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Quantification of lupus anti-ribosome P antibodies using a recombinant P2 fusion protein and determination of the predicted amino acid sequence of the autoantigen in patients' mononuclear cells

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SUMMARY

The cDNA encoding the ribosomal protein P2 antigen was cloned from a human cDNA library constructed in the lambda gt11 expression vector. A β-galactosidase-P2 fusion protein was purified to near homogeneity and used to develop an ELISA which was highly specific for anti-P antibodies produced in murine and human SLE. The median concentration of human IgG anti-P antibodies in serum was estimated to be 100 μg/ml (range 6–450 μg/ml). Pre-incubation of human anti-P sera with a synthetic peptide, corresponding to the C-terminal 22 amino acids of P2, completely inhibited reactivity with the fusion protein in the ELISA. These findings confirm that lupus anti-P sera show a striking restriction in epitope specificity and indicate that the P2 fusion protein is a useful alternative to the synthetic peptide antigen for detection and quantification of anti-P antibodies. To investigate the possibility that anti-P antibodies were induced by 'altered-self', cDNA encoding P2 were also cloned from lupus patients and control mononuclear cells. The predicted amino acid sequences of the patients' P2 were identical to that of the normal controls indicating that a primary structural abnormality of the P2 autoantigen was unlikely.

Keywords ribosomal P protein systemic lupus erythematosus autoantibodies

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by serum antibodies directed against intracellular proteins and nucleic acids (reviewed by Christian & Elkon, 1986). Approximately 15% of unselected SLE patients (Bonfa & Elkon, 1986), but a high proportion of patients with lupus psychosis (Bonfa *et al.*, 1987), produce antibodies against three ribosomal phosphoproteins P0, P1, and P2. A minority of MRL/lpr mice also produce anti-ribosome P antibodies that bind to all three P proteins on immunoblots (Bonfa *et al.*, 1988). The human (Elkon *et al.*, 1986) and most murine (Bonfa *et al.*, 1988) lupus anti-P sera recognize a highly conserved, linear epitope contained within the carboxyl termini of all three P proteins. Recent cloning and sequencing of human cDNAs encoding the P proteins has confirmed that the carboxyl terminal 17 amino acids of P0, P1, and P2 are identical, except for one conservative substitution (Rich & Steitz, 1987). Using a synthetic peptide corresponding to the 22 carboxy-terminal amino acids of P2 (C-22) (Elkon *et al.*, 1986), we determined that all SLE anti-P antisera tested bound to the synthetic peptide (Bonfa *et al.*, 1987) but the peptide could not completely absorb anti-P binding to the antigens in the native state (Elkon *et al.*, 1986). This finding suggested that the anti-P antibodies recognized additional determinants on the P proteins. In order to perform further mapping of antigenic determinants, and to develop an immunoassay using a full-length human P protein, we cloned the cDNA encoding the P2 protein and compared the sensitivity and specificity of ELISAs using either the C-22 synthetic peptide or recombinant fusion protein.

In multisystem autoimmune disorders, considerable evidence also suggests that autoantibody production is antigen-driven: (i) there is a limited number of protein antigens (Gharavi, Chu & Elkon, 1988), many of which are found in the same ribonucleoprotein complexes (Lerner & Steitz, 1979); (ii) multiple epitopes may be recognized on a single protein antigen (Earnshaw *et al.*, 1987) and the properties of the autoantigen epitopes are similar to the properties of epitopes on foreign

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protein antigens (Elkon *et al.*, 1988); (iii) there is limited clonal diversity with extensive somatic mutation of genes encoding individual autoantibodies (Schlomchik *et al.*, 1987); (iv) injected self antigen augments the autoantibody response and T helper cells are necessary (Cohen & Eisenberg, 1982; Londei, Battazo & Feldmann, 1985; Wofsy & Seaman, 1985). Despite all of these observations, the target autoantigens have not been studied in detail. To determine whether variation in the predicted primary sequence of the P2 protein could explain autoantibody production in patients with SLE, we compared the nucleotide sequences of cDNA obtained from peripheral blood mononuclear cells (PBM) of SLE patients and normal controls.

MATERIALS AND METHODS

Patients

Serum was obtained from the following groups: 20 normal, healthy subjects; 18 SLE patients with anti-P antibodies as defined by radioimunoassay employing the synthetic peptide as antigen (Bonfa *et al.*, 1987); 16 SLE patients without anti-P antibodies as defined by the same assay; 40 disease controls (eight patients each with the following diseases: Sjögren's syndrome, rheumatoid arthritis, polymyositis, malaria, melanoma).

Mice

MRL/lpr mice were obtained from Jackson Laboratories, Bar Harbor, ME; New Zealand B/W F1 and NIH/Swiss mice were bred at the Hospital for Special Surgery, New York.

cDNA cloning and screening

HeLa cytoplasmic extracts were prepared by Dounce homogenization as described (Elkon & Jankowski, 1985). RNA was extracted by the guanidinium thiocyanate caesium chloride method (Glisin, Crkvenjakov & Byus, 1974; Chirgwin *et al.*, 1979) and poly(A)$^+$ RNA obtained by oligo(-dT) cellulose chromatography. Cloning into lambda gt11 was performed by standard procedures (Huynh, Young & Davis, 1985). Recombinant phages were plated on *E. coli* strain Y1090 at 2×10^4 plaques per 15 cm diameter plate. The expression of β-galactosidase fusion proteins was induced with 10 mM isopropyl thiogalactopyranoside (IPTG). Subsequently the filters were screened with an apparently monospecific anti-P serum followed by ^{125}I-labelled protein A. Positive regions were excised, the phage eluted and re-screened until the plaques were uniformly positive. Phage inserts were subcloned into the Eco R1 restriction site of pGem 3 (Promega Biotec, Madison, WI) and DNA sequencing performed by the dideoxy chain termination method (Sanger, Nickleu & Coulson, 1977).

Expression of a β-galactosidase fusion protein

The lysogenic *E. coli* strain Y 1089 was infected with a recombinant phage containing the cDNA encoding the full-length P2 protein. The cells were grown in NZCYM medium at 30°C to an OD at 605 nm of 0·6 and then incubated at 45°C for 20 min. IPTG was added to 10 mM and the culture incubated at 37°C for another 1–2 h. The cells were harvested by centrifugation, and the insoluble fusion protein recovered by the method of Adam *et al.* (1986). Total protein was measured by the method of Bradford (1976).

ELISA assays

Microtitre wells were coated with 200 ng of P2 fusion protein or the C-22 synthetic peptide conjugate (Elkon *et al.* (1986) and see below) at 4°C overnight. The plates were washed with PBS (0·15 M NaCl buffered with 10 mM phosphate, pH 7·4), and then blocked with 1% bovine serum albumin (BSA-PBS) for 1 h at room temperature. Serum samples (diluted 1:500 in PBS containing 0·05% Tween-20 and 10% normal goat serum) were added to the wells. The plates were incubated at 37°C for 3 h. Alkaline phosphatase-conjugated goat anti-human or goat anti-mouse IgG, diluted 1:1000 in PBS containing 10% normal goat serum, was then added to the plates and incubation was performed for an additional hour at 37°C. The reaction was developed with *p*-nitrophenylphosphate and the optical density (OD) read at 405 nm with a Dynatech Multiscanner (Flow Laboratories). Values greater than 3 standard deviations above the mean of 20 normal controls were considered positive.

Affinity purification of anti-P antibodies

A 22 amino acid peptide corresponding to the carboxyl terminus of the sequence predicted for the HeLa P2 cDNA was synthesized by a solid phase method (Barany & Merrifield, 1980). The peptide was isolated by high pressure liquid chromatography and the composition confirmed by amino acid sequence analysis. All but one SLE anti-P serum bound equally well to peptides corresponding to the *Artemia salina* or human P2 sequence (Elkon *et al.*, 1988). The C-22 synthetic peptide was coupled to Affigel 10 (Biorad, Richmond, CA) according to the manufacturer's instructions at a ratio of 2 μM/ml of gel. A high titre anti-P serum was passed through the column and anti-P peptide antibodies eluted with 0·2 M glycine HCl, pH 2·8. Anti-P activity

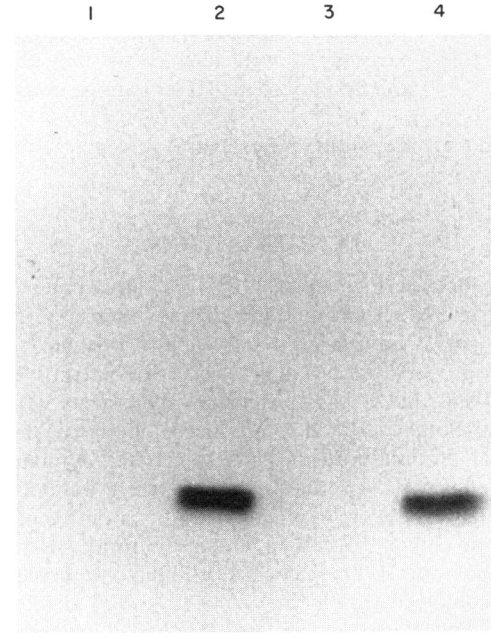

Fig. 1. Immunoprecipitation of P2 synthesized *in vitro*. The plasmid pTP2, containing the P2 cDNA, was transcribed by SP 6 polymerase. The RNA was used to program a rabbit reticulocyte lysate and immunoprecipitation of the ^{35}S-methionine labelled product was attempted with either SLE anti-P sera (lanes 2 and 4), a normal human serum (lane 1) or a serum from a SLE patient with antibodies against Ro and La (lane 3).

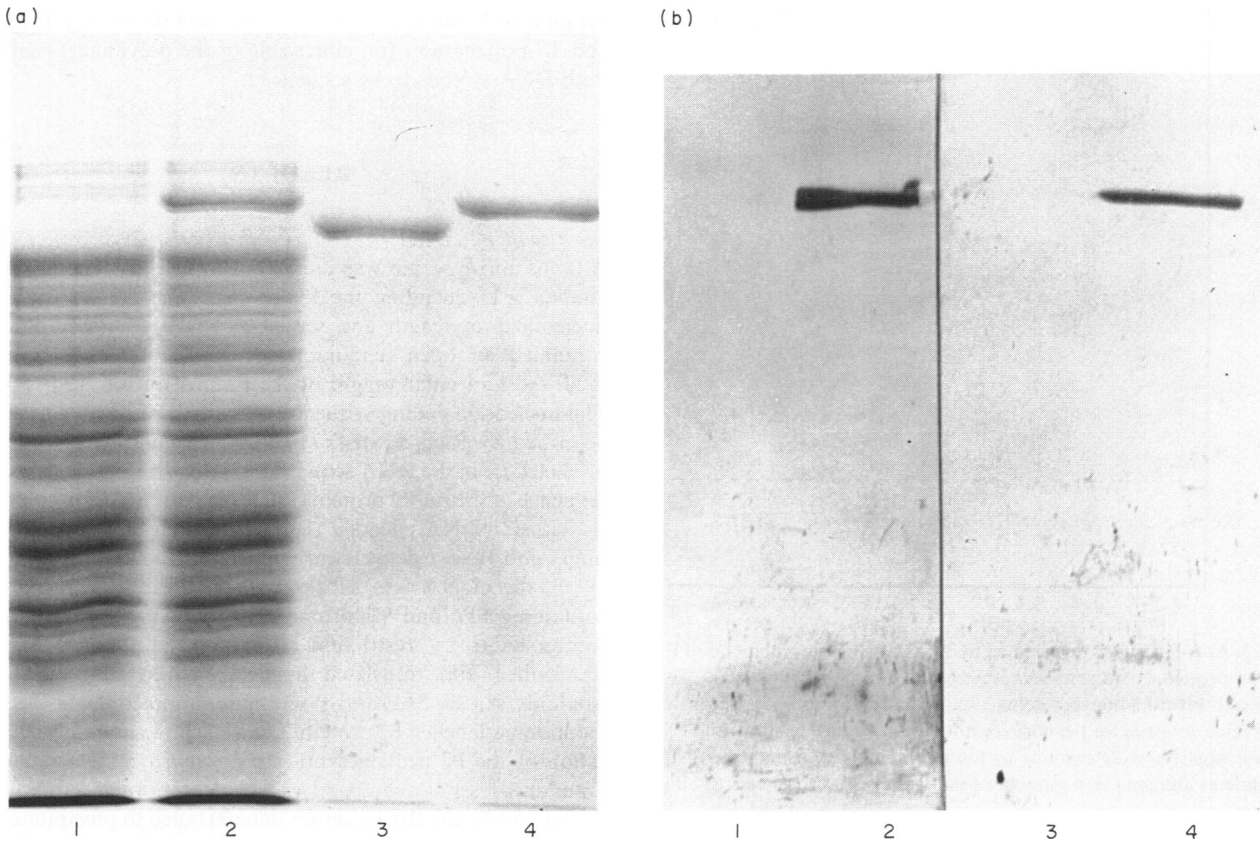

Fig. 2. Coomassie blue stain of an 8% polyacrylamide SDS gel (a). The samples loaded were: *E. coli* Y1089 whole cell lysate before (lane 1) and after (lane 2) induction with IPTG, β-glactosidase (lane 3) and the isolated β-galactosidase-P2 fusion protein (lane 4). Immunoblots of β-glactosidase and the β-galactosidase-P2 fusion protein (b). Protein was applied, 500 ng/lane, and, following electrophoresis on an 8% polyacrylamide SDS gel, transferred to nitrocellulose paper. Lanes 1 and 2 and lanes 3 and 4 were probed with two different SLE anti-P sera (diluted 1/200) followed by alkaline phosphatase-conjugated goat anti-human IgG. Lanes 1 and 3, β-galactosidase. Lanes 2 and 4, β-galactosidase-P2 fusion protein.

in the eluate was measured by the ELISA described above and total IgG in the eluate as well as in the starting serum by a solid phase radioimmunoassay (Elkon *et al.*, 1983).

In-vitro transcription and translation

One microgram of pGem 3 containing the P2 cDNA was linearized and transcribed *in vitro* with SP-6 polymerase in the presence of 7 methylguanosine 5′triphosphate to cap the 5′ end of the mRNA (Melton *et al.*, 1984). Template DNA was digested with DNase and the RNA extracted by phenol-chloroform. The RNA was used to programme micrococcal nuclease-treated rabbit reticulocyte extracts (Pelham & Jackson, 1976) in the presence of ^{35}S-methionine in order to radioactively label the synthesized proteins. To determine whether anti-P antibodies would bind to the *in vitro* synthesized proteins, protein-A-facilitated immuno-precipitation was performed as described (Elkon *et al.*, 1986). The eluted proteins were resolved on a 15% polyacrylamide-SDS (sodium dodecyl sulphate) gel and autoradiographs developed by fluorography at −70°C.

Analysis of the predicted sequence of the autoantigen

Blood was obtained from two SLE patients with high titres of anti-P antibodies and two healthy controls. Peripheral blood mononuclear (PBM) cells were separated by density gradient centrifugation on Ficoll-Hypaque and poly (A)$^+$ RNA isolated as described above. To specifically prime first strand cDNA synthesis, a 22-mer oligonucleotide

(5′-ATTCCTGCTCCCCTGCTGCAAATAA-3′)

complementary to a region in the 3′ non-coding region of P2 was synthesized on an Applied Biosystems 380B DNA synthesizer. The oligonucleotide was annealed to poly (A)$^+$ RNA at 90°C for 1 min and first and second strand DNA synthesis performed by standard methods (Maniatis, Fritsch & Sambrook, 1982). The cDNA was size-fractionated on a Sepharose CL 6B (Pharmacia) column and cDNA greater than 200 nucleotides was ligated to Eco-R1-digested lambda gt11 bacteriophage arms. The recombinant DNA was packaged and used to infect *E. coli* Y1090. Following induction of protein expression with IPTG, plaques were screened with an anti-P serum until the plaques were uniformly positive. Phage DNA was purified and Eco R1 inserts

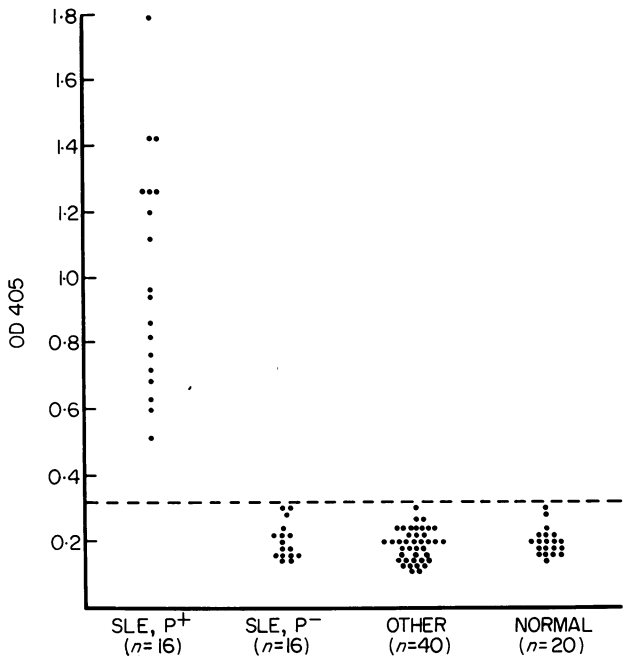

Fig. 3. Anti-P levels as determined by ELISA using the recombinant P2 fusion protein as antigen. Sera were tested at a dilution of 1/500. The dashed horizontal line represents 3 standard deviations above the mean OD value obtained for the normal controls. P^+, anti-P positive, and P^-, anti-P negative as determined by radioimmunoassay using a synthetic peptide as antigen (3). *n* number of individuals studied in each group.

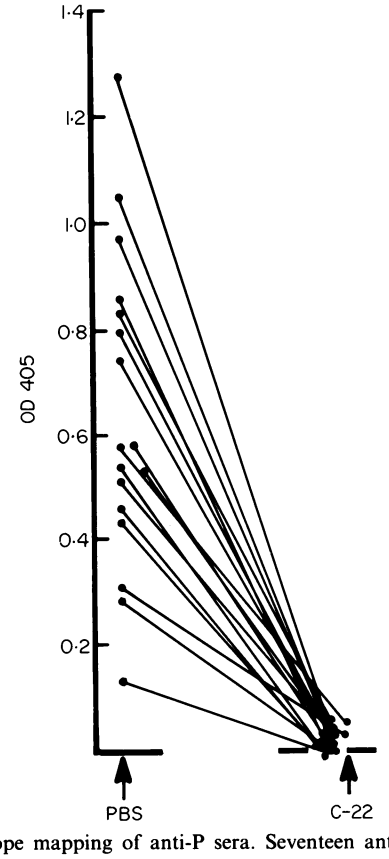

Fig. 4. Epitope mapping of anti-P sera. Seventeen anti-P sera were incubated in buffer alone (PBS) or with 2·5 μg of C-22 peptide conjugate (C-22). The standard ELISA was then performed using the P2-fusion protein as antigen.

were subcloned into the Eco R1 site of the plasmid pGem3. Priming with oligonucleotides complementary to both the SP6 and T7 polymerases (on either side of the polylinker) enabled both DNA strands to be sequenced.

RESULTS

Identity of P2 cDNA

A lupus anti-P serum was used to identify a human cDNA in lambda gt11 encoding the P2 protein. The cDNA was 366 nucleotides in length and sequence analysis showed that it contained an open reading frame of 345 nucleotides. The predicted molecular weight of the P2 protein was 12·075 kD. The nucleotide coding sequence was identical to that recently described by Rich & Steitz (1987). The amino acid sequence predicted from the DNA sequence was 93% homologous to the sequence of the rat P2 protein (Lin *et al.*, 1982). To confirm that the cloned cDNA encoded the P2 autoantigen recognized by lupus anti-P sera defined earlier (Elkon, Parnassa & Foster, 1985), the cDNA was subcloned into the pGem 3 plasmid containing SP6 and T7 promoters. Following orientation of recombinants by restriction mapping, the P2 cDNA was transcribed and translated as described in Materials and Methods. All six SLE anti-P sera immunoprecipitated the ^{35}S-methionine-labelled P2 protein. Figure 1 shows immunoprecipitation of the P2 protein synthesized *in vitro* by two different lupus anti-P sera (lanes 2 and 4), whereas a normal serum (lane 1) and a SLE anti-Ro/La serum (lane 3) failed to precipitate the protein. The apparent molecular weight of P2 (17 kD) on SDS-polyacrylamide gels agrees with earlier results showing a migration slower than expected (Lin *et al.*, 1982).

P2 fusion protein and ELISA

To develop a more convenient ELISA for measuring anti-P antibodies in SLE patients' sera, a β-galactosidase-P2 fusion protein was isolated from *E. coli*. The *E. coli* strain Y 1089 was infected with the recombinant bacteriophage and approximately 10 mg of fusion protein was obtained per litre of culture. The purity of the isolated fusion protein was >90% as determined by densitometry. Figure 2a shows a Coomassie blue stain of the cell lysates before (lane 1) and after (lane 2) induction with IPTG and the isolated fusion protein (lane 4). Immunoblots of the isolated protein (Fig. 2b) revealed that anti-P antisera recognized the fusion protein (lanes 2 and 4), but did not bind to β-galactosidase alone (lanes 1 and 3).

The results of the ELISA using the isolated fusion protein as antigen (referred to as ELISA (FP)) are shown in Fig. 3. All 18 SLE sera that bound to the synthetic peptide comprising the C terminal 22 amino acids of the P proteins were found to be positive in this assay. In contrast, none of the sera from normals, SLE patients without anti-P peptide antibodies (Bonfa *et al.*, 1987), patients with other autoimmune disorders or patients with chronic infections contained antibodies to the fusion protein. Similarly, all four MRL/lpr mice identified as having anti-P antibodies by immunoblotting (Bonfa *et al.*, 1988) produced ELISA (FP) values of >0·120 (3 s.d. above the mean obtained for the normal control NIH/Swiss mice) and the 25 NZB/W mice that were negative for anti-P by immunoblotting (n= 4) were negative by the ELISA (FP).

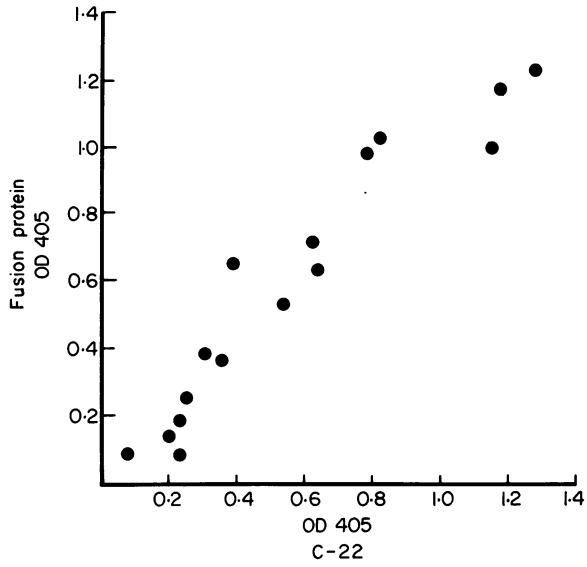

Fig. 5. Correlation between values obtained by ELISA using the synthetic peptide (C-22) or P2 fusion protein as antigen. Sixteen sera were studied and a correlation coefficient (r) of 0·92 was obtained ($P < 0·001$).

Comparison between anti-peptide and anti-fusion protein antibodies

To determine whether human lupus anti-P sera bound to several epitopes on the P2 fusion protein, an inhibition study was performed. Each serum was diluted so that the ELISA (FP) OD_{405} reading was $\leqslant 1·3$. The diluted serum was then incubated with either buffer alone or 2·5 μg of C-22 peptide conjugated to thyroglobulin. After 2 h at room temperature, the ELISA (FP) was performed. Figure 4 shows that pre-incubation of all 17 sera tested with the synthetic peptide completely abrogated binding to the fusion protein. Since this indicated that the C terminal epitope was also recognized in the ELISA (FP), the two ELISAs (FP and C-22) were compared directly. Sera were diluted between 1/100 and 1/5000 and incubated on ELISA plates coated with either the fusion protein or C-22 peptide under identical conditions. As shown in Fig. 5, a highly significant correlation was observed between the two assays ($r = 0·92$, $P < 0·001$).

Quantification of anti-P antibodies in SLE sera

In both the ELISA (FP) and the ELISA (C-22), positive results could still be obtained after diluting high titre sera by 1/50 000. In order to determine the approximate serum levels of anti-P antibodies in SLE sera, an affinity-purified anti-P antibody was used to develop a standard curve. The median anti-P concentration in 16 SLE sera was estimated to be 100 μg/ml (range 6–450 μg/ml). This reflected between 0·03 and 2·25% of total serum IgG.

The nucleotide sequences of cDNAs encoding P2 in patients' cells

On average, 2 μg of poly $(A)^+$ RNA was obtained from 3×10^7 PMN cells. In all cases this was sufficient to produce at least 5×10^4 plaques per cDNA synthesis. Approximately 4×10^4 plaques were screened for each preparation yielding between four and 15 positive plaques. Agarose gel electrophoresis revealed that the cDNA inserts derived from the two patients and one control PMN cells were sufficient to encode the full-length P2 protein ($\geqslant 340$ base pairs), whereas the cDNA inserts from PMN cells from one control were approximately 240 b.p. in length indicating partial degradation of mRNA. Nucleotide sequencing revealed that the coding sequences from clones derived from two SLE patients and one control PMN cells were identical. The nucleotide sequence obtained from the second control P2 cDNA was also identical except that the sequence from base 1 to 95 was absent from the clone. The sequences were also identical to the nucleotide sequence of HeLa P2 cDNA (Rich & Steitz, 1987).

DISCUSSION

We report cloning the cDNA encoding the human ribosomal P2 protein, isolation of a β-galactosidase-P2 fusion protein and determination of the predicted amino acid sequence of the P2 autoantigen from the mononuclear cells of SLE patients with anti-P antibodies. The P2 fusion protein was used to develop a sensitive ELISA to detect and quantify anti-P antibodies in SLE sera. In addition, epitope mapping of P2 was performed with the P2 fusion protein and the synthetic peptide corresponding to the C-terminus of human P2.

The ELISA using the P2 fusion protein as antigen (ELISA FP) proved to be highly specific for anti-P. SLE patients without anti-P peptide antibodies and patients with other autoimmune disorders were negative in this assay. These results confirm previous observations indicating that anti-P antibodies are found predominantly, if not exclusively, in patients with SLE (Bonfa & Elkon, 1986). The ELISA (FP) also confirmed the presence of anti-P antibodies in a minority of MRL/lpr sera (Bonfa *et al.*, 1988), but not in sera obtained from NZB/NZW F1 or normal mice. Using an affinity-purified anti-P antibody as a standard, human SLE sera contained between 6 and 450 μg/ml of IgG anti-P antibodies. These values are similar to those obtained by affinity purification of anti-DNA-histone antibodies (Townes, Stewart & Osler, 1962), but lower than estimates of anti-Sm/RNP antibodies isolated by immunoprecipitation (Maddison & Reichlin, 1977). Whether these results reflect true differences in the serum concentrations of specific autoantibodies, are due to methods of isolation of the antibodies, or are influenced by the conformation of the antigen (see below), remains to be determined.

We have previously shown that all human SLE (Bonfa *et al.*, 1987; Elkon *et al.*, 1986) and most MRL/lpr mouse (Bonfa *et al.*, 1988) anti-P antisera so far tested bind to the conserved C-terminus shared by all three P proteins. This epitope appears to be the only epitope recognized by anti-P antibodies under the denaturing conditions of immunoblotting (Elkon *et al.*, 1986). The properties of this epitope (terminal location, hydrophilicity and accessibility) are similar to those described for epitopes on foreign protein antigens (Elkon *et al.*, 1988). The strong correlation between the ELISA values obtained with the synthetic peptide, C-22, and the P2 fusion protein as antigens suggested that anti-P sera bound to similar epitopes in the two assays. This was confirmed by an inhibition assay where the synthetic peptide completely inhibited binding of anti-P antibodies to the fusion protein. This reinforces the view that the C-terminus contains a dominant epitope recognized by SLE antisera (Elkon *et al.*, 1986; Bonfa *et al.*, 1987). Since anti-P sera have also previously been shown to contain additional antibodies to the P proteins (Elkon *et al.*, 1986), inability to detect

antibodies to other potential epitopes on the recombinant P2 protein may be explained by unfolding of P2 fused to β-galactosidase, by reactivity of conformational antibodies with the pentameric complex of P0, P1, and P2 (Elkon *et al.*, 1986; Rich & Steitz, 1987; Uchiumi, Wahha & Traut, 1987) or by anti-P antibodies specific for phosphate groups on the authentic, but not recombinant or synthetic antigens.

Although several lupus autoantigens have now been cloned, sensitive and specific immunoassays have so far only been reported for anti-centromere (Earnshaw *et al.*, 1987), anti-La (St. Clair *et al.*, 1988), and anti-U1 RNP 68 000 mw protein (Netter *et al.*, 1988) antibodies. Factors influencing the ability to develop quantitative immunoassays include using cDNA encoding the full-length protein, levels of expression and degradation in the bacterial host, ease of isolation and, possibly, the degree to which the protein antigen retains its native conformation. Since the P2 cDNA described here encodes the full-length P2 protein and large amounts of the fusion protein were isolated, an ELISA was readily developed. Considering that anti-P antibodies were detected with similar sensitivity and specificity using either the ELISA (FP) or the ELISA (C-22), utilization of the recombinant FP as antigen may offer considerable advantages in terms of lower cost and rapid production of a lupus autoantigen.

The possibility that 'altered-self' is responsible for autoantibody production has long been considered (Witebsky & Rose, 1956; Weigle, 1965; Allison, Denman & Barnes, 1971). This hypothesis is supported by experiments showing that immunization with modified autologous or unmodified heterologous proteins induce autoantibodies and, in some cases, autoimmune diseases in animals (Witebsky & Rose, 1956; Weigle, 1965). Evidence that the target organ is abnormal (Sundick & Wick, 1974; Truden *et al.*, 1983) and that the presence of the autoantigen is necessary for the maintenance of autoimmunity (Pontes de Carvalho *et al.*, 1982) has been provided in one spontaneous autoimmune disease, thyroiditis of the obese strain of chickens. Despite these findings, Roitt and co-workers were unable to detect serological evidence of abnormalities in the autoantigen obtained from diseased animals (Pontes de Carvalho *et al.*, 1982). Using recombinant DNA technology, we showed the predicted sequences of the P2 protein antigen were normal in two patients with anti-P antibodies. Although this makes a structural abnormality of P2 unlikely, the possibility cannot be excluded. A small proportion of the protein could be abnormal or the abnormal protein could be produced in cells other than mononuclear cells. Since only the nucleotide sequence for P2 was evaluated and anti-P antibodies bind to all three P proteins, differences in the DNA encoding P0 or P1 would not have been detected. Finally, these results do not address possible modifications of the protein antigen arising either from post-transitional changes or binding to foreign proteins or nucleic acid.

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