Antibodies to protein P in systemic lupus erythematosus

L S Teh, A E Bedwell, D A Isenberg, C Gordon, P Emery, P J Charles, M Harper, N Amos, B D Williams

Abstract

A synthetic peptide was used to develop an enzyme linked immunosorbent assay (ELISA) to detect antibodies to the ribosomal proteins P₀, P₁, and P₂. Significantly increased levels of IgG antibodies to protein P were found in 16% (18/116) of patients with systemic lupus erythematosus but slightly increased levels were detected in 2% (2/98) of patients with rheumatoid arthritis and one normal control subject. No association was observed between the presence of IgG antibodies to protein P and either lupus psychosis or depression. Sequential studies in individual patients failed to show an association between antibody levels and the development of psychosis.

Systemic lupus erythematosus (SLE) is a multi-system autoimmune rheumatic disease, characterised by serum antibodies directed against intracellular proteins and nucleic acids.¹ Neuropsychiatric (psychiatric or neurological) abnormalities are common, occurring in 50% of patients,²⁻⁴ and contribute substantially to the morbidity and mortality of patients with SLE.^{5,6} Psychiatric diseases with or without psychosis are the most common manifestations and may resemble de novo affective disorders or schizophrenia.⁷ Although the presentations in patients with SLE with psychosis can be acute and dramatic, the patients often respond rapidly and completely to treatment with no residual damage to the nervous system. The diagnosis is based on clinical features and the exclusion of other diseases. There is no single laboratory test of sufficient sensitivity and specificity to be of use in the diagnosis of lupus neuropsychiatric disease.⁸

Much interest has been focused on antibodies to ribosomal protein P, the target antigen of which is intracellular in origin and not restricted to neuronal cells. Antibodies to ribosomal protein P have been reported to be specific for SLE.^{9,10} These antibodies react with three 60S ribosomal phosphoprotein subunits P₀, (38 kilodaltons), P₁ (19 kilodaltons), and P₂ (17 kilodaltons), and recognise a common epitope contained within a linear sequence of 22 amino acids from the carboxyl terminal.¹¹ Using a synthetic peptide corresponding to the terminal 22 amino acids, a radioimmunoassay was developed to detect these antibodies and a striking association was noted between IgG antibodies to protein P and lupus psychosis.¹² Antibodies to protein P were reported in 90% (18/20) of patients with SLE with lupus psychosis and, in sequential studies, a five to 30 fold increase in the levels of these

antibodies was noted before and during the development of psychosis.¹² In another study, IgG and IgM antibodies to protein P were shown to occur in 7/8 patients with severe depression (requiring admission to hospital and treatment with antidepressants), but in only 13/29 patients with psychosis.¹³

This paper describes the development of an enzyme linked immunosorbent assay (ELISA) to detect antibodies to ribosomal protein P and examines their association with psychosis, depression, and other neuropsychiatric manifestations of SLE.

Patients and methods

PATIENTS

Serum samples were obtained from the following patient groups: patients with SLE (n=116), rheumatoid arthritis (n=98), osteoarthritis (n=5), psoriatic arthritis (n=8), ankylosing spondylitis (n=4), mixed connective tissue disease (n=8), primary Sjögren's syndrome (n=24), and non-SLE neuropsychiatric disease (n=25). The patients with SLE and rheumatoid arthritis fulfilled the American Rheumatism Association criteria.¹⁴ Lupus psychosis was defined by a severe behavioural disturbance requiring referral to a hospital and treatment, a disturbance lasting two weeks or longer, and the exclusion of the effects of drugs, infections, or metabolic derangements. With one exception, all patients with mixed connective tissue disease had positive antibodies for ribonucleoprotein P, antinuclear factor, and rheumatoid factor; clinically they had Raynaud's phenomenon, features of scleroderma, and a non-erosive polyarthritis. There were 11 patients with a variety of neurological diseases who did not have SLE (four with cerebral vasculitis, two with mononeuritis multiplex, two with dementia, one each with multiple sclerosis, hemiparesis, and visual loss). Fourteen patients with psychosis not due to SLE (11 with manic depressive psychosis and three with schizophrenia) were also studied. We also examined serum samples from 72 normal subjects.

A history of neuropsychiatric disease was present in 34% (39/116) of the patients with SLE: 13 with lupus psychosis, 10 with depression, seven with epilepsy (five had grand mal epilepsy, one each with myoclonic jerks and temporal lobe epilepsy), and nine with focal neurological disease (two had a left hemiparesis, one each with transverse myelitis, transient weakness of the left arm, dysaesthesiae, diplopia, subarachnoid haemorrhage, optic neuritis, and brain stem lesion).

Rheumatology Research Centre,
University Hospital of Wales,
Heath Park, Cardiff, UK

L S Teh
A E Bedwell
N Amos
B D Williams

Bloomsbury Rheumatology Unit,
University College and Middlesex School of Medicine,
London, UK

D A Isenberg

Department of Rheumatology,
University of Birmingham,
Birmingham, UK

C Gordon
P Emery

Department of Rheumatology,
Charing Cross Hospital,
London, UK

P J Charles

Department of Psychological Medicine,
University Hospital of Wales,
Heath Park, Cardiff, UK
M Harper

Correspondence to:
Dr L S Teh,
Rheumatology Research Centre,
University Hospital of Wales,
Heath Park, Cardiff CF4 4XW,
United Kingdom

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Where possible samples were obtained from patients with neuropsychiatric disease during the episodes of psychosis or depression (active disease), but some samples were obtained from patients with a previous history of psychosis or depression (inactive disease). Serial samples were also obtained from 27 patients with SLE.

REAGENTS

Peroxidase conjugated rabbit antihuman IgG and IgM were purchased from Dako (High Wycombe, UK). Peroxidase conjugated rabbit antihuman IgA, mouse monoclonal antihuman IgG₁, IgG₂, IgG₃, and IgG₄, and peroxidase conjugated antimouse IgG and IgM were purchased from Sigma Chemical (Poole, UK). Calf thymus DNA was obtained from Sigma.

The synthetic 22 amino acid P peptide was purchased from Professor B Dunn (University of Florida, Gainesville, FL, USA).

CONJUGATION OF PEPTIDE P TO RABBIT SERUM ALBUMIN

The 22 amino acid synthetic peptide P was conjugated to rabbit serum albumin with glutaraldehyde at a weight ratio of peptide P: rabbit serum albumin of 2:1.¹⁵ This peptide P-rabbit serum albumin conjugate was stable at -70°C for at least three months.

MEASUREMENT OF ANTIBODIES TO PROTEIN P

Antibodies to protein P were measured using a standard ELISA method. Plastic microtitre plates (Nunc-Immunoplate) were coated with either conjugated protein P-rabbit serum albumin or rabbit serum albumin alone (100 µl/well of 1:2000 dilution of the substrate in phosphate buffered saline) overnight at 4°C. The plates were incubated with 1% bovine serum albumin and, after washing the wells, were sequentially incubated with serum samples diluted at 1:100 followed by peroxidase conjugated isotype specific antihuman antibody. The plates were developed with *o*-phenylenediamine. Assays were carried out in triplicate and the level of antibody to protein P was determined by subtracting the absorbance of the rabbit serum albumin plate from that of the protein P-rabbit serum albumin plate. This value was then expressed with respect to a reference positive serum sample (arbitrarily assigned 100 U/ml). This serum sample has been previously shown by radioimmunoassay to contain IgG antibodies to protein P. Values three standard deviations above the mean derived from the normal subjects were regarded as positive for antibodies to protein P and these values were +18 U/ml for IgG, +9 U/ml for IgA and -1 U/ml for IgM. To ensure consistency between assays each plate contained a known positive and negative sample. The assays were performed without knowledge of the clinical status of each subject. The interplate variation was 9% and the intraplate variation 5%.

MEASUREMENT OF IgG SUBCLASSES BY ELISA

The ELISA used to detect IgG subclasses was

similar to the assay for antibodies to protein P. The following modifications were made. After incubation with test serum samples, mouse monoclonal antihuman IgG₁ (1:1000 dilution), IgG₂ (1:1000 dilution), IgG₃ (1:300 dilution) or IgG₄ (1:1000 dilution) were added to the plates overnight followed by peroxidase conjugated antimouse IgG antibody (1:1000 dilution) for subclasses 1, 2, and 4, and IgM (1:1000 dilution) for subclass 3. The plates were developed with *o*-phenylenediamine.

INHIBITION OF ANTIBODIES TO PROTEIN P

To determine the specificity of the binding of antibodies to protein P, the reference positive serum sample was preincubated with peptide P before testing by the ELISA or by western blotting analysis.

WESTERN BLOTTING

Hep-2 cells were used as a source of P₀, P₁, and P₂ antigens. Following sodium dodecylsulphate polyacrylamide gel electrophoresis in 12% gel, the antigens were electrotransferred to nitrocellulose. Nitrocellulose strips were incubated with test serum samples at 1:100 dilution

Figure 1 (A) Inhibition of binding of IgG antibodies to protein P by P peptide. Anti-P=antibodies to protein P; RSA=rabbit serum albumin; NHS=normal human serum. (B) Inhibition of binding of IgG antibodies to protein P analysed by western blotting in 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis. 1=normal serum, 2=serum containing antibodies to protein P, 3=serum 2 preincubated with P peptide, 4=reference serum containing antibodies to protein P, 5=serum 4 preincubated with P peptide.

followed by peroxidase conjugated antihuman IgG, diluted 1:1000 before development with 3', 3-diaminobenzidine tetrahydrochloride.

MEASUREMENT OF IgG ANTIBODIES TO DNA BY ELISA
Antibodies to DNA were also measured in 61 patients with SLE by an ELISA. The plates were coated with DNA extracts (without removal of single stranded DNA), blocked with 0.2% bovine serum albumin, incubated sequentially with serum samples and peroxidase conjugated antihuman IgG before development

Figure 2 (A) Distribution of IgG antibodies to protein P in the studied patients. (B) Distribution of IgM antibodies to protein P. RA=rheumatoid arthritis; SS=Sjögren's syndrome; OA=osteoarthritis; AS=ankylosing spondylitis; PsA=psoriatic arthritis; MCTD=mixed connective tissue disease; SLE=systemic lupus erythematosus; Psych=psychosis; Neuro=neurological disease.

Patterns of neuropsychiatric disease (NPD) in patients with systemic lupus erythematosus with or without IgG antibodies to protein P

	Positive antibodies to protein P		Negative antibodies to protein P		Total
	Active disease	Inactive disease	Active disease	Inactive disease	
Psychosis	3	0	6	4	13
Depression	1	0	4	5	10
Epilepsy	1	2	2	1	6
Focal neurology	1	0	7	2	10
Subtotal with NPD	6	2	19	12	39
No NPD	10		67		77

with o-phenylenediamine.¹⁰ The results were compared with a reference serum sample known to contain antibodies to DNA. Levels greater than 25 U/ml were regarded as positive.

Results
The specificity of the ELISA used to detect antibodies to protein P was confirmed by preincubating the reference positive serum samples with peptide P before testing in the ELISA. Figure 1A shows that after preincubation with free peptide P or peptide P-rabbit serum albumin there was a dose dependent inhibition of the binding of antibodies to protein P to the peptide P-rabbit serum albumin coated on the plate. Preincubation with rabbit serum albumin produced no inhibition. By western blotting analysis, preincubation of two positive serum samples specifically inhibited the binding of the antibodies to protein P to bands in the 38 and 19 kilodalton regions (fig 1B). We were unable to show a band in the 17 kilodalton region and this may be because whole Hep-2 cells were used as the source for protein antigens in this study. In other studies which clearly showed all three bands on western immunoblotting, ribosomes were used.^{17 18}
Figure 2 shows the occurrence of IgG and IgM antibodies to protein P in the different patients groups. The most often detectable isotype was IgG, which was present in 16% (18/116) of patients with SLE, followed by IgM in 11% and IgA in 5% of patients with SLE. However, these antibodies were not confined to patients with SLE; low levels of IgG antibodies to protein P were detected in 2/98 serum samples from patients with rheumatoid arthritis and one from a normal subject. IgM antibodies to protein P were also present in two serum samples from patients with mixed connective tissue disease and one normal subject, also at low levels. By western blotting analysis the serum samples from two patients with rheumatoid arthritis and two patients with mixed connective tissue disease also reacted to bands in the 38 and 19 kilodalton regions. Only three patients had all three isotypes and four patients had two isotypes (two with IgG and IgM, and two with IgG and IgA). All the remaining patients were negative for all three isotypes.
Of the 18 patients with SLE with increased levels of IgG antibodies to protein P, only eight had neuropsychiatric diseases (three with psychosis, one with depression, three with epilepsy, and one with focal neurological disease). The remaining 10 patients did not have a history of neuropsychiatric disease. The table shows that 31 of the 77 patients without IgG antibodies to protein P also had a variety of neuropsychiatric diseases. There was no significant differences in the occurrence of IgG antibodies to protein P in patients with or without neuropsychiatric diseases ($p>0.1$, χ^2 test). Similar results were obtained when comparing the occurrence of IgM and IgA antibodies to protein P with psychosis or depression or other neuropsychiatric diseases (data not shown). Subdivision of the patients into those with active or inactive neuropsychiatric diseases

also failed to show any difference in the occurrence of IgG, IgM, or IgA P antibodies to protein P between the two groups ($p>0.1$, Fisher's exact test). Four of nine patients with active psychosis and one of five with depression (mild and severe) had IgG or IgM antibodies to protein P, compared with 4/16 with non-psychiatric neurological diseases. Selecting patients with increased levels of IgG antibodies to protein P and then subdividing the patients into those with and without neuropsychiatric diseases failed to show any difference in the antibody levels ($p>0.05$, Wilcoxon's rank sum test).

IgG subclasses were measured in 16 patients who had increased levels of IgG antibodies to protein P and all four subclasses could be detected in five patients. In four patients, only one subclass was present and in three of these it was subclass 3. Furthermore, IgG was the only isotype detectable in these three serum samples. The most common subclasses produced by patients with SLE with neuropsychiatric diseases were IgG₁ and IgG₃, and in patients without neuropsychiatric disease IgG₂ and IgG₃. This

difference in the occurrence of subclasses was not significant ($p>0.1$, Fisher's exact test).

IgG antibodies to DNA were also measured in 61 patients with SLE and correlated with IgG antibodies to protein P. Thirty three patients were positive for IgG antibodies to DNA, whereas only eight were positive for IgG antibodies to protein P. There was no correlation between IgG antibodies to protein P and DNA ($r=0.16$, $p>0.1$).

SERIAL STUDIES OF ANTIBODIES TO PROTEIN P IN PATIENTS WITH SLE

Levels of IgG antibodies to protein P and DNA were studied serially in five patients with SLE with increased levels of IgG antibodies to protein P. Three of these had lupus psychosis, one had depression, and one had no neuropsychiatric disease.

Figure 3 shows the fluctuations in the level of IgG antibodies to protein P in a white woman with a four year history of SLE. At presentation the level of IgG antibodies to protein P was increased at 177 units, but was undetectable in the cerebrospinal fluid. Antibodies to protein P decreased with treatment, but the reappearance of the lupus psychosis at weeks 54 and 72 were associated with only a small increase in the level of antibodies to protein P. During her second relapse of psychosis the patient was uncooperative and refused venesection. IgM antibodies to protein P were negative throughout the illness whereas IgA antibodies to protein P were weakly positive during the first episode of psychosis but negative thereafter. IgG subclasses 1 and 2 were the main subclasses detected throughout the illness.

Figure 4 shows that in two patients, serum levels of IgG antibodies to protein P were increased three months before the psychotic episodes and decreased to normal levels with treatment. However, psychosis persisted in one

Figure 3 Serial study of IgG antibodies to protein P (closed circles) and antibodies to DNA (open circles) in the reference patient. Black boxes represent episodes of psychosis; doses of prednisolone (mg) are shown at the top of the graph.

Figure 4 Serial studies of IgG antibodies to protein P (closed circles) and antibodies to DNA (open circles) by enzyme linked immunosorbent assay. Black boxes represent episodes of psychosis; doses of prednisolone (mg) are shown at the top of the graphs.

patient (fig 4B), though the level of antibodies to protein P returned to normal.

Despite a four fold increase in serum levels of IgG antibodies to protein P in two other patients with SLE (fig 4C and D), psychosis did not develop. Figure 4C is of a white woman with a history of SLE for 30 years and endogenous depression for 20 years. The other patient (fig 4D) is a white 29 year old with a 14 year history of SLE and renal disease, but no neuropsychiatric disease.

Serum samples from 22 patients with SLE who were negative for IgG antibodies to protein P in the single samples tested were also studied serially. These patients included those with a variety of neuropsychiatric diseases and also those without any neuropsychiatric disease. The levels of antibodies to protein P remained negative in 20 patients but in two patients, one with myoclonic jerks and one with focal neurological disease, there was an increase in IgG antibodies to protein P during remission of the neuropsychiatric disease (data not shown).

Discussion

An ELISA has been developed to detect antibodies to ribosomal protein P; this assay is specific, reproducible and detects antibodies reported in previous studies.^{12 13} In serum samples from patients with SLE, IgG antibodies to protein P were the predominant isotype; the occurrence noted in this study was similar to that reported by others.^{12 13 18–20} Slightly increased levels of antibodies to protein P were found in patients with rheumatoid arthritis, mixed connective tissue disease, and some normal subjects; significantly increased levels, however, appear to be a specific marker antibody for SLE. IgM and IgA antibodies to protein P were present less often.²¹

We failed to confirm the previously described association between IgG antibodies to protein P and the presence of psychosis or depression.^{12 13} In this study IgG antibodies to protein P were found in 21% (8/39) of patients with active or inactive neuropsychiatric diseases but 13% (10/77) of patients who had no history of neuropsychiatric disease also had increased levels (table). These differences were not statistically significant. Similar observations were found for IgM and IgA antibodies to protein P. The reason for the discrepancy between our findings and those reported previously are unclear, but it is unlikely to be due to differences in the sensitivity of the assays used to detect these antibodies. Our reference serum sample contains antibodies to protein P, confirmed using the radioimmunoassay of Bonfa *et al*¹² and an identical ELISA has been used by another group to show antibody activity against ribosomal protein P.¹³

Differences in the ethnic origins of the patients studied could be relevant; in our patients most of those with SLE were white, whereas in one published study many of the patients were black.¹² Our unpublished observations on the occurrence of antibodies to protein P in Malaysian patients with SLE, where the presence of antibodies to protein P is

much higher, lends some support to this conclusion.

Another factor which may contribute to the discrepancy is the selection of patients with more severe depression by one of the studies,¹³ whereas most of our patients with depression did not require admission to hospital for treatment. Our observations agree with a study which also failed to show an association between increased levels of antibodies to protein P and the development of neuropsychiatric disease.²⁰

As determining the level of antibodies to protein P in single samples was uninformative, sequential studies were subsequently carried out. In five patients, three of whom provided a large number of samples over a substantial period of time, no association could be shown between antibody levels and the development of specific neuropsychiatric features. In some subjects, levels of antibodies to protein P decreased during psychosis (fig 4B), whereas in others the levels, if they did increase before or during psychosis, only increased by less than three fold (figs 3 and 4A). Our findings are similar to those made in two Indonesian sisters who developed lupus psychosis, in whom no clear relation between changes in the levels of antibodies to protein P and the development of psychotic episodes could be shown.¹⁷

In certain diseases isotype switching or an alteration in the IgG subclass of antibodies plays an important part in the development of disease.^{22 23} IgM antibodies to neutrophilic cytoplasm have been shown to be present during systemic vasculitis and severe pulmonary haemorrhage, but an isotype switch to IgG occurs during recovery.²² In the development of antibodies to glomerular basement membrane, active disease is associated with IgG₁ antibody, whereas recovery is associated with a subclass switch to IgG₄.²³ In several of our reference samples IgG, IgM, and IgA antibodies to protein P were measured but no isotype switching could be shown. Subclass identification of IgG antibodies to protein P also failed to show any association between a particular subclass and the development of psychosis or depression. Our study, however, has not excluded the possibility that changes in the affinity of the antibodies to protein P for the target antigen may occur during the disease and this enhanced binding to the target antigen may be responsible for the relapse of neuropsychiatric diseases.

Confusion also exists as to whether the determination of levels of antibodies to protein P in cerebrospinal fluid is a better predictor of lupus psychosis than the measurement of levels in serum. In one study there was selective enrichment of antibodies to protein P in the cerebrospinal fluid of a patient with lupus psychosis.²⁴ However, contradictory results were obtained in another study where the cerebrospinal fluid obtained from eight patients with active neuropsychiatric disease had only low levels of antibodies to protein P.¹³ In the reference patient in our study, antibodies to protein P were undetectable in the cerebrospinal fluid despite there being a considerable increase in the level in serum samples.

Rather than being directly involved, antibodies to protein P may serve as markers for other more relevant neuronal binding antibodies which initiate neuropsychiatric disease, such as the neuronal or brain cross reactive antibodies to lymphocytes. These antibodies target antigens on the surface of neuronal tissue, and a temporal association between the development of neuropsychiatric lupus and an increase in the titre of these antibodies has been described.²⁵⁻³⁰ These antibodies may be more relevant as IgG antineuronal antibodies in the cerebrospinal fluid have been shown to be associated with diffuse non-focal neuropsychiatric diseases but not with focal diseases.²⁷ Serum IgG antineuronal antibodies are also correlated with the impairment of cognitive function and diffuse non-focal neuropsychiatric disease in patients with SLE.²⁸

In conclusion, our observations fail to confirm the association between IgG antibodies to protein P and neuropsychiatric diseases. Single point estimations or sequential data are not helpful as predictive assays to identify those patients with neuropsychiatric disease or to predict the likelihood of developing neuropsychiatric diseases. The reasons for the varied results reported elsewhere are not clear at present, but may relate to the different ethnic groups present in the patients studied.

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