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Estimation of amounts of anti-La(SS-B) antibody directed against immunodominant epitopes of the La(SS-B) autoantigen

T. P. GORDON, M. GREER, P. REYNOLDS, A. GUIDOLIN & L. J. MCNEILAGE *Department of Clinical Immunology, Flinders Medical Centre, Bedford Park, South Australia*

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SUMMARY

The contribution of circulating anti-La(SS-B) antibody to the hypergammaglobulinaemia seen in primary Sjögren's syndrome is unknown. In this study levels of anti-La(SS-B) antibody directed against three immunodominant epitopes of the anti-La(SS-B) autoantigen were measured by ELISA in 84 anti-La(SS-B)$^+$ sera using purified recombinant protein and antibody affinity-purified against the three anti-La(SS-B) fusion proteins. There was marked variation in the amounts of IgG anti-La(SS-B) antibody detected, with levels ranging from 0·02 mg/ml to 11 mg/ml. The anti-La(SS-B) levels were >1 mg/ml in 61% of patients; in 18% of sera the anti-La(SS-B) level constituted 10% or more of the total serum IgG. However, other patients were seen with marked hypergammaglobulinaemia and low anti-La(SS-B) concentrations. These results support an antigen-driven mechanism for the anti-La(SS-B) response and suggest that anti-La(SS-B) antibody production is regulated independently of other immunoglobulins.

Keywords anti-La(SS-B) antibody concentration immunodominant autoepitopes

INTRODUCTION

Autoantibodies to the anti-La(SS-B) ribonucleoprotein together with hypergammaglobulinaemia and rheumatoid factor are characteristic serological features of primary Sjögren's syndrome (Moutsopoulos, 1980; Fox *et al.*, 1986). Although serum autoantibodies are believed to contribute significantly towards the elevation in serum IgG levels (Moutsopoulos & Manoussakis, 1989), measurement of the concentration of circulating anti-La(SS-B) has been hampered by difficulties preparing the purified autoantigen. The availability of full-length complementary DNA (cDNA) clones encoding La(SS-B) (Chambers *et al.*, 1988; St. Clair *et al.*, 1988; Rauh, Hornig & Luhrmann, 1988; Bini, Chu & Elkon, 1990; Kohsaka *et al.*, 1990; McNeilage, MacMillan & Whittingham, 1990) now enables synthesis of large amounts of recombinant protein that may be used for epitope mapping and antibody quantification.

Using soluble recombinant La(SS-B) proteins McNeilage *et al.* (1990) identified three immunodominant regions: LaA (aa 1-107); LaC (aa 111-242); and LaL2/3 (aa 346-408). The order of immunodominance based on the number of sera reacting with each region and the strength of reactivity was LaA > LaC $\gg$ LaL2/3. We have used affinity-purified antibody specific for each of these La(SS-B) fragments together with purified recombinant La(SS-B) to determine the concentration of autoantibody in a panel of 84 anti-La(SS-B) positive sera. The magnitude of the observed anti-La(SS-B) antibody response together with recognition of multiple regions of the molecule are in keeping with an autoantigen-driven immune response, with anti-La(SS-B) levels often contributing significantly to the hypergammaglobulinaemia.

Correspondence: Dr T. P. Gordon, Department of Clinical Immunology, Flinders Medical Centre, Bedford Park, South Australia 5042.

MATERIALS AND METHODS

Purification of recombinant fusion protein from La(SS-B) DNA fragments

The three La(SS-B) subfragments, LaA, LaC and LaL2/3 were sub-cloned into the pGEX-2 vector and expressed in *Escherichia coli* as fusions with the glutathione-S-transferase (GST) gene (McNeilage *et al.*, 1990). The La–GST fusion proteins were prepared by affinity purification according to the method of Smith & Johnson (1988).

Preparation of affinity-purified anti-La(SS-B) antibody

Soluble recombinant fusion protein (15–20 mg) was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. The efficiency of coupling for all three fragments was >95%. Ten anti-La(SS-B) antibody$^+$ sera were pooled and their immunoglobulin fractions isolated by caprylic acid precipitation (Stein-

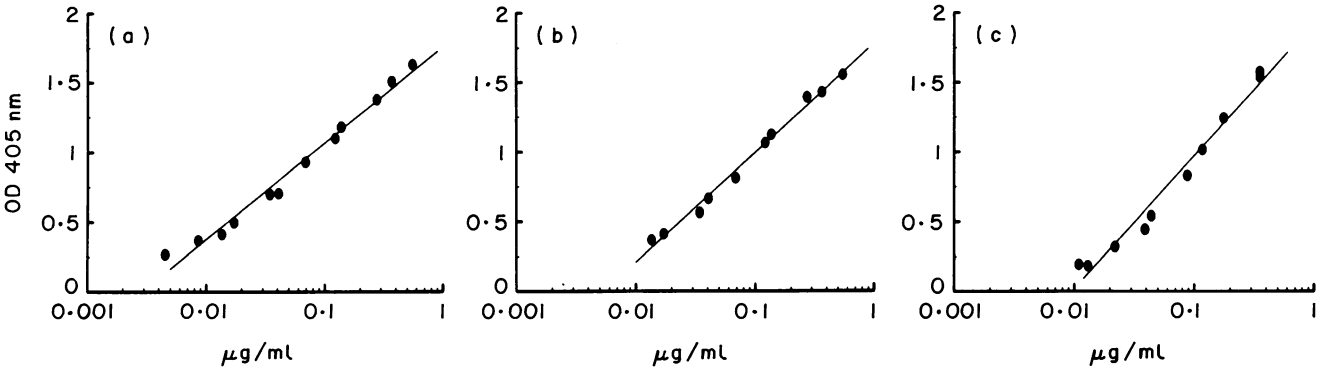

Fig. 1. Representative standard curves for calculation of the concentration of anti-LaA (α LaA), anti-LaC (αLaC) and anti-LaL2/3 (αLaL2/3) in anti-La(SS-B)$^+$ human sera. Equivalent amounts of LaA (a), LaC (b) and LaL2/3 (c) fusion proteins were coated onto ELISA plates and probed with two-fold dilutions of αLaA, αLaC and αLaL2/3 affinity-purified antibodies respectively. Optical density (OD) was read at 405 nm.

buch & Audran, 1969). Two millilitres of the immunoglobulin fraction in phosphate-buffered saline (PBS; 0·05 M sodium phosphate, 0·15 M NaCl, pH 7·4) were loaded onto each of the LaA, LaC, and LaL2/3 immunoabsorbent columns and, after washing with 300 ml of PBS, the bound anti-La(SS-B) was eluted with 0·1 M glycine containing 0·5 M NaCl, pH 2·3 and neutralized with 2 M Tris pH 7·5. The eluted fractions were pooled, dialysed against PBS and IgG levels were measured by rate nephelometry on a Beckman ICS II.

Quantification of anti-La(SS-B) antibody specific for the different La(SS-B) fusion proteins

Affinity-purified preparations of LaA-, LaC- and LaL2/3-GST fusion proteins (Smith & Johnson, 1988) were assessed for purity on SDS–PAGE (Laemmli, 1970). All three proteins gave single bands of the expected Mr when stained with Coomassie blue. For standardization of the three antigens, coating concentrations were chosen that gave the same reactivity on the plates with a polyclonal rabbit anti-GST antibody. One hundred microlitres of each recombinant protein diluted in 0·03 M carbonate, pH 9·6, was coated onto microwell ELISA plates (Nunc, Roskilde, Denmark) overnight at 4°C. Wells were blocked with 200 μl of 3% bovine serum albumin (BSA) for 60 min at 37°C and the LaA-, LaC- and LaL2/3-coated plates incubated for 60 min at 37°C with 150 μl of two-fold dilutions of the corresponding affinity-purified anti-La antibody.

The plates were washed four times with PBS–0·05% Tween 20 and bound IgG detected using alkaline phosphatase-conjugated goat anti-human IgG (Sigma Chemical Co., St Louis, MO) as described previously (Gordon *et al.*, 1990). Standard curves were constructed by plotting optical density (OD 405 nm) against antibody concentration (mg/ml).

Duplicate samples of serum diluted 1/1000 were assayed for binding to the different La(SS-B) fragments as described above and anti-La(SS-B) concentrations read from the appropriate standard curves. Further dilutions were made for those sera whose absorbance readings did not fall initially within the linear portion of the standard curve.

Potassium thiocyanate (KSCN) elution of anti-La(SS-B) antibodies bound to La(SS-B) fragments

Anti-La(SS-B)$^+$ sera diluted 1/200 or anti-La(SS-B) affinity purified from the three La–GST fusion proteins were bound to fusion protein-coated ELISA plates as described above. After a 60 min incubation the plates were washed and 100 μl of PBS containing either 0, 0·5, 1·0, 2·0 or 3·0 M KSCN were added to each well for 15 min, followed by washing and detection of non-eluted antibody. Results were expressed as a percentage of absorbance readings in the absence of thiocyanate (Pullen, Fitzgerald & Hosking, 1986).

Human sera

Anti-La(SS-B) antibody concentrations were determined in 84 sera positive for anti-La(SS-B) by immunodiffusion and Western blotting (McNeilage *et al.*, 1990). Serum IgG concentrations were measured by nephelometry (Beckman ICS II).

RESULTS

Construction of standard curves to measure anti-La(SS-B) concentration

Figure 1 shows representative standard curves of OD 405 nm plotted against affinity-purified anti-La(SS-B) antibody concentrations from which the amount of anti-LaA, anti-LaC and anti-LaL2/3 were determined. All anti-La(SS-B)$^+$ sera gave OD readings of $>0·25$. The OD value was always $<0·2$ when a panel of 45 normal sera were tested on LaA-, LaC- and LaL2/3-coated ELISA plates. Affinity-purified antibodies were of the IgG isotype and no contaminating proteins were observed on SDS-PAGE. The intra-assay coefficient of variation for the ELISA was 5·3% ($n=15$) and the interassay variation for 15 assays on separate days was 17·0%. Analysis of unbound fractions from the affinity columns showed removal of specific anti-La(SS-B) activity by each column, whereas reactivity to the other La(SS-B) fragments in the flow-through remained intact. The specificity of each of the three affinity-purified antibodies was confirmend by their lack of cross-reactivity with the other two La–GST fusion proteins at the concentrations used in the standard curves (Fig. 2).

To determine whether our affinity-purified antibodies were of similar relative avidity to the population of anti-La(SS-B) antibodies in the pool of 10 anti-La(SS-B)$^+$ sera, resistance to thiocyanate elution was measured as described (Pullen *et al.*, 1986). Figure 3 shows that the elution profiles of both populations of antibodies were similar over a range (1·5–3·0 M) of KSCN concentrations, supporting the view that anti-La(SS-B)

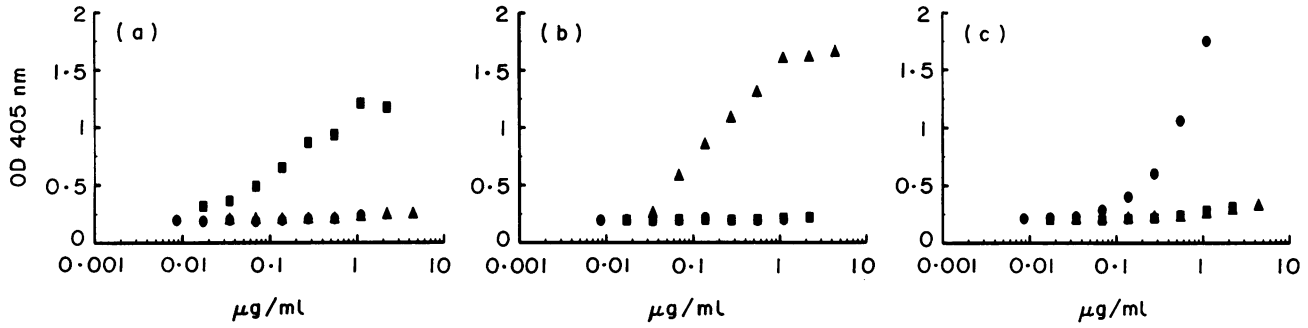

Fig. 2. Specificity of the affinity-purified anti-La(SS-B) antibodies. LaA (a), LaC (b) and LaL2/3 (c) fusion proteins were coated onto ELISA plates and probed with two-fold dilutions of the three affinity-purified antibodies αLaA (■), αLaC (▲), and αLaL2/3 (●). Optical density (OD) was read at 405 nm.

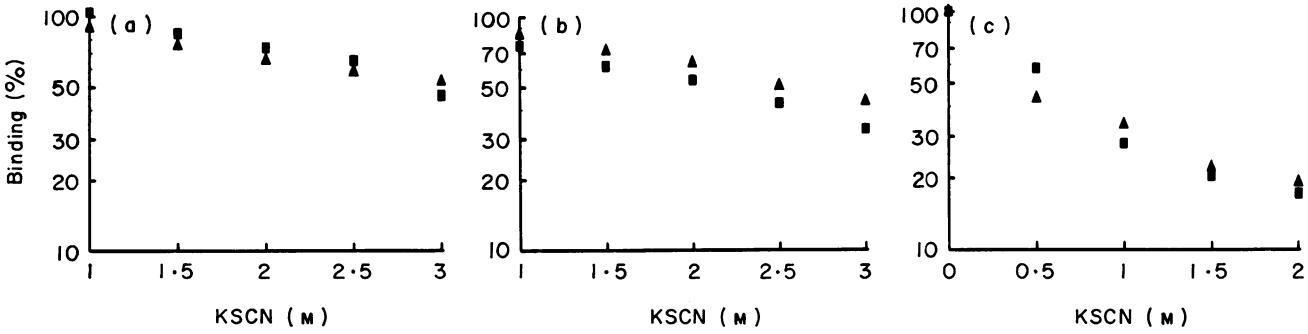

Fig. 3. Potassium thiocyanate (KSCN) elution profiles of affinity-purified anti-La(SS-B) antibodies (αLaA, αLaC, αLaL2/3) compared with a pool of 10 anti-La(SS-B)$^+$ human sera (αLa). Affinity-purified antibody or pooled serum were bound to LaA (a), LaC (b) or LaL2/3 (c) fusion proteins coated onto ELISA plates and then incubated for 15 min with varying concentrations of KSCN. Results were expressed as a percentage of total binding of specific antibody. ■, αLaA, αLaC, αLaL2/3; ▲, αLa.

antibodies of both high and low affinities are eluted from the immunoaffinity columns. It therefore seems unlikely that measurements taken from the standard curves are overestimating autoantibody levels.

Concentrations of serum anti-La(SS-B) antibody reacting with immunodominant epitopes of the La(SS-B) autoantigen

The box plots in Fig. 4 summarize results from the 84 anti-La(SS-B)$^+$ sera tested against the LaA, LaC and LaL2/3 fusion proteins. The line in the box represents the 50th percentile, and upper and lower edges of the box the 25th and 75th percentile. Outlays to the 10th and 90th percentile are shown as open circles. The majority (85%) of sera tested had hypergammaglobulinaemia (median serum IgG 26·0 mg/ml, range 4·7–57·9 mg/ml; normal range 5–19 mg/ml). The concentrations of total IgG anti-La(SS-B) antibody (taken as the sum of A, C and L2/3) measured in the 84 sera varied over a wide range (0·02–11 mg/ml) with a medial value of 1·1 mg/ml. There was considerable variation in the amounts of specific anti-La(SS-B) antibody directed against the three fusion proteins, such that anti-LaA (median 0·70 mg/ml, range 0·002–7·68 mg/ml) > anti-LaC (median 0·3 mg/ml; range 0·002–8·96 mg/ml) > anti-LaL2/3 (median 0·034 mg/ml, range 0·001–0·4 mg/ml). However, concentrations of 1 mg/ml or greater were observed in 61% of sera tested. Eighteen percent of patients' sera contained anti-La(SS-B) levels which contributed over 10% of the total serum IgG, 42% of the patients had greater than 5% anti-La(SS-B) and 80% had greater than 1% anti-La(SS-B) (Fig. 4b). When anti-La(SS-B) antibody concentrations in the 84 sera were compared with total serum IgG concentrations by linear regression there was no significant correlation of the IgG level with the anti-LaA, anti-LaC, anti-LaL2/3 or total anti-La levels. Some patients with marked hypergammaglobulinaemia were observed to have low levels of anti-La(SS-B), e.g. of the 10 patients with IgG levels of >40 mg/ml, four had total anti-La(SS-B) concentrations of $<0·5$ mg/ml.

DISCUSSION

This study measured the serum concentrations of anti-La (SS-B) autoantibody reacting with the three major antigenic regions of the La(SS-B) polypeptide in a panel of 84 anti-La(SS-B)$^+$ sera. The quantities of specifc anti-La(SS-B) autoantibody were often in the milligram range, thereby contributing to the hypergammaglobulinaemia. This was consistent with an earlier study in which milligram per millilitre quantities of anti-La(SS-B) were purified from patients' sera (Horsfall *et al.*, 1986). In one fifth of the patients the IgG anti-La(SS-B) response constituted 10% or more of the serum IgG level, whilst in five patients the ratio was greater than 20%. However, there was a lack of correlation between anti-La(SS-B) levels and the total IgG, with some patients showing hypergammaglobulinaemia with low amounts of circulating anti-La(SS-B). Moreover, preliminary studies in this unit on serial samples have shown that fluctuations in anti-La(SS-B) concentrations are not linked to changes in serum IgG levels (unpublished data). This suggests that anti-

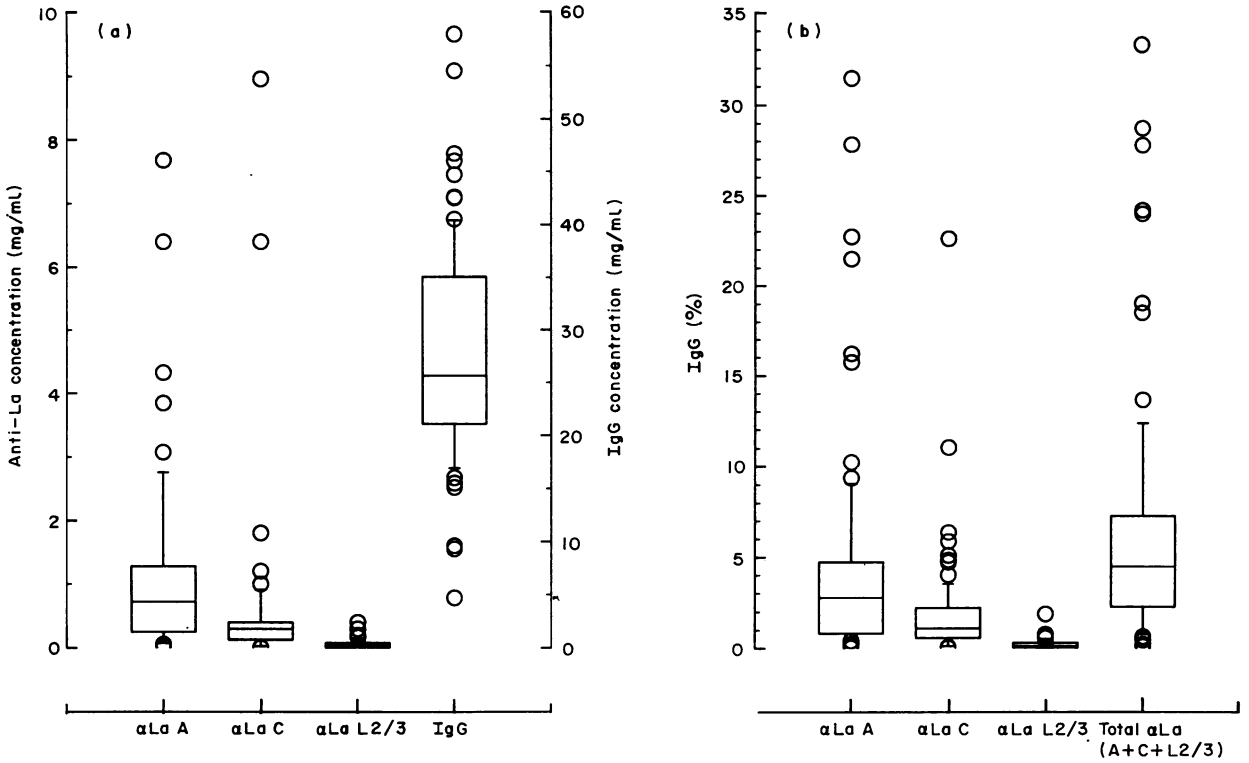

Fig. 4. Box plots showing the concentrations of specific antibody binding to LaA (αLaA), LaC (αLaC) and anti-LaL2/3 (αLaL2/3) fusion proteins together with IgG levels in 84 anti-La(SS-B)$^+$ sera (a). The contribution of specific antibody to the serum IgG level (per cent IgG) is shown in (b). The boxes represent the 25 to 75 percentiles, the bars show the median and the circles represent outlays to the 10 and 90 percentiles.

La(SS-B) antibodies may be regulated independently from other immunoglobulins.

Using immune precipitation with calf thymus extract Maddison & Reichlin (1977) estimated that in some cases anti-RNP antibody accounted for over 20% of the serum IgG and 33% of the total immunoglobulin in one case; these values are comparable with those reported here for anti-La(SS-B). However, instead of using a mixture of antigens we have used purified recombinant proteins together with known concentrations of affinity-purified antibody to measure directly the anti-La(SS-B) antibody concentrations.

Our results indicate that there is a wide range in the concentrations of anti-La(SS-B) antibody reacting with La(SS-B), with the estimated levels similar to and often higher than would be expected from immunisation with foreign antigen. As noted by Maddison & Reichlin (1977) for anti-RNP, the anti-La(SS-B) levels occasionally accounted for the majority of the hypergammaglobulinaemia. Why this was true for some but not all anti-La(SS-B) sera is unclear, as is the relationship of the anti-La(SS-B) response and raised IgG levels to the pathogenesis of Sjögren's syndrome. These cross-sectional studies suggest that factors contributing to anti-La(SS-B) antibody production may be independent of those controlling other immunoglobulins, and taken together with the multiple epitope distribution, support the concept that these autoantibodies are generated by self-immunisation rather than indiscriminate polyclonal B cell activation (reviewed in Tan, 1989).

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