
Η ανοσολογική απάντηση έναντι των κύριων B αντιγονικών επιτόπων της La/SSB μπορεί να ανασταλεί από το ενεργό δίκτυο ιδιοτυπικών/αντι-ιδιοτυπικών αντισωμάτων. Στην παρούσα μελέτη εκτιμήθηκε ο ρόλος αυτού του δικτύου σε εγκύους με anti-Ro/SSA και/ή anti-La/SSB αντισώματα σε σχέση με την εμφάνιση του συνδρόμου των νεογνικών λύκων (ΣΝΛ). Δείγματα ορών ασθενών με anti-Ro/La αντισώματα, που συλλέχθηκαν κατά την εγκυμοσύνη ή και 6 μήνες μετά τον τοκετό, λήφθηκαν από το Ερευνητικό Μητρώο για το Νεογνικό Λύκο. Με εφαρμογή της τεχνικής ELISA, διαπιστώθηκε ότι οι μητέρες που γέννησαν αποκλειστικά υγιή παιδιά χωρίς ιστορικό γέννησης παιδιού με ΣΝΛ παρουσίαζαν υψηλότερα ποσοστά αντι-ιδιοτυπικών αντισωμάτων σε σύγκριση με αυτές που κυοφορούσαν παιδί με ΣΝΛ (P<0.0001) ή με αυτές που κυοφορούσαν υγιές παιδί, αλλά είχαν ιστορικό γέννησης παιδιού με ΣΝΛ (P=0.0151). Η παρουσία αυτή των αντι-ιδιοτυπικών αντισωμάτων έναντι των anti-La/SSB αυτοαντισωμάτων δρα πιθανώς προστατευτικά για το έμβρυο, παρεμποδίζοντας τη δράση των παθογενετικών μητρικών αυτοαντισωμάτων, ενώ η ευρύτερη εφαρμογή της μεθόδου μπορεί να βοηθήσει στον προσδιορισμό των κυοφοριών με χαμηλή επικινδυνότητα για την εμφάνιση νεογνικού λύκου.
Anti-La/SSB Antiidiotypic Antibodies in Maternal Serum

A Marker of Low Risk for Neonatal Lupus in an Offspring

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Objective. The anti-La/SSB response to major B cell epitopes of La/SSB can be blocked by an active idiotypic/antiidiotypic network, which can be identified using synthetic complementary epitopes deduced from the sequence of the major B cell epitopes of the molecule. This study evaluated the role of this network in pregnant women with anti-Ro/SSA and/or anti-La/SSB antibodies in the development of neonatal lupus syndrome (NLS).

Methods. Sixty-three serum samples collected from anti-Ro/anti-La–positive women during pregnancy or within 6 months after delivery were obtained from the Research Registry for Neonatal Lupus and the PR Interval Dexamethasone Evaluation study. These samples, as well as 30 sera from healthy individuals, were tested in a blinded manner by enzyme-linked immunosorbent assay against synthetic peptides corresponding to major B cell epitopes and complementary epitopes of La/SSB.

Results. Sera from mothers giving birth to a healthy child and having no history of a child with NLS exhibited higher antiidiotypic antibody activity compared with mothers carrying a child with NLS (P < 0.0001) or mothers giving birth to a healthy child but who previously gave birth to a child with NLS (P = 0.0151). Sera from mothers of healthy children, which exhibited no apparent epitope activity against amino acids 349–364, revealed a significantly greater frequency of hidden anti–349–364aa epitope responses, blocked by antiidiotypic antibodies, as compared with sera from women pregnant with an affected child (P = 0.0094).

Conclusion. The presence of antiidiotypic antibodies to autoantibodies against La/SSB may protect the fetus by blocking pathogenic maternal autoantibodies. Testing for these antiidiotypic responses may be useful in predicting a decreased risk of NLS.

Neonatal lupus syndrome (NLS) is considered a model of passively acquired systemic autoimmune disease. Placentally transported maternal IgG autoantibodies against Ro/SSA and/or La/SSB are strongly implicated in the pathogenesis of the disease. NLS is characterized by 2 dominant manifestations, cutaneous rash and congenital heart block (CHB), the latter being most often of third-degree severity in a structurally normal heart (1), but the syndrome also involves hematologic and hepatic features (2). The true incidence of NLS is unknown (3), but CHB is estimated to occur in ~2% of anti-Ro/SSA–positive mothers (4). The presence of anti-La/SSB antibodies increases the risk of CHB in the fetus to 5% as compared with the presence of anti-Ro/SSA alone (5). A putative role for the candidate antibodies in the pathogenesis of this disease derives from in vitro and in vivo data, demonstrating that maternal anti-Ro/SSA and/or anti-La/SSB antibodies opsonize fetal apoptotic cardiomyocytes, which in turn induces a proinflammatory/profibrotic response by
phagocytosing macrophages, ultimately leading to tissue injury (6).

In previous studies, investigators in our laboratory mapped the linear B cell epitopes of both Ro/SSA (60 kd) and La/SSB. La/SSB contains 4 linear epitopes spanning the amino acid sequences 145–164 aa, 289–308 aa, 301–320 aa, and 349–364 aa of the molecule (7). The dominant epitopes of the La/SSB response in patients with primary Sjögren’s syndrome or patients with systemic lupus erythematosus (SLE) span sequences 289–308 aa and 349–364 aa. These reactivities are strongly linked to the HLA–DQA1*0501 genotype (8). Moreover, animal immunization experiments revealed that epitope 289–308 aa is an immunodominant T cell/minor B cell epitope, while epitope 349–364 aa is a subdominant T cell/major B cell epitope. Animal immunization with peptides of either 289–308 aa or 349–364 aa revealed intra- and intermolecular spreading of the immune response to Ro/La RNP. Thus, the formation of autoantibodies against La/SSB is an antigen-driven process in which epitopes 289–308 and 349–364 are actively involved (9).

The autoimmune response can be regulated by either external factors, such as the tissue microenvironment (10), or factors internal to the immune system, such as antiidiotype antibodies (11) or regulatory T cells (12). Antiidiotypic antibodies react with the idiotypes within the F(\(\text{ab'}\))\(_2\) fragment of autoantibodies. The presence of antiidiotypic antibodies has been described in association with a variety of autoantibodies, but their role in the perpetuation or the regulation of autoimmune diseases, as well as their overall clinical utility, have not been fully elucidated.

Complementary peptides have the potential to adopt structures that are complementary to B cell epitopes and mimic the shape of the paratopes of the antibodies recognizing these epitopes. Therefore, complementary peptides can be efficiently used for the detection of antiidiotypic antibodies (13) (Figure 1). Previous studies in our laboratory (14) disclosed that 1) both epitopes 289–308 and 349–364 and complementary epitopes 289–308 and 349–364 of La/SSB are recognized by sera containing anti-La/SSB antibodies, 2) antibodies to these complementary epitopes are antiidiotypic antibodies directed to the epitope antibodies (anti-La/SSB), and 3) by using the complementary epitopes as inhibitors of the antiidiotypic antibodies, the anti-La/SSB reactivity can be recovered in anti-Ro/SSA–positive, anti-La/SSB–negative autoimmune sera.

Among the systemic autoimmune diseases, NLS is the ideal model for studying antiidiotypic antibodies, since pathogenetic autoantibodies to Ro/SSA and/or La/SSB may be directly involved in tissue injury (15,16). Accordingly, the aim of this study was to evaluate the idiotypic/antiidiotypic network of antibodies targeting the dominant epitopes of La/SSB in mothers positive for anti-Ro and/or anti-La/SSB antibodies, and to define the role of this network in the development of NLS. To accomplish this goal, peptides and complementary peptides deduced from the sequences 289–308 aa and 349–364 aa of La/SSB were synthesized and tested against maternal sera. Test material was obtained from the Research Registry for Neonatal Lupus (RRNL) and the PR Interval and Dexamethasone Evaluation (PRIDE) study group.

**SUBJECTS AND METHODS**

**Human sera.** Sixty-three maternal serum samples positive for anti-Ro and/or anti-La autoantibodies (confirmed on serial echocardiographs of anti-Ro–positive women) were provided by the RRNL and the PRIDE study group. Sera were collected from women during pregnancy or within 6 months after delivery, and were coded. All assays on serum samples were performed in a blinded manner. Thirty sera from healthy female blood donors served as normal controls. The mean age of the control group did not differ significantly from that of the mothers included in the study.

After completion of the assays, the samples were decoded and individuals were divided into 3 groups. Group A (n = 29) comprised sera from mothers pregnant with a child with NLS (CHB or cutaneous rash), group B (n = 10) comprised sera from mothers who had given birth to a child with NLS but whose subsequent pregnancy resulted in a healthy child with exposure to the current antibodies, and
modified ELISA that was optimized for each synthetic peptide. Antipeptide and anti-complementary peptide epitopes of La/SSB were purified from human auto-

Table 1. Demographic features and the clinical and serologic profiles of the 3 groups under study

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Group A (n = 29)</th>
<th>Group B (n = 10)</th>
<th>Group C (n = 24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD years</td>
<td>34 ± 5</td>
<td>31.5 ± 5.7</td>
<td>33 ± 4.9</td>
</tr>
<tr>
<td>SLE</td>
<td>17.2</td>
<td>20</td>
<td>33.3</td>
</tr>
<tr>
<td>SS</td>
<td>10.4</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>SLE/SS</td>
<td>10.4</td>
<td>10</td>
<td>4.15</td>
</tr>
<tr>
<td>MCTD or scleroderma</td>
<td>3.4</td>
<td>0</td>
<td>4.15</td>
</tr>
<tr>
<td>UAS</td>
<td>20.7</td>
<td>20</td>
<td>16.7</td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>27.6</td>
<td>20</td>
<td>16.7</td>
</tr>
<tr>
<td>NA</td>
<td>10.3</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Autoantibody profile, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNP</td>
<td>6.9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ro60</td>
<td>62.1</td>
<td>60</td>
<td>79.2</td>
</tr>
<tr>
<td>Ro52</td>
<td>86.7</td>
<td>80</td>
<td>95.8</td>
</tr>
<tr>
<td>La/SSB</td>
<td>51.7</td>
<td>70</td>
<td>45.8</td>
</tr>
</tbody>
</table>

* Group A comprised mothers pregnant with a child with neonatal lupus syndrome (NLS), group B comprised mothers who had given birth to a child with NLS but whose subsequent pregnancy resulted in a healthy child with exposure to the antibodies, and group C comprised mothers giving birth to a healthy child and having no history of a child with NLS. The mean (±SD) age of the mothers was not statistically significantly different among the 3 groups (ages 34 ± 5 years, 31.5 ± 5.7 years, and 33 ± 4.9 years in groups A, B, and C, respectively). In Table 1, demographic data as well as the clinical and serologic profiles of the mothers are shown.

**Peptide synthesis.** Synthetic peptides corresponding to the 2 B cell/T cell epitopes of La/SSB, A \(^{289}\) NNGLQLR-NKEVTWEVLEG \(^{308}\) (pep289–308) and G \(^{498}\) SGKGVQFOQGKKTF \(^{516}\) (pep349–364), as well as their complementary peptides, S \(^{308}\) FEYPSPHFVFPELEVTTIC \(^{328}\) (cpep289–308) and K \(^{364}\) FRFLALKLYSFSFRP \(^{383}\) (cpep349–364), were purchased from Biosynthesis (Lewisville, TX).

**Specificity of anti-complementary peptide antibodies for the detection of antiidiotypic antibodies.** The specificity of anti-complementary peptides for the detection of antiidiotypic antibodies against anti-La/SSB has been described elsewhere (14). Briefly, antibodies against the 2 epitopes and complementary epitopes of La/SSB were purified from human autoimmune sera. These antibodies specifically recognized the peptide against which they were purified. Purified antibodies against the 2 major epitopes exhibited high anti-La/SSB activity against recombinant La/SSB. In addition, F(ab') \(^{2}\) fragments of antibodies against the complementary peptide of the major epitope of La/SSB (cpep349–364) bound to a common idiotypic of antibodies against the major epitope of La/SSB (pep349–364), and these inhibited the binding of anti-La/SSB antibodies to recombinant La/SSB by 91%.

**Antipeptide and anti-complementary peptide enzyme-linked immunosorosent assays (ELISAs).** Antipeptide and anti-complementary peptide epitopes of La/SSB were detected by a modified ELISA that was optimized for each synthetic peptide. Costar high-binding (peptide assays) and Nunc Multisorp (complementary peptide assays) microtiter plates were coated overnight at 4°C with 100 µl of the appropriate peptide solution, comprising pep289–308 (minor B cell epitope) and cpep289–308 (15 µg/ml in carbonate/bicarbonate buffer, pH 9.1) or pep349–364 (major B cell epitope) and cpep349–364 (10 µg/ml in phosphate buffer, pH 7.1). After blocking the remaining binding sites with blocking buffer (2% bovine serum albumin in phosphate buffered saline [PBS]) for 1 hour at room temperature and washing with PBS–0.05% Tween 20, the plates were incubated overnight with 100 µl of sera (diluted 1:150 in blocking buffer at 4°C). The wells were then washed and goat anti-human IgG conjugated to alkaline phosphatase (1:1,200 in blocking buffer) (Jackson ImmunoResearch, Avondale, PA) was added. The plates were incubated for 1 hour at room temperature. Subsequently, the wells were washed and 100 µl p-nitrophenol substrate (Sigma-Aldrich, Munich, Germany) was added. After incubation at 37°C for 20 minutes, absorbance was measured at 405 nm using a 190 Spectramax reader (Molecular Devices, Sunnyvale, CA). The cutoff point for a positive response in each assay was calculated as the mean (+3 SD) optical density (OD) value obtained from the sera of the 30 healthy individuals. The cutoff point was arbitrarily set at 100 binding units (BU) and each OD value was accordingly converted to BU. The inter- and intraassay coefficients of variation were less than 10% in all experiments.

**Detection of hidden anti-pep349–364 activity by ELISA.** Sera (diluted 1:200 in blocking buffer) not reactive with the major B cell epitope of La/SSB (pep349–364) or its complementary counterpart (cpep349–364) were heated at 55°C for the dissociation of idiotypic/antiidiotypic complexes. Subsequently, cpep349–364 was added as an antiidiotypic blocking agent, at a final concentration of 60 µg/ml. The mixture was submitted at slow cooling (from 55°C to 25°C in 3 hours) in order to establish a new equilibrium in which the complementary peptide served as an antiidiotypic inhibitor by binding to antiidiotypic antibodies and eventually unmasking the idiotypic antibodies. Sera were then tested in ELISA against the major B cell epitope of La/SSB (pep349–364) in the same manner as described above.

**Statistical analysis.** Differences in the percentage activity among the 3 groups were analyzed with Fisher’s exact test. Differences between the median OD values were evaluated by Mann-Whitney U test.

**RESULTS**

**Idiotypic activity.** The idiotypic activity in each serum group is shown in Figures 2a and b. In 67% of anti-La/SSB–positive mothers as compared with 17.8% of anti-La/SSB–negative mothers (each assessed by ELISA or immunoblot against recombinant La/SSB [as part of the RRNL]), antibodies to the major epitope of La/SSB (pep349–364) were detected. Reactivity against the major B cell epitope (pep349–364) of La/SSB was found in 14 of 29 mothers of group A (48.3%), 5 of 10 mothers of group B (50%), and 9 of 24 mothers of group
C (37.5%). Reactivity against the minor epitope of La/SSB (pep289–308) was found in 7 of 29 mothers of group A (24.1%), 3 of 10 mothers of group B (30%), and 12 of 24 mothers of group C (50%). The idiotypic response was not statistically significantly different among the 3 groups.

**Antidiotypic response.** The antidiotypic activity in each serum group is shown in Figures 2c and d. Reactivity against the complementary epitope of the major B cell epitope of La/SSB (cpep349–364) was found in 7 of 29 mothers of group A (24.1%), 3 of 10 mothers of group B (30%), and 19 of 24 mothers of group C (79.2%). Reactivity against the complementary epitope of the minor B cell epitope of La/SSB (cpep289–308) was found in 8 of 29 mothers of group A (27.6%), 3 of 10 mothers of group B (30%), and 11 of 24 mothers of group C (45.8%).

Sera from group C, comprising mothers giving birth to a healthy child and having no history of a child with NLS, exhibited higher antidiotypic activity toward the complementary epitope of the major B cell epitope of La/SSB (cpep349–364) compared with sera from mothers carrying a child with NLS (group A) (P < 0.0001) or sera from mothers previously giving birth to a child with NLS but then having a healthy child exposed to the current antibodies (group B) (P = 0.0151) (Figure 3). Interestingly, mothers of group B, who were expected to exhibit a serologic pattern similar to that in the mothers of group C, had a lower frequency of antidiotypic antibodies compared with group C and exhibited a similar pattern of response as that in group A. No remarkable correlations were observed between the levels of antipeptide antibodies and anti-complementary peptide antibodies in positive sera.
Recovery of the hidden anti–pep349–364 activity by specific blocking of antiidiotypic antibodies. Antiidiotypic antibodies to La/SSB have previously been found to compete with the antigen for binding to the idiotypes of anti-La/SSB antibodies and to be involved in their serologic detection, leading to false-negative results. To investigate the functional blocking of antibodies to the major B cell epitope of La/SSB (pep349–364) by antiidiotypic antibodies, a procedure to overcome this antiidiotypic interference was developed. We applied a heat and complementary peptide treatment, as described in Subjects and Methods, to 23 serum samples (14 from group A, 4 from group B, and 5 from group C) that did not exhibit antiidiotypic and idiotypic activity against the major B cell epitope of La/SSB (pep349–364).

Only 4 of 14 mothers (28.5%) carrying a child with NLS (group A) exhibited hidden anti–pep349–364 responses, in contrast to 8 of 9 women (88.8%) having healthy children (merged data from groups B and C) ($P = 0.0094$) (Figure 4). The median BU values for the activity of the antibodies against the major B cell epitope of La/SSB (pep349–364) were not significantly different between sera from group A and sera from the combined group B and C ($P = 0.833$) before treatment, but were significantly different after treatment ($P = 0.0407$), with higher BU values in the combined group, indicating that an active antiidiotypic response was present in women having a healthy child.

**DISCUSSION**

The presence of an active network of antiidiotypic/idiotypic antibodies directed to the major B cell epitope of La/SSB in association with the development of NLS has been demonstrated for the first time in this study. In fact it was shown that mothers carrying a healthy child have a larger proportion of antiidiotypic antibodies in their sera, detected either directly or indirectly, compared with those carrying a child with NLS.

In the 1970s Jerne proposed the network hypothesis, in which complementary interactions involving idiotypes and antiidiotypes were suggested to contribute to the homeostasis of the adaptive immune response (17). Antibodies produced against an infectious agent can elicit antiidiotypic antibodies that may have the incidental property of being antibodies to the host structure (18). The idiotypic/antiidiotypic network was, in the past, a field of intensive research in autoimmunity. It has been shown that antiidiotypic antibodies can act as regulators of the autoimmune response in SLE (19). The detection of antiidiotypic antibodies in clinical specimens is challenging, since most autoimmune diseases involve polyclonal responses to self antigen. Moreover, some idiotypes are unique to each patient, and therefore the performance of general studies is not possible. The isolation of a nonhomogenous population of antibodies and the possibility of cross-reactions adds to the difficulties of this task.

An alternative strategy to probe the antiidiotypic antibodies is through the use of complementary epitopes of the major B cell epitopes of autoantigens (20–23). Peptides with complementary structure can be generated in animals by interacting pairs of idiotypic and antiidiotypic antibodies with complementary combining sites (13).

Patients with inflammatory vascular disease associated with antineutrophil cytoplasmic autoantibodies with specificity for proteinase 3 (PR-3) also synthesize antibodies against a peptide translated from the antisense DNA strand of PR-3 (cPR-3). Human and mice anti-PR3 and anti–cPR-3 antibodies bind each other, indicating an idiotypic/antiidiotypic relationship (24).

In our previous studies (14) it was shown that purified antipeptide and anti–complementary peptide IgG and their F(ab')$_2$ fragments isolated from the sera of patients with SLE and patients with Sjögren’s syndrome recognized, specifically, the peptide against which they were purified. Antibodies to the minor B cell epitope (pep289–308) and to the major B cell epitope (pep349–364) of La/SSB, as well as their F(ab')$_2$ fragments, exhibited high anti-La/SSB activity against recombinant La/SSB in ELISA and on immunoblot, and gave a positive speckled nuclear immunofluorescence.
staining on HEp-2 cells. Purified anti-complementary peptide F(ab')2 fragments bound to a common idiotype located within or spatially close to the antigen combining site of anti-La/SSB antibodies (anti-pep349–364) and inhibited the binding of anti-La/SSB antibodies onto recombinant La/SSB. Specific antiidiotype antibodies were found to be able to mask the anti-La/SSB response; the dissociation of idiotype/antiidiotype immune complexes and the subsequent blocking of antiidiotype antibodies with the complementary epitope revealed hidden idiotype antibodies. Immunization experiments in nonautoimmune mice with the synthetic peptide of the 2 main epitopes of La/SSB or their complementary epitopes led to an antigen-driven appearance of antibodies, not only against the immunizing peptide but also against their complementary counterparts (25). Affinity purification of both antibodies revealed their idiotypic/antiidiotypic activity.

Antiidiotype antibodies that have been generated by animal immunization experiments have been utilized in the study of NLS in order to detect maternal autoantibodies against Ro/SSA and La/SSB bound to affected fetal hearts (15), but their presence and role in maternal sera have never been investigated. In the present study, antiidiotype antibodies were detected in maternal sera. The prevalence of antiidiotype antibodies targeting antibodies to the major B cell epitope of La/SSB was higher in mothers giving birth to a healthy child and having no history of a child with NLS (group C) as compared with mothers carrying a child with NLS (group A). Mothers who gave birth to a child with NLS but whose subsequent pregnancy resulted in an unaffected child (group B) exhibited an unexpected low frequency of antiidiotype antibodies. Several explanations may account for this low frequency of antiidiotype antibodies in group B. 1) A second event leading to impairment of the antiidiotype response may be necessary for induction of the disease. 2) Genetic factors, such as TGF-β polymorphisms or HLA class II genotypes, might augment fetal events subsequent to the initial inflammatory response triggered by the maternal autoantibodies. 3) Other epitopes of La/SSB or Ro/SSA (52 kd or 60 kd) not included in this study might be involved. Previous studies have shown that anti–52-kd Ro/SSA antibodies are causally related to the development of CHB (26) and that maternal autoantibodies directed to a specific epitope within the leucine zipper amino acid sequence 200–239 (p200) of the Ro52 protein correlate with prolongation of fetal atrioventricular time and heart block (27). Recent data from a large study of sera from anti-Ro–positive women demonstrated a high prevalence of reactivity to p200, but the frequencies were similar between women who had children with CHB and women who had children who were healthy (28). 4) Mothers of group B actually possess antiidiotype antibodies, but either these are neutralized by idiotype antibodies or the affinity of the antiidiotype antibodies may be lower and, therefore, antiidiotype antibodies cannot be detected by conventional ELISA.

In this study it was also shown that mothers pregnant with healthy children, whose sera exhibited no apparent antiidiotype or idiotype activity against the major B cell epitope of La/SSB, contained hidden antibodies against this epitope in their sera, which was not evident in mothers of affected children. In fact, unmasked antiidiotype antibodies were present in mothers of both group B and group C, suggesting that antiidiotype antibodies bind to and neutralize the antibodies to the major epitope of La/SSB. Taken together, the hypothesis generated from these data is that antiidiotype antibodies in maternal sera protect the fetus from the pathogenic effects of antibodies targeting the major B cell epitope of La/SSB.

There is a clear need to identify an early marker of CHB, given that third-degree block has not been reversed to date (28). Despite a large effort to identify a single marker, such as a specific epitope of Ro/SSA or La/SSB, that would elucidate the risk of disease development, this has yet to be accomplished. The present study is limited by the fact that the level of antiidiotype response cannot predict the risk of neonatal lupus. However, the presence of antiidiotype antibodies against antibodies to the major B cell epitope of La/SSB could be used as a serologic marker of low-risk pregnancies.

REFERENCES

7. Tzioufas AG, Yiannaki E, Sakarellos-Daitisiotis M, Routsias JG,


