Serological abnormalities, including common idiotype PR4, in families with rheumatoid arthritis

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SUMMARY A broad range of autoreactivity among a group of 12 French patients with rheumatoid arthritis (RA) and 58 of their healthy first degree relatives has been identified. Over 15% of the patients were found to have antibodies to ssDNA, histone H1, H2A, and H2bB. Among the relatives, IgG and IgM rheumatoid factor and antibodies to ssDNA, H2A, and H4 were present in more than 10%. Even more remarkable, a common anti-DNA antibody idiotype designated PR4, known to be present in 70% of patients with systemic lupus erythematosus (SLE), was found in approximately 30% of both patients with RA and their healthy relatives. This is quite different from its lack of increased expression in relatives of patients with SLE and suggests that in the family members of those with RA a particular combination of environmental influence on germline gene expression is responsible.

Rheumatoid arthritis (RA) is an inflammatory autoimmune disorder of unknown aetiology characterised by joint inflammation and erosion. Among a wide range of immunological abnormalities, the presence of high titres of rheumatoid factors (RFs) is often notable. There is a well established association between RA and HLA-DR4 in most white populations,¹ and more recently an association with the immunoglobulin heavy chain allotype Glm(x) has been described.^{2,3} Several family and twin studies have attempted to elucidate an environmental or genetic predisposition for the development of the disease, but no clear markers have been found apart from HLA and immunoglobulin allotype associations.

In one of the family studies, which focused on possible serological abnormalities, no significant differences in the antinuclear activity or in natural antibody production in relatives and controls compared with patients could be detected.⁴ In another study antinuclear antibodies were shown to correlate with RF positive patients with RA but not with affected RF negative or unaffected members of

these families.⁵ Pasquali *et al* showed that an idiotype determinant carried only on the RF immunoglobulin of a patient with RA was detectable in the serum samples of four family members spanning three generations.⁶ Two of these family members were RA seropositive or had definite mild RA. All four family members were RF positive, and the idiotype was shown to be present on the RFs of two of these relatives.

Other idiotypic studies of patients with RA have shown the presence of common rheumatoid factor associated idiotypes Wa, Po, and Bla in up to 80% of human RFs studied.^{7,8} These idiotypes, however, were found on myeloma proteins with RF activity rather than on RFs isolated from patients with RA. These results suggest the inheritance of antibody genes related to the idiotype determinants. Our interest in familial idiotypic studies of patients with RA developed from the finding of a cross reactive idiotype PR4 in the serum samples of patients with RA. This idiotype, originally identified on a leprosy derived human monoclonal antibody,⁹ was subsequently found in patients with leprosy (70%), systemic lupus erythematosus (SLE) (70%), RA (40%), and Sjögren's syndrome (15%) compared with 3% of healthy controls, spouses of patients with SLE, and healthy relatives.⁹ In the population of

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rheumatoid patients screened there seemed to be a subset of patients with greatly increased levels of the idiotype (much higher than in any of the other groups studied), which did not correlate with RF.

This study reports the frequency of the PR4 idiotype in 12 patients with classical RA and in 58 relatives of these patients and provides a detailed analysis of the autoantigen binding profiles of the patients and relatives.

Patients, materials, and methods

PATIENTS

Twelve patients with classical RA, diagnosed according to the revised criteria of the American Rheumatism Association,¹⁰ attending the University of Brest rheumatology department were included in the study. Fifty eight healthy first degree relatives of these 12 patients were included in the study. Fifty unrelated healthy controls with no history of autoimmune diseases in the family were included in the study to provide negative controls in each test (only 30 normal serum samples were used in the PR4 assay).

DETECTION OF THE PR4 IDIOTYPE

Serum samples were collected, stored at -70°C , and screened using the following procedures: the PR4 idiotype was measured by an enzyme linked immunosorbent assay (ELISA).¹¹ Briefly, affinity purified rabbit anti-PR4 idiotype (4 $\mu\text{g}/\text{ml}$) or purified rabbit immunoglobulin as a control (4 $\mu\text{g}/\text{ml}$) in 0.1 M bicarbonate buffer pH 9.6 was coated overnight at 4°C onto 96 well polystyrene Immulon I (Dynatech) plates. The plates were washed with phosphate buffered saline (PBS), blocked with 2% bovine serum albumin-PBS for one hour at 37°C , and washed again with PBS-Tween. Patients' serum samples were diluted 1:640 in PBS-Tween containing 1% bovine serum albumin. The diluted sera were added to the plates and incubated for one hour at 37°C . The plates were washed with PBS-Tween. Goat F(ab')₂ antihuman polyvalent immunoglobulin alkaline phosphatase conjugate (Sigma) was added and the plates incubated for one hour at 37°C . The plates were washed in PBS-Tween followed by one rinse in 0.1 M bicarbonate buffer pH 6.9. Alkaline phosphatase substrate disodium *p*-nitrophenyl phosphate (Sigma) in 0.1 M bicarbonate buffer pH 6.9 containing 2 mM MgCl₂ was added. When the colour reaction was complete the plates were read in a Dynatech MR 580 micro-ELISA reader at 405 nm. The arithmetic mean of the duplicate values was subtracted from the mean of the background values and the idiotype level calculated with reference to a standard curve of PR4 idiotype diluted in sera

included on each plate. Serum samples were considered positive if the PR4 idiotype level was above the upper limit of normal, which was set at the mean +2 standard deviations (SD) of 30 healthy controls.

RHEUMATOID FACTOR

Rheumatoid factor activity was determined by an ELISA.¹² Briefly, 100 μl of 10 $\mu\text{g}/\text{ml}$ of Fc fragments of IgG prepared from Cohn fraction II (Sigma) were added to 96 well microtitre plates (Nunc, Gibco, Paisley, Scotland). The plates were incubated for four hours at 37°C , washed three times, blocked with 5% fetal calf serum in PBS for another hour at 37°C , and again washed three times. Serum samples diluted 1:100 were incubated for three hours and IgM RFs were detected with peroxidase conjugated F(ab')₂ goat antihuman IgM (Cappel Laboratories, Cochranville, PA) absorbed with IgG. Several monoclonal IgM RFs, isolated from the serum of patients with Waldenström's macroglobulinaemia or produced as described elsewhere,¹³ served as positive controls. IgG RFs were detected with peroxidase conjugated F(ab')₂ goat antihuman Fd γ (Cappel) extensively absorbed with IgM and free light chains, as described by Carson *et al.*¹⁴ Several polyclonal IgG RFs, isolated from the serum of patients with RA, served as positive controls. Optical densities more than 2SD above the mean of background levels (medium only on irrelevant proteins) were considered positive for IgM or IgG RF activity.

Table 1 Numbers and percentages (%) of individuals with raised levels of immunoglobulin, antihistone and antinuclear antibodies, rheumatoid factor, and PR4 idiotype

	Patients with RA (n=12)	Relatives of patients with RA (n=58)	Normal controls (n=50; PR4 n=30)
Total IgG	1 (8)	4 (7)	0
Total IgM	2 (17)	9 (16)	0
Total IgA	6 (50)	14 (24)	0
Rheumatoid factor:			
IgG	7 (58)	16 (28)	0
IgM	11 (92)	9 (16)	1 (2)
Anti-ssDNA	3 (25)	9 (16)	2 (4)
Anti-dsDNA	2 (17)	6 (10)	0
Anti-poly-dAdT	1 (8)	2 (3)	0
Antihistones:			
1	2 (17)	3 (5)	1 (2)
2a	2 (17)	7 (12)	0
2b	1 (8)	4 (7)	2 (4)
3	4 (33)	18 (31)	1 (2)
4	1 (8)	1 (2)	0
PR4 idiotype	4 (33)	17 (29)	1 (3)

ANTINUCLEAR ANTIBODIES

Antibodies to single stranded (ss) DNA, double stranded (ds) DNA, histones H1, H2A, H2B, H3, and H4 were measured with a newly developed ELISA kit (Neosystem, Strasbourg, France).¹⁵ Briefly, serum samples diluted 1:500 for anti-DNA assays and 1:500 for the antihistone assays were included on antigen coated plates for one hour at 37°C, washed three times with PBS containing 1% bovine serum albumin and 0.05% Tween, incubated with biotin conjugated F(ab')₂ anti-IgG and anti-IgM for another hour at 37°C, washed, and incubated with peroxidase conjugated streptavidin for one hour. After the washing, 2, 2'-azinobis (3-ethylbenzthiazoline) sulphonic acid (Boehringer, Mannheim, West Germany) was added as substrate, incubated for one hour at 37°C, and optical densities

were read at 405 nm on a Titertek multiscanphotometer (Flow Laboratories, Twine, Scotland).

MONOCLONAL IMMUNOGLOBULIN

Serum samples were tested by a high resolution electrophoresis technique developed by Papadopoulos *et al.*¹⁶ Immunofixation was used to identify monoclonal bands.¹⁷ Goat, bovine, and rabbit antibodies (Kallestad, Austin, TX) were used for independent confirmation of specificity.

STATISTICS

Comparisons were made by the χ^2 test with Yates's correction when required, Spearman's rank correlation, and the Mann-Whitney U test for unpaired data.

Fig. 1 Autoantibody expression in the families with rheumatoid arthritis.

Results

The patients with RA showed a wide range of serological abnormalities. These included raised levels of total immunoglobulins, IgG, IgM, and IgA, antibodies binding to ssDNA and dsDNA, histones, polynucleotides, IgG and IgM rheumatoid factor activity, and presence of the common DNA associated idiotype PR4 (Table 1). These serological abnormalities were not, however, restricted to the patients, their healthy family members showed a similar broad range and level of reactivities (Table 1). In contrast, the control sera from unrelated

healthy individuals had a low frequency of reactivity in the tests.

Within individual families marked variation in autoantibody expression was observed, and Figs 1A-L show the family pedigrees. From an analysis of these family pedigrees it is clear that family members may possess an autoantibody profile which differs from that of the affected member of the family. It is not known whether the treatment or stage of disease in the affected member of the family may play a part in these differences when comparisons between healthy family members and affected members are made. Clearly the results from one

Fig. 2 Levels of PR4 idiotype in the patients with rheumatoid arthritis and their relatives and in the healthy controls.

serum sample are not necessarily representative of a disease whose activity fluctuates over many years. No significant associations between any of the antibody reactivities studied was evident (data not shown).

Idiotypic analysis of the serum samples showed that raised levels of the PR4 idiotype were found in four of the 12 patients with RA (33%) compared with one of 30 healthy controls (3%). The PR4 idiotype was also detected at raised levels in 17 of the 58 family members (29%). Figure 2 shows the levels in the patients, relatives, and normal subjects. The idiotype was found in patients and family members both with and without rheumatoid factor activity, and no significant correlation between the idiotype and IgG or IgM rheumatoid factor or with total immunoglobulin levels was noted (χ^2 test, $p>0.5$; by Spearman's rank correlation PR4 ν IgG $r=-0.12$, PR4 ν IgA $r=0.004$, PR4 ν IgM $r=0.01$). These observations imply that rheumatoid factor activity present in the serum samples did not compromise the idiotype detection by ELISA. No significant correlation of the PR4 idiotype level and antinuclear activity expressed as combined antinuclear reactivities was found (χ^2 test, $p>0.5$). Neither was there a correlation between the

presence/absence of idiotype with increased concentrations of serum immunoglobulin on any single or combination of the immunoglobulins detected in the patients or family members.

Discussion

The results clearly show that autoantibody activity is present in healthy family members of patients with RA. The marked range and level of these reactivities is as broad as that in the patients with RA themselves.

The ELISA used to detect the antinuclear antibodies is probably the reason that we have found a higher frequency of these autoantibodies in patients with RA and their relatives than previously reported.⁴ This method identifies both low and high affinity antibodies, whereas other, older techniques, such as the Farr assay, select for high affinity antibodies.¹⁸

Intriguingly, family members may exhibit a wider range of autoreactivity than that of the affected family member. Comparisons of relatives and patients with RA in this analysis are based on a single serum sample, however, and the serum samples of the patients may of course be affected by drug treatment or duration of disease.

The autoreactivity detected is not an artefact of the assay procedure as the healthy controls tested did not show such a range of reactivity. No simple patterns of association of autoreactivities were evident, implying that the coexpression of individual autoreactive antibodies is not simply due to a linked response, and is most probably due to individual factors together with any polyclonal activation.

The expression of such a wide range of autoreactivity in individuals who do not exhibit any clinical signs of RA clearly shows that possession of autoantibodies alone is insufficient to induce clinical symptoms of the disease. Other factors, either genetic or environmental, must play a part in the clinical expression of RA.

The pattern of expression of the PR4 idiotype in the patients with RA and their healthy relatives is particularly interesting and contrasts with our previous experience with this idiotype in family members of patients with SLE. The PR4 idiotype was originally identified on a human hybridoma derived monoclonal antibody which bound DNA, the major *M Leprae* coat protein PGL-1 and a range of other autoreactivities.⁹ It was derived from a patient with leprosy. This idiotype was subsequently found in serum samples of patients with leprosy (70%), systemic lupus erythematosus (70%), Sjögren's syndrome (15%), and rheumatoid arthritis (40%), and at a frequency of 3% in healthy normal controls.¹¹

Unlike other common idiotypes associated with anti-DNA antibodies (see Ref 19 for review) it was *not* found more frequently in the healthy relatives of patients with SLE. Thus the 16/6 idiotypic, for example, found in 50 to 70% of patients with SLE and 3% of healthy controls¹⁹ was identified in the serum samples of 24% of healthy relatives.²⁰ Even more remarkably the 3I idiotypic described by Betty Diamond and colleagues in 85% of patients with SLE was present in 15/19 (79%) of healthy first degree relatives.²¹ More general autoantibody reactivity has also been found in the serum of healthy relatives of patients with SLE. For example, Isenberg *et al*, in a study of 147 lupus relatives of multiethnic origin, showed that antinuclear activity and antibodies to ssDNA and dsDNA were found in 17%, 20%, and 1.3% respectively.²⁰

The PR4 idiotypic was found at a frequency in the relatives equivalent to that detected in the patients with RA and similar to that originally described.¹¹ The idiotypic showed no association of expression with antinuclear activity, rheumatoid factor activity, or immunoglobulin concentration, and was present in six of the healthy family members (10%), who showed no detectable levels of autoreactivity, in addition to 11 healthy family members with autoreactivity (19%). The PR4 idiotypic has been shown to be carried by DNA reactive antibodies in lupus patients, but is clearly not found only on DNA reactive antibodies in these patients with RA or their family members. The lack of association with total immunoglobulin concentrations confirms our earlier finding, which used a different method to measure the total immunoglobulin concentrations.¹¹

The reason for the relatively high frequency of the PR4 idiotypic in the first degree relatives of patients with RA as compared with relatives of those with SLE is unclear. It has been established that both the 16/6 and PR4 idiotypes are not confined to antibodies binding DNA.^{11 23 24} Whereas the specificity of some 16/6 idiotypic positive, non-DNA-binding immunoglobulins is known—the idiotypic is found, for example, on antibodies to the klebsiella polysaccharide K30^{22 23}—the other reactivities of PR4 idiotypic positive antibodies remain to be determined.

Recently, mRNA sequencing of the V_H regions of several antibodies carrying the 16/6 idiotypic has shown them to have more than 99% homology with a known human germline gene sequence, V_H26.²⁴ Thus it is reasonable to postulate that this idiotypic is indeed germline encoded and that genetic influences are paramount in its expression in the lupus relatives, albeit on non-DNA binding antibodies. In this context it is relevant to note that 4% of lines or clones of antibodies produced by Epstein-Barr virus stimulated cord-blood lymphocytes expressed the

16/6 idiotypic (Lydyard *et al*, unpublished data). In contrast, none of them expressed the PR4 idiotypic.

A variety of observations have suggested links between mycobacterial disease and RA.²⁵ Recently for example, an abnormal glycosylation pattern, first identified in patients with RA, was also found in untreated patients with tuberculosis, but not in a wide variety of other infectious diseases.²⁶ It is thus of interest, and possibly relevant, that the PR4 idiotypic was first found on a monoclonal antibody derived from a patient with leprosy.⁹ It is also present in the serum samples of nearly 40% of patients with tuberculosis (Williams and Isenberg, unpublished observations). Perhaps therefore the expression of PR4 idiotypic in the healthy relatives of patients with RA is evidence of a breakdown in the immune network where germline gene expression and response to mycobacterial or other infection intersect. Although the PR4 idiotypic has not yet been sequenced to confirm its germline gene origin, given its presence in many patients with a variety of infectious and autoimmune disease, this seems more likely than envisaging identical somatic mutation.

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