recombinant Ro60. In addition, antibodies eluted from recombinant Ro60, but not recombinant La, specifically stained human cell lines overexpressing Ro60 antigen (ref. 19 and data not shown). Therefore spreading of the antibody response was not due to the appearance of cross-reactive antibodies.

To determine whether immunization of mice with recombinant La or Ro60 induced nonspecific anti-nuclear antibodies, sera were tested for immunoblot reactivity with a panel of defined nuclear antigens. As shown in Fig. 3*C*, immune mouse sera specifically recognized both La and Ro60 but did not react with Scl-70, histones, or the Sm polypeptides. Immune sera from La-immunized mice also reacted with the La antigen present in the rabbit thymus extract (Sm extract) used as a source of the Sm antigen. Sera from Ro60-immunized mice reacted with recombinant La but did not react with rabbit La present in the Sm extract presumably because of the low titer of the anti-mLa response and the low abundance of La in rabbit thymus preparations. Sera from mice immunized with purified histones or recombinant 6×His-HEL did not react

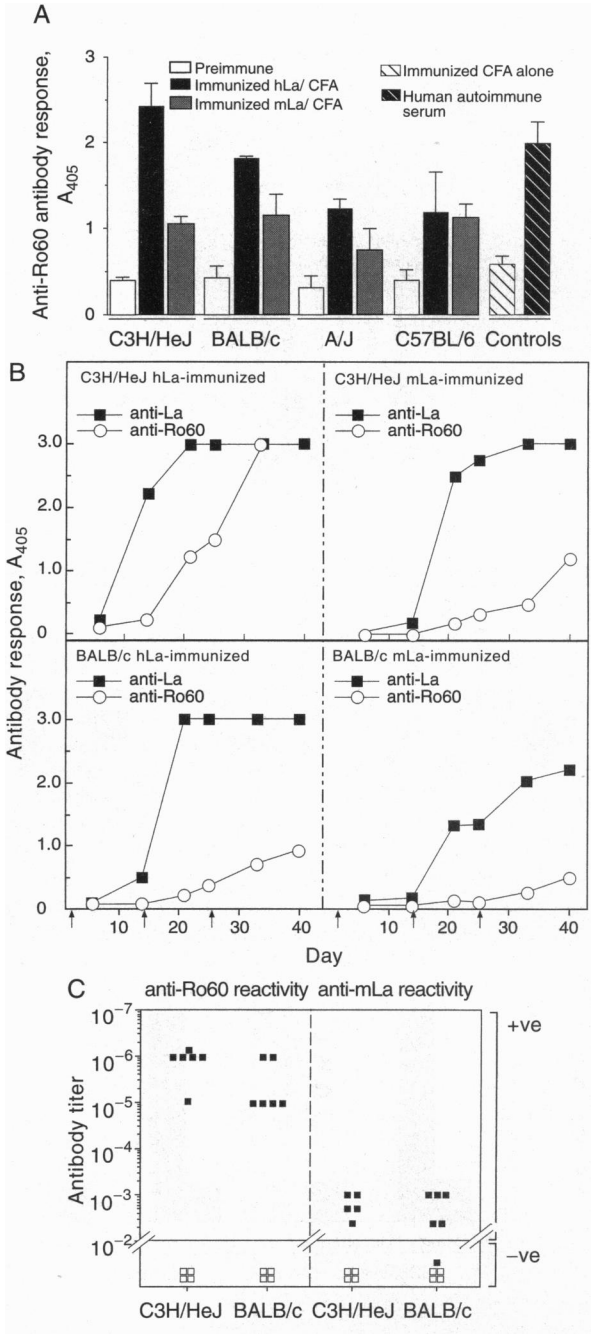

FIG. 2. Intermolecular spreading of the autoimmune response after immunization with either La or Ro60. (*A*) Pooled preimmune and immune sera (diluted 1:1000) from mice immunized with 6×His-hLa or 6×His-mLa were tested by ELISA for reactivity with recombinant human 6×His-Ro60. Sera from mice immunized with CFA alone and human anti-Ro60 autoimmune sera are shown as controls. Error bars indicate 2 SD. (*B*) Kinetics of appearance of anti-Ro60 antibodies. C3H/HeJ and BALB/c mice were immunized with either 6×His-hLa or 6×His-mLa as described in Fig. 1. Sera were collected on days 6, 14, 21, 26, 33, and 40; pooled; and then tested by ELISA (1:1000) for reactivity with recombinant GST-La derived from the same species used for immunization (■) and with 6×His-human Ro60 (○). Vertical arrows along the *x* axis indicate immunization and booster times. (*C*) C3H/HeJ and BALB/c mice were immunized with 6×His-human Ro60, and sera from individual mice were tested by ELISA for reactivity with 6×His-Ro60 (anti-Ro60) and a GST-mLa fusion protein (anti-mLa). End-point titrations of sera were determined using a positive cutoff value ≥ 3 SD above the mean value of preimmunization sera. Sera did not react with recombinant GST alone or with 6×His-HEL (data not shown). ■, Immune sera; □, preimmune sera; +ve, positive titration value; −ve, negative titration value.

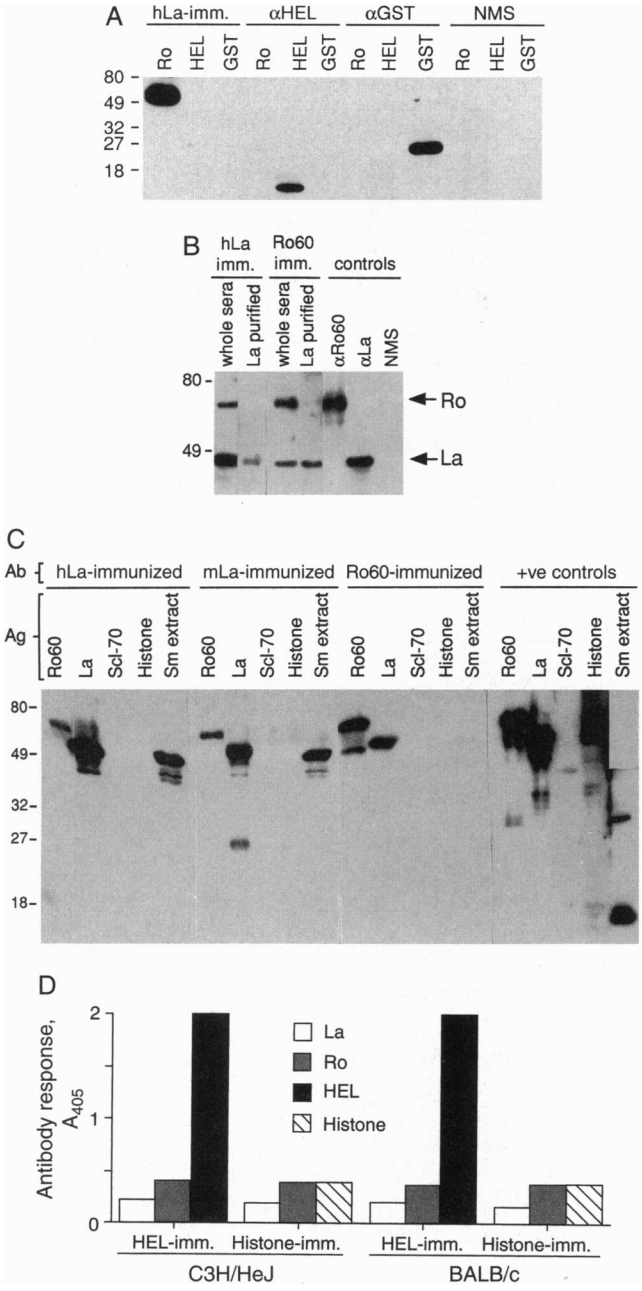

FIG. 3. Intermolecular spreading of the autoantibody response to La/Ro autoantigens is specific. (*A*) Pooled sera (diluted 1:1000) from C3H/HeJ mice immunized with 6×His-hLa (hLa-imm.) were tested for immunoblot reactivity with 6×His-human Ro60, HEL (Sigma), and recombinant GST (0.5–1 μg per lane). Bound antibodies were detected by ECL using an anti-IgG second reagent. Molecular masses are shown in kDa. NMS, normal mouse sera; αHEL and αGST, specific rabbit antisera. (*B*) Anti-La autoantibodies do not cross-react with Ro autoantigen. Anti-La antibodies from the sera of mice immunized with 6×His-hLa (hLa imm.) and 6×His-human Ro60 (Ro60 imm.) were affinity-purified on 6×His-hLa bound to nitrocellulose (18). The bound antibodies were eluted (La purified) and tested alongside the original serum (whole sera) for immunoblot reactivity with recombinant La and Ro (each ≈1 μg per lane). Controls using monoclonal antibodies to Ro60 (αRo60 mAb), La (αLa mAb), and normal mouse sera (NMS) are shown. Ag, antigen; Ab, antibody. (*C*) Anti-La and anti-Ro60 autoantibodies do not react with other nuclear autoantigens. Pooled sera from La-immunized and Ro60-immunized mice (diluted 1:500) were tested by immunoblot for reactivity with a panel of nuclear autoantigens including 6×His-mLa, 6×His-human Ro60, Scl-70, commercial histones, and a rabbit thymus extract containing SmB and SmD antigens (Sm extract). The upper half of the Sm extract control lane was removed and blotted separately with anti-La monoclonal antibody to confirm the identity of the La band in this extract (not shown). (*D*) Immunization with histones or 6×His-HEL does not result in an anti-La/Ro autoantibody response. Groups of four C3H/HeJ and BALB/c mice were immunized as described with a commercial preparation of histones (Histone-imm.) or recombinant 6×His-HEL (HEL-imm.). Sera from individual mice were pooled and tested for reactivity to recombinant La, Ro60, commercial HEL, and histones by ELISA. Anti-HEL sera were diluted 1:2000 and anti-histone sera were diluted 1:500 for ELISA estimations.

with either 6×His-La or 6×His-Ro60 (Fig. 3*D*). Importantly, immune self-tolerance to histones was not broken after immunization in CFA, indicating differences in the extent of tolerance to distinct nuclear components in normal mice.

DISCUSSION

Despite extensive investigations into the specificity of human anti-La and anti-Ro60 autoimmune sera (1, 2, 20), little is known about the extent of immunological tolerance to the La and Ro autoantigens in normal individuals (3). The data presented here demonstrate that anti-La and anti-Ro autoantibody production can be elicited after immunization of normal mice with recombinant mLa in adjuvant. Moreover, the antibody response was T-helper cell dependent as the autoantibodies were class switched. Therefore T- and B-cell tolerance to La and Ro autoantigens is incomplete even in normal mice.

Immune self-tolerance after immunization with purified intracellular self-antigens such as cytochrome *c* (21) and histones (22) is well established, though immunity to these molecules can be elicited under some circumstances (22–24). Our data confirm the lack of immune reactivity to purified histones in normal mice and highlight the incomplete nature of tolerance to the La and Ro polypeptides, which can initiate autoimmunity in their own right. The relatively low abundance of the La/Ro proteins and their nuclear/cytoplasmic sequestration probably accounts for the apparent lack of T- and B-cell tolerance to these antigens (25, 26). Thus the immune system is likely to be ignorant of many self-determinants encoded within the La and Ro molecules as well as other intracellularly sequestered antigens (27–29). In contrast, ubiquitously expressed nuclear proteins such as histones may induce more efficient T-cell tolerance because of their relative abundance.

Partial self-tolerance to the La antigen is reflected in the lower magnitude of the anti-mLa IgG response and slower kinetics of anti-Ro recruitment after immunization with mLa compared with hLa. The 23% sequence nonidentity of hLa and mLa (15) provides species polymorphisms sufficient for a significant xenogeneic anti-hLa T-helper response (data not shown), which might augment the autoimmune response leading to anti-La/Ro immunity.

Intramolecular spreading of autoimmune responses has been demonstrated after immunization of mice with an ovarian self-peptide in a model of murine oophoritis (30). Intermolecular spreading of autoimmunity occurs in nonobese diabetic (NOD) mice, where initial reactivity to the glutamic acid decarboxylase protein coincides with the onset of insulitis, with subsequent appearance of autoantibodies to carboxypeptidase H and peripherin within 14 days and autoantibodies to heat shock protein within 30 days (31, 32). The time taken for the appearance of anti-islet cell antibodies in NOD mice mirrors that seen for development of anti-Ro60 antibodies after immunization of mice with La. Spreading of T-cell autoimmunity has also been described in NOD mice (32, 33) and models of murine experimental allergic encephalomyelitis (34).

These model autoimmune systems (30–34) involve genetically susceptible animals and relatively organ-specific target structures where spreading of autoimmunity is likely to be

facilitated by inefficient mechanisms of self-tolerance (27, 35). In contrast, the initiation and spreading of autoimmunity to La and Ro autoantigens represents non-organ-specific immunity and occurs in several different strains of healthy mice. Accordingly, once initiated the spreading of self-reactivity to La and Ro probably does not require special pathological mechanisms or particular genetic characteristics. Rather, our findings suggest that even in normal mice there is incomplete tolerance to these ubiquitously expressed proteins, so that initiation of immunity to a single component of the autoantigen potentially leads to a persistent autoimmune response involving additional linked self-components.

What are the possible mechanisms of intra- and intermolecular spreading? We propose that immunization of mice with La or Ro activates antigen-specific T-helper cells capable of stimulating B cells with specificity for distinct endogenous structures associated with the priming antigen. Intra- and intermolecular spreading of the immune response could occur because helper signals are provided to clonally distinct B cells targeting different components of the La/Ro RNP particle. This model proposes that B cells with specificity for any component of the La/Ro RNP complex will constitutively capture and present determinants from endogenously derived La/Ro RNPs to T-helper cells with specificity for the priming antigen. A similar mechanism of immune spreading operates during viral immune responses when T cells recognizing only one component of a virus provide T help for B cells reactive against any structure within the virus particle (36–40). Specific B cells are very efficient at presenting cognate antigens to T cells because capture and uptake of the specific antigen is facilitated by the presence of surface immunoglobulin even at very low concentrations of the antigen (41). Moreover, constitutive presentation of self-antigen by autoreactive B cells occurs *in vivo* (42) and can potentially stimulate autoreactive T cells (42, 43). In addition RNP particles including those containing La and Ro are known to cluster as blebs near the membrane of apoptotic cells, possibly enhancing their endogenous availability to the immune system during normal cell turnover (44). Although antigen presentation by resting B cells can induce tolerance in T cells (45, 46), activation of specific B cells is likely to occur in the presence of mature antigen-specific T cells and following repeated immunization with antigen.

Alternatively, spreading of autoimmunity could follow from enhanced Fc receptor-mediated uptake and antigen presentation of La/Ro RNP immune complexes by professional antigen-presenting cells (47–50) facilitated by autoantibody recognizing any component of the La/Ro RNP complex. Regardless of whether endogenous La/Ro RNP antigens are presented by specific B cells or professional antigen-presenting cells, spreading of the autoimmune response potentially also involves T-helper cells recognizing determinants from different protein components of the La/Ro RNP.

The findings presented here suggest how an autoimmune response to a single region of a self-antigen could trigger intra- and intermolecular autoimmune spreading to involve associated self-components. Indeed, initiation of immunity might also involve a response to foreign antigens linked to self-components as has been described (8, 51). These concepts begin to explain how mixed diagnostic patterns of autoantibody specificity might arise in systemic autoimmunity.

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