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Immunology

Properties of the ribosomal P2 protein autoantigen are similar to those of foreign protein antigens

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ABSTRACT **Approximately 15% of patients with systemic lupus erythematosus have autoantibodies that bind to a shared epitope previously shown to be located on the carboxyl-terminal 22 amino acids of three 60S ribosomal proteins, P0, P1, and P2 ("P proteins"). A hydrophilicity plot and fine epitope mapping with seven synthetic peptides revealed that the properties of the antigenic site were similar to certain properties of epitopes on foreign protein antigens—namely, the epitope was located in the most hydrophilic portion of the P2 protein and also in the terminal region of the molecule. However, this site has been highly conserved during evolution. A mouse monoclonal antibody induced by immunization with ribosomal proteins had a fine specificity similar to the lupus antibodies. This finding indicates that a highly conserved region of a lupus autoantigen may also be antigenic in some normal animals. Therefore, lupus autoantibodies may be similar in most, if not all respects, to antibodies produced by immunization.**

A number of factors determine which regions of a protein are recognized by antibodies as antigenic sites (epitopes) after immunization with a foreign protein (reviewed in refs. 1 and 2). Host factors include the B- and T-cell repertoire and major histocompatibility complex determinants. Those regions of the immunogen that differ from the homologous host protein and which are accessible and flexible are most likely to be targeted by the host's antibodies. Although accessibility on the surface of a protein is usually required for antigenicity (and therefore is largely predictable by hydrophilicity values), the degree of foreignness and flexibility may be more important discriminators (2–5). Terminal regions of proteins are usually accessible and flexible (6) and therefore are frequently antigenic (4).

In contrast to the "rules" governing antigenicity of foreign proteins, little is known about the epitopes of autoantigens in multisystem autoimmune diseases. Elkon *et al.* (7) recently localized the major linear (sequential/continuous) epitope on three phosphoproteins (P0, P1, and P2) from the large ribosomal subunit that is recognized by sera from patients with systemic lupus erythematosus (SLE). The epitope is highly conserved and is contained within the carboxyl-terminal 22 amino acids of all three P proteins. A synthetic peptide corresponding to the carboxyl-terminal 22 amino acids of P2 from *Artemia salina* was able to absorb completely the reactivity of SLE anti-P sera with all three P proteins on an immunoblot (7). To compare the properties of the autoantigen to those of foreign protein epitopes, we performed fine specificity mapping of the amino acids recognized by SLE anti-P sera, using synthetic peptides and a site-specific amino acid modification.

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MATERIALS AND METHODS

Hydrophilicity and Variability Plots. The amino acid sequence of eL12, the *A. salina* equivalent of the human protein P2, was obtained from Amons *et al.* (8). Each residue was assigned a numerical value related to the free energy of water-vapor transfer and the interior–exterior distribution of amino acid side chains (9); the average value of nine consecutive amino acids was plotted by using the IBI Pustell program. A variability plot of P2 proteins from yeast (10), *Drosophilia* (11), *A. salina* (8), rat (12), and human (13) was performed as described by Wu and Kabat (14).

Synthetic Peptides. The peptides used for epitope mapping were synthesized by a solid-phase method (15). The composition of each peptide was confirmed by amino acid analysis. Peptides were conjugated to bovine thyroglobulin or rabbit serum albumin at a 50:1 molar ratio by using glutaraldehyde as described (7). A specific modification of the single methionine residue within the peptide was made by oxidation with chloramine T (16). Thin-layer chromatography confirmed that the methionine in the peptide had been quantitatively oxidized to methionine sulfoxide.

Antisera and Tissue Antigens. SLE sera containing anti-P activity have been described elsewhere (17, 18). An IgM mouse monoclonal antibody produced by immunizing a BALB/c mouse with total chicken ribosomal proteins followed by whole chicken ribosomes (19) was a gift from H. Towbin (CIBA–Geigy). Saline-soluble mouse and chicken spleen extracts as well as salt-washed ribosomes were isolated and used for immunoblotting as described (17, 20).

Epitope Mapping. Antibody binding to the synthetic peptides was measured by a solid-phase ELISA. The ELISA was a minor modification of the radioimmunoassay described elsewhere (21). Briefly, microtiter wells were coated with peptide conjugates, blocked with bovine serum albumin, and sequentially probed with dilutions of SLE anti-P serum followed by alkaline phosphatase-conjugated goat anti-human IgG (Sigma). The reaction was developed with *p*-nitrophenyl phosphate, and the OD at 405 nm was read on a Dynatech ELISA reader. In all cases, serial dilutions of patients' sera were made to ensure that, at the dilution tested, reactivity to peptide C-22, which comprises the 22 carboxyl-terminal residues of *A. salina* P2, was on the linear portion of the titration curve. The OD_{405} value represents binding of IgG to the peptide after subtraction of binding of the same serum to glutaraldehyde-coupled carrier alone. Results expressed as a percentage were calculated from: (binding to test peptide ÷ binding to C-22) × 100. ELISA for the mouse monoclonal anti-P antibody was developed with alkaline phosphatase-conjugated goat anti-mouse IgM (Sigma).

Abbreviation: SLE, systemic lupus erythematosus.

RESULTS

Hydrophilicity and Variability. A hydrophilicity plot of the *A. Salina* P2 protein reveals a long hydrophobic stretch followed by a hydrophilic carboxyl-terminal segment (Fig. 1). The hydrophobic region is rich in alanine residues and is predicted to consist of α helices (12). The linear epitope recognized by SLE anti-P antibodies, between amino acids 90 and 111 (7), is contained within the most hydrophilic portion of the protein. The secondary structure of this region is uncertain (12). A variability plot shows that the amino and carboxyl termini are the most highly conserved sites on the P2 protein (Fig. 2).

Epitope Mapping of SLE Anti-P Antibodies. The composition of the synthetic peptide antigens used in this study is given in Fig. 3. Fig. 4 shows the pattern of binding of anti-P antibodies from 6 of 12 SLE sera analyzed to five synthetic peptides that correspond to the carboxyl-terminal 3 (peptide C-3), 5 (peptide C-5), 7 (peptide C-7), 11 (peptide C-11), and 22 (peptide C-22) residues of *A. salina* P2. The binding of all 12 sera was virtually identical when peptide C-11 or C-22 was used as antigen. Therefore, the epitope was localized to the terminal 11 residues. Although no serum bound significantly to peptide C-3, considerable heterogeneity was noted in the binding of each individual serum to peptides C-5 and C-7. The most striking difference was the virtual absence of (serum A) or low (21%, serum B) binding of two sera to the terminal seven residues compared to a >70% binding of two other anti-P sera (D and E) to C-7. To determine whether a discrete epitope could be located within the internal (I) portion of the peptide, immunoassays were repeated using the peptides I-5 and I-7 (see Fig. 3) as antigens. None of the SLE anti-P sera bound to the I-5 peptide conjugate, whereas variable binding (between 11% and 43%) was observed for peptide I-7. Since the single methionine at position 105 is present in both antigenic peptides C-11 and C-7, the effect of oxidation of methionine [to methionine sulfoxide, Met(O)] on antibody binding was tested. The modified peptide [Met(O)-containing C-11] was coupled to rabbit serum albumin (RSA) as described in Fig. 4, and the ELISA was repeated with 17 SLE anti-P sera. Thirteen sera reacted as well to Met(O)-containing C-11 as to the unmodified peptide C-11, whereas four sera showed significantly decreased binding (12%, 39%, 40%, and 63% of control values).

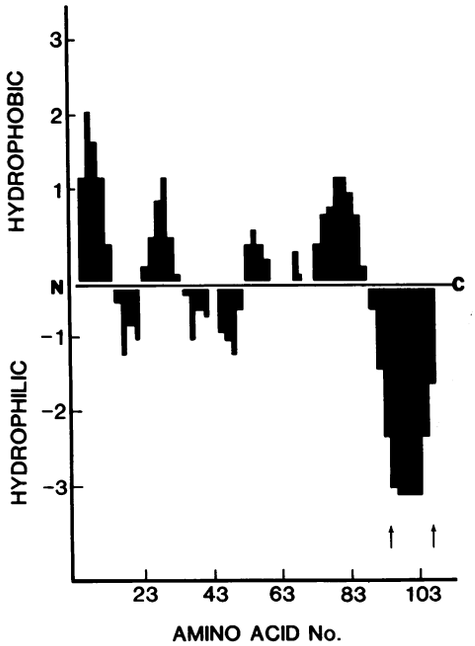

FIG. 1. Hydrophilicity plot of the *A. salina* P2 protein as described by Kyte and Doolittle (9). The amino (N) and carboxyl (C) regions are indicated as is the carboxyl-terminal 22 residues (between arrows) previously shown to contain the antigenic determinant(s) (7). The hydrophilicity plot was performed with an IBI Pustell program.

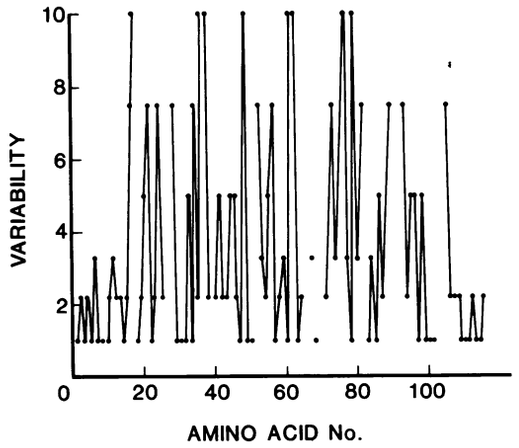

FIG. 2. Variability plot of yeast, *Drosophila, Artemia*, rat, and human P2 proteins. Gaps were introduced into some sequences to maximize homology, and these regions were excluded from evaluation. Variability was calculated by dividing the number of different amino acids at a given position by the frequency of the most common amino acid at that position (14).

The known and predicted carboxyl-terminal amino acid sequence of the P2 proteins from *A. salina* (8) and human (13) sources, respectively, are identical except for the conservative replacements of glutamic acid by aspartic acid at residues 91 and 103 and of glutamic acid by serine at residue 10[illegible] (see Fig. 3). Comparison between the binding of 12 anti-P sera to the human or *A. saline* C-11 synthetic peptides revealed that most sera bound equally well (<15% difference) to either antigen by ELISA. However, one serum (A in Fig. 4) showed greater binding to the *A. salina* C-11 peptide than to human C-11. Preferential binding to the *A. salina* sequence was confirmed by an inhibition assay in which significantly more human C-11 peptide was required to inhibit binding to either the human or *A. salina* C-11 peptide (not shown).

A mouse monoclonal anti-P antibody induced by immunization with chicken ribosomes has been shown (19) to bind to all three P proteins on an immunoblot and to the P proteins from all species tested. These findings suggest that the mouse antibody, like SLE anti-P antibodies (17, 22), recognizes the conserved carboxyl terminus of P2. Fig. 5 shows that the immunoblot profiles of the mouse and human anti-P antibodies are the same and that the mouse monoclonal reactivity against chicken (the immunogen, lane d) and mouse (autoan-

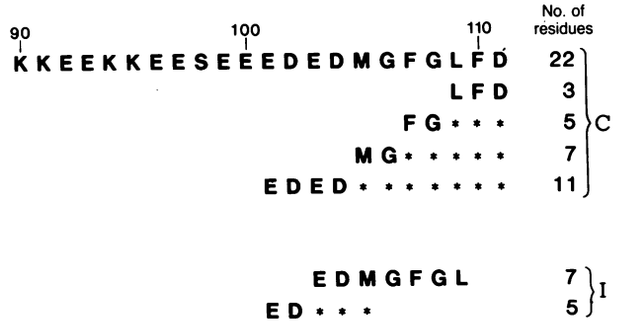

FIG. 3. Amino acid sequences (single-letter code) of seven synthetic peptides used to map epitopes recognized by SLE anti-P antibodies. Peptides were synthesized by solid-phase methods and conjugated to bovine thyroglobulin at a 50:1 molar ratio as described. C, peptides terminating at the carboxyl end; I, internal peptides; *, identical residue to the amino acid above.

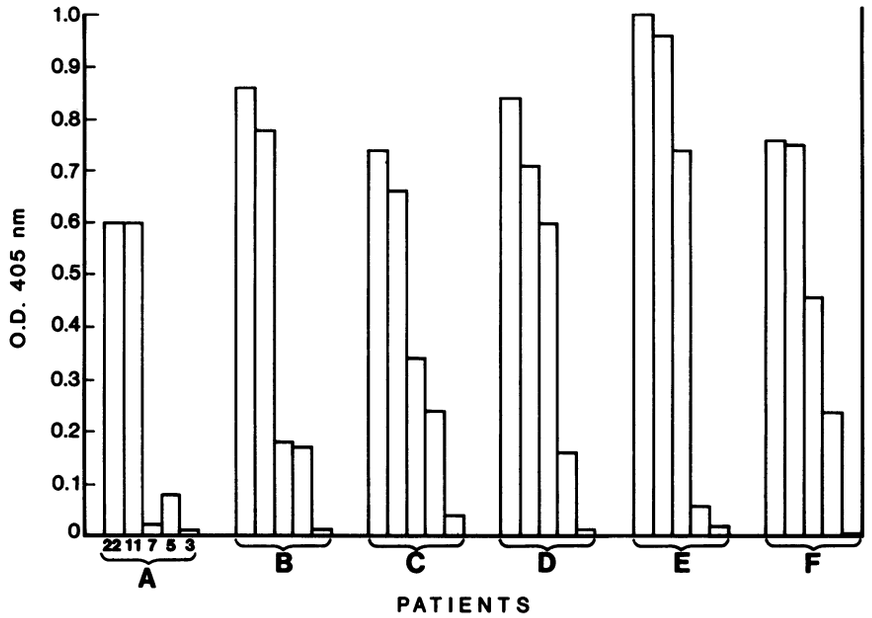

FIG. 4. Patterns of binding of six anti-P SLE sera to five different synthetic peptides, C-3, C-5, C-7, C-11, and C-22. Peptide conjugates were absorbed to microtiter wells of an ELISA plate and sequentially incubated with dilutions of patient sera and alkaline phosphatase-conjugated goat anti-human IgG. The OD_{405} reading represents binding to the peptide after subtraction of binding to glutaraldehyde-treated bovine thyroglobulin in the absence of peptide.

tigen, lane e) P proteins are similar. The ELISA profile of the monoclonal anti-P was also similar to that of SLE anti-P antibodies. The OD_{405} values for peptides were: C-22, 0.7; C-11, 0.76; C-7, 0.68; C-5, 0.2; C-3, 0.

DISCUSSION

In SLE and related diseases, autoantibodies bind to a limited number of intracellular proteins (23). It is not known why these proteins become antigens or what role they play in the initiation and perpetuation of antibody synthesis. According to some studies, autoantibodies in SLE arise through polyclonal B-cell activation secondary either to intrinsic B-cell hyperreactivity or suppressor T-cell deficiencies (24–26). These models predict that the autoantibodies would be produced against multiple unrelated self and foreign proteins. The relatively small number of protein antigens, multiple epitopes on the same antigen, autoantibodies reactive against linear and conformational determinants (7), and the limited number of B-cell clonal precursors (27) are not in accord with these predictions. Further, immunization of lupus patients (28, 29) or mice (30) with protein antigens fails to induce an abnormal response or to augment autoreactivity, and SLE

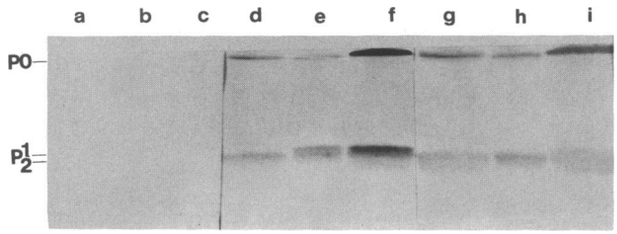

FIG. 5. Immunoblot analysis of a normal mouse serum (lanes a–c), a mouse monoclonal anti-P antibody (lanes d–f), and an SLE anti-P serum (lanes g–i). Sixty micrograms of chicken (lanes a, d, and g) or mouse (lanes b, e, and h) spleen extracts and 2 μg of rabbit ribosomal protein (lanes c, f, and i) were used as sources of P proteins. The reactions were developed with alkaline phosphatase-conjugated goat anti-mouse IgM (lanes a–f) and goat anti-human IgG (lanes g–i).

sera bind to fewer foreign (*Escherichia coli*) proteins compared to sera from normal controls (31).

To further characterize the nature of the autoantibody response in SLE, the properties of the P2 autoantigen were compared with those described for foreign protein antigenic sites. The major linear epitope of the P2 protein identified previously (7) was located within the most hydrophilic region of the protein. Fine epitope mapping with seven synthetic peptides indicated that, for all anti-P sera tested, the epitope(s) resided within the carboxyl-terminal 11 residues. Therefore, these two properties, hydrophilicity and terminal location, are similar for the P2 autoantigen and foreign protein epitopes. In addition, the epitope on P2 is likely to be accessible because of its hydrophilicity and the ability of anti-P antibodies to inhibit protein synthesis *in vitro* and when injected into the living cell (data not shown). Although all anti-P antibodies tested bound to the terminal 11 residues of the P2 protein, there appeared to be at least two overlapping epitopes within this region—one almost completely contained within peptide C-7 and another requiring residues further toward the amino end of the C-11 peptide. Individual serum also showed considerable variation in binding to shorter peptides or to a peptide oxidized at the methonine residue. Whether this heterogeneity is due to the random way in which the B-cell repertoire is generated (32) or is related to possible stimuli for antibody production remains to be determined. Similarly, the "heteroclitic" activity [greater reactivity with a protein antigen of a species different from the presumed immunogen (33, 34)] noted for one anti-P serum may be explained by one of these mechanisms. Regardless of the mechanisms involved, multiple overlapping epitopes and microheterogeneity in individual recognition sequences are also characteristic of the immune response to foreign proteins (1, 2).

The single property of the epitope recognized by anti-P antibodies that differs from the properties described for epitopes on foreign proteins is the conserved nature of peptide C-11. As shown in Fig. 2, the amino and carboxyl termini of the P2 proteins have the least variability on a Wu and Kabat plot. Most, if not all, autoantibodies to intracellular proteins in the autoimmune diseases bind to antigens

from multiple mammalian species as well as invertebrates, indicating that the antibodies recognize conserved regions of the proteins (35). This raises the question as to whether antibodies that react with highly conserved epitopes can be induced by immunization. Whereas rabbit anti-self cytochrome C antibodies preferentially bound to nonconserved regions of the protein (36, 37) and several monoclonal antibodies produced by immunization failed to react with self (38, 39), both polyclonal (40–43) and monoclonal (44, 45) autoantibodies have been produced by immunization. The finding that a monoclonal anti-P antibody derived from nonautoimmune mouse strain BALB/c bound to murine P proteins and had a fine specificity indistinguishable from SLE anti-sera when tested against a panel of synthetic peptides indicates that SLE autoantibodies may be similar in all respects to antibodies produced by immunization. Whether autoantibodies to conserved epitopes are a frequent result of immunization with SLE autoantigens and what the relative contributions of antigen and host factors are to autoantibody production in spontaneous SLE remain to be determined.

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