Characterisation of non-histone nuclear proteins cross reactive with purified rheumatoid factors

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SUMMARY In order to examine the interactions between isolated rheumatoid factors (RFs) and cell nuclear antigens we have prepared 10 RFs by affinity chromatography against IgG coupled to Sepharose. Of these, seven cross reacted with cell nuclei on indirect immunofluorescence. The nuclear antigen appeared to be DNA histones by indirect immunofluorescence on rat liver, though on immunoblotting the rheumatoid factors also reacted with three non-histone polypeptides which were identified in the soluble fraction of nuclear extracts. We were unable to show any relationship between these polypeptides and rheumatoid arthritis nuclear antigen. These reactions represent a hitherto unrecognised phenomenon, which extends the range of antigens recognised by rheumatoid factors. We suggest that the immunopathogenic significance of RFs may not be restricted to their reactivity with IgG, and that non-histone nuclear proteins merit further investigation.

Key words: cross-reactive rheumatoid factors, immunoblotting.

The cross reactivity of isolated IgM rheumatoid factors (IgM RFs) with both the Fc portion of IgG and a cellular nuclear antigen has been reported by a number of groups.^{1–5} On indirect immunofluorescence IgM RFs react with rat liver cell nuclei to give a homogeneous staining pattern which can be inhibited with aggregated human IgG, Fc fragments, and rat liver chromatin,^{4,6} suggesting that the reaction is due to RFs rather than contaminating IgM antinuclear antibodies (ANA). Several workers^{1–5} have also provided evidence against the possibility that IgG ANA copurifying with RF may act as a bridge for the secondary binding of RF to the Fc portion of IgG ANA. The only evidence against the participation of C1q, which binds directly to DNA⁷ and to IgG affinity columns,⁸ has been the lack of effect of heating the RF preparations to 56°C and lack of reactivity when anti-C1q conjugates were used.^{1,6}

There is good evidence that deoxyribonucleic acid (DNA) histones bind IgM RFs, as shown by inhibition studies with nucleosomes and DNA-histones,^{3,9,10} by acid elution¹¹ and reconstitution on

tissue sections,¹ and by direct binding in an enzyme-linked immunoabsorbent assay.¹⁰ However, Johnson¹² has suggested that the cross reaction may be an ionic interaction with free basic groups and may be of little immunopathological significance.

Recently the technique of immunoblotting has been used to show the reactivity of polypeptides with sera containing antibodies to soluble non-histone proteins,^{13,14} including rheumatoid arthritis nuclear antigen (RANA) which migrates as a doublet with a molecular weight of 80 000 and is present in Epstein-Barr virus (EBV) transformed cells.^{15,16} However, the nature of the antigen(s) reactive with RFs has not been examined by this method, and there is no data about a possible relationship with RANA.

In this study immunofluorescence was used to investigate a possible role for C1q and other immune reactants in the binding of rheumatoid factors to nuclei. All investigations were performed in isotonic saline at a physiological pH in an attempt to minimise non-specific interactions. In addition we have adapted the immunoblot technique to examine the possibility that a non-histone protein may represent the specific antigen reactive with RFs and to investigate whether it is related to RANA.

Materials and methods

IGM RF PREPARATIONS

Sera from eight patients with rheumatoid arthritis (RA), and one matched synovial fluid were selected on the basis of high titre rheumatoid factors. Serum from a patient with a polymyositis/scleroderma overlap syndrome, i.e. mixed connective tissue disease (MCTD), was chosen as a control on the basis of an IgG ANA titre of greater than 1/128 000 and a RF titre of 1/160 by latex agglutination. Cyanogen bromide activated Sepharose (Pharmacia Great Britain, Hounslow, Middlesex, UK) was prepared according to the manufacturer's instructions, and purified IgG (Miles, Stoke Poges, UK) was coupled at 20 mg IgG/g Sepharose for two hours at room temperature. After blocking for two hours at room temperature with 0.2 M glycine pH 8.0 the gel was packed as 15 ml columns in 30 ml syringes. The columns were washed with three cycles of 0.1 M bicarbonate, 0.5 M NaCl, pH 8.3; 0.1 M glycine, 0.5 M NaCl, pH 2.5; and 3 M guanidine hydrochloride. Immediately before use the columns were washed extensively with 0.15 M phosphate-buffered saline (PBS) pH 7.4. 5 ml of serum was applied to the column over two hours and washed slowly through with PBS. The column was washed with three column volumes of PBS, and the RF was eluted with 20 ml 1.5 M guanidine hydrochloride. After dialysis at 4°C against PBS with three buffer changes the RFs were analysed by latex agglutination, and IgM and IgG concentrations were determined by radial immunodiffusion.

IMMUNOFLUORESCENCE

Fluorescein isothiocyanate conjugated (FITC) goat antisera specific for human immunoglobulin, γ and μ chains and C1q (Behringwerke, Marburg, FRG) were obtained commercially. FITC rabbit antihuman fibrinogen, C1s, and C3, were kind gifts from Dr David Woodrow, Experimental Pathology, Charing Cross Hospital Medical School. Human C1q was purified by the method of Zubler *et al.*¹⁷ ANA activity was studied by indirect immunofluorescence on cryostat tissue sections of rat liver (Bio-Dx), mouse kidney (Kallestad, FRG), and acetone/methanol fixed Hep₂ cells as previously described.¹⁸ 20 μ l of isolated IgM RF was incubated at room temperature in a moist chamber for 30 minutes. After incubation slides were washed for 10 minutes with PBS pH 7.4, incubated as before with the appropriate FITC antisera for 30 minutes, and then washed for 10 minutes in PBS. Slides were mounted in 90% glycerol/PBS and viewed with a Leitz Laborlux 12 UV microscope. In order to investigate possible direct binding of C1q the

method was repeated using purified C1q (10 mg–1 g/l) as the first layer and developed with an anti-C1q FITC conjugate.

Inhibition studies were performed with an equal volume of IgG, aggregated IgG (heated at 63°C for 30 minutes), C1q, washed and pelleted latex IgG, protamine sulphate, and purified histones (Sigma Poole, Dorset, UK), all in PBS, by prior incubation with IgM RF for 30 min at 37°C. They were then examined for ANA activity with FITC antihuman IgM. Rat liver sections were depleted of histones by washing with 0.1 M HCl and reconstituted with 25 mg/l of histones as described by Tan *et al.*¹¹

PREPARATION OF HISTONE IMMUNOSORBENT COLUMNS

2.5 g of DNA–cellulose (Sigma) was washed in 1 M NaCl for five minutes at room temperature and for 40 minutes in PBS. The DNA–cellulose was mixed for one hour with a 1 g/l solution of whole histones (Sigma) in PBS. After a further wash in PBS the gel was packed into a 10 ml column. 10 ml of isolated RF was passed through the column for two hours, washed through with PBS, and a 10 ml eluate was collected. The column was eluted with bicarbonate buffer pH 8.3, 0.5 M NaCl and glycine buffer pH 2.5, and 0.5 M NaCl, and the 10 ml sample was dialysed overnight against PBS. Both the flow-through, containing unbound material and the eluate containing RF which had bound histones were analysed for RF titre by latex agglutination and for cross reactivity by immunofluorescence and the Western blot.

SODIUM DODECYL SULPHATE (SDS) POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE) AND WESTERN BLOT

5–15% gradient gels (pH 8.8) with a 4% stack (pH 6.8) were run as vertical slabs 170 mm $\times$ 150 mm $\times$ 1.5 mm according to the method of Laemmli.¹⁹ Nuclear or whole cell extracts for blotting were prepared by sonication in PBS as previously described.¹⁵ After centrifugation at 20 000 g the supernatant of the sonicate was retained as the normal saline extract, and the pellet then sonicated in 1 M NaCl to provide a histone-enriched (high salt) extract. Samples for electrophoresis were boiled for five minutes in sample buffer containing 1% SDS and 0.1 M dithiothreitol.

Proteins separated by SDS PAGE were transferred to nitrocellulose paper (Schleicher and Schull, Dassel, Germany) in a Biorad Transblot cell (Biorad Laboratories, Watford, UK) at 30 V for 18 hours in 0.025 M trometamol (TRIS) and 0.192 M glycine containing 20% methanol by a method based on that of Burnette.²⁰ After blocking with 2% casein

in PBS for two hours the sheets were incubated with isolated IgM RF diluted 1:2 with PBS/casein for one hour at room temperature. The sheets were washed four times for 15 min in PBS and incubated for one hour in antihuman IgM (Dako, Copenhagen, Denmark) labelled by the chloramine-T method²¹ and diluted in PBS/casein. After a further four washes in PBS the sheets were dried and developed by autoradiography for 72 hours.

For identification of proteins within the IgM RF preparations the eluates were separated by SDS PAGE, transferred, and incubated as above with antihuman IgM, IgG, or IgA, antihuman C1q, antihuman C3, or antihuman fibrinogen diluted 1:100, washed as above, and incubated for one hour with ¹²⁵I-protein A labelled by chloramine-T, followed by further washes and development by autoradiography for 18 hours.

Results

The protein concentrations of the isolated IgM RFs were from 0.02 to 0.35 g/l and they were all positive

by latex agglutination, with titres varying from 1:32 to 1:256. On indirect immunofluorescence for anti-nuclear antibody activity with FITC antihuman IgM seven (including the RF from the synovial fluid) out of 10 RFs gave nuclear staining, with a mean titre of 1/64. The nuclear staining pattern was homogeneous on rat liver and mouse kidney sections, but a more granular pattern was seen with Hep₂ cells (Fig. 1). Latex IgG and aggregated IgG at two different concentrations completely inhibited the cross reaction on indirect immunofluorescence (Fig. 1c). Monomeric IgG, free histones, and protamine partially inhibited the cross reaction, and C1q had no effect. Passage of the IgM RF down a DNA-cellulose-histone column before immunofluorescence abolished cross reactivity, which could be recovered from the eluate as homogeneous nuclear staining. Two RFs, both negative for IgM ANA, showed evidence of IgG ANA at titres of 1/2 and 1/8. One of these was from the MCTD patient whose serum IgG ANA titre was greater than 1/128 000.

The concentration of IgM in the dialysed eluates

Fig. 1a

Fig. 1b

Fig. 1c

Fig. 1 Indirect immunofluorescence with a purified rheumatoid factor developed with FITC anti-IgM: (a) homogeneous nuclear staining on rat liver; (b) an amorphous granular staining pattern on Hep₂ cells; (c) complete inhibition by aggregated IgG of nuclear staining shown as unstained 'holes' against background cytoplasmic staining on rat liver. ($\times 425$).

varied from 0.0135 to 0.29 g/l in approximately 20 ml, with the mean total IgM yield being 0.79 mg, accounting for approximately 70% of the protein in the purified material. The heavy and light chains of IgM were identified as two major bands on polyacrylamide gel electrophoresis, with molecular weights of 78 000 and 32 000 (Fig. 2). The identities of other minor components seen on the gel were confirmed by the Western blot with specific antisera (not shown). RF I and II contained the C3 complement component at 75 000. The minor band at 62 000 was the IgA heavy chain, and the 52 000 band in the IgM RFs corresponded to the IgG heavy chain. All the RFs contained an unidentified 41 000 band which did not react with an antifibrinogen serum. RFs I, II, and III showed a 31 000 band corresponding to C1q. Because RFs I, II, and III

Fig. 2 Polyacrylamide gel electrophoresis, samples stained with Coomassie Blue, showing molecular weight standards (stds), the polypeptides of purified C1q (the same material that was used in the inhibition and absorption experiments), IgG, and five purified rheumatoid factor preparations.

also contained the highest concentration of IgM they were selected for further studies with specific conjugates.

No nuclear staining was seen with either fluorescein conjugates of antibodies to the complement components C1q, C1s, and C3 or with FITC antifibrinogen. Heating the RFs to 56°C did not diminish their ANA activity with the anti-IgM conjugate. No specific staining was seen when the slides were overlaid with purified C1q in a range of dilutions from 10 mg/l to 1 g/l and developed with anti-C1q.

ANTIGENS REACTIVE WITH RHEUMATOID FACTORS

Comparison of acid eluted and untreated rat liver sections as a substrate for detection of the cross reaction of IgM RF showed that the cross reaction was completely inhibited by acid elution and partially restored by reconstitution with histones (Fig. 3).

On Western blotting only one rheumatoid factor RF I, showed any reaction with histones, seen as faint bands between 14 000 and 23 000 in the

Fig. 3a

Fig. 3b

Fig. 3c

Fig. 3 Demonstration of reactivity between a purified rheumatoid factor and histones by depletion and reconstitution on rat liver using indirect immunofluorescence. (a) Untreated section showing homogeneous and rim staining pattern by purified rheumatoid factor; (b) acid eluted section; (c) acid eluted section reconstituted with histones. ($\times 480$).

purified histones and in the high salt cell extract. However, all three reacted strongly in the isotonic saline extract with a higher molecular weight (94 000) polypeptide and relatively weakly with polypeptides of molecular weight 200 000 and 48 000 (Fig. 4). These polypeptides were seen in the EBV transformed B lymphoblastoid cell lines WiL2, Daudi, B958, Raji and in the EBV negative lines Ramos and Molt-4. The reactivity was the same with isolated nuclei as with whole cell extracts. Identical polypeptides were blotted in fresh rabbit thymus extract but not in a commercially available rabbit thymus acetone powder. The bands did not react with an isolated rheumatoid factor which had no ANA activity on immunofluorescence nor with the conjugate alone. No reactivity was seen when the blots were developed with anti-IgG or staphylococcal protein A.

Recognition of the three bands by the rheumatoid

Fig. 4 Western blots by three purified rheumatoid factors RFs I-III on histones (lanes 1), isotonic saline extract of Molt-4 cells (lanes 2), and high salt histone-enriched extract (lanes 3).

factors was completely inhibited by aggregated IgG but not monomeric IgG. Absorption of RF I on the DNA-cellulose-histone column completely abolished the reaction, and the activity was recovered from the eluate.

Discussion

In this study we found cross-reactive RFs in six out of eight RA patients studied. This frequency (approximately 75%) is in keeping with reports from Johnson⁴ but is much higher than that described by previous authors.¹⁻³ However, our study was designed to examine immunochemical interactions between RFs and nuclear antigens rather than to investigate the prevalence of this phenomenon in an RA population, and we suggest that our high frequency was due to the deliberate selection of sera from patients with severe RA with high titre RFs and which (with one exception) were ANA positive.

Surprisingly, no previous study has published data on the examination of affinity purified rheumatoid factors by polyacrylamide gel electrophoresis. We found that in addition to IgM, IgG and complement components could be identified in our preparations.

We have confirmed previous work¹⁻⁶ which showed that the reactions were not due to IgG, by using anti-IgG conjugates and by the lack of cross reactivity of a rheumatoid factor purified from a patient with MCTD deliberately selected for the presence of very high titre IgG ANA. It is of interest that the low titre of ANA (1:8) within this RF preparation compared with serum IgG ANA suggests that the maximum contamination of IgG without RF activity in these eluates from the affinity columns was less than 0.1% after a single purification step. With purified C1q we were unable to show any direct binding to cell nuclei. Heating the RF to 56°C had no effect on the reaction, and there was no nuclear fluorescence when FITC anti-C1q, anti-C1s, or anti-C3 were used as conjugates. This confirms previous work¹⁻⁶ that provided evidence against C1q mediating in a false positive reaction.

On indirect immunofluorescence the rheumatoid factors reacted with mouse kidney or rat liver nuclei to give a predominantly homogeneous or rim pattern compatible with antibodies to DNA-histones. This reaction was abolished by prewashing the slides with 0.1 M HCl, a procedure which elutes histones (as well as other nuclear antigens), and could be partially restored by reconstitution with purified histones. This supports previously published work which has shown interaction with DNA histones both by immunofluorescence¹⁻⁵ and by solid phase assays.¹⁻¹⁰ However, the granular pattern on Hep₂ cells was quite unlike that seen with antihistone antibodies and suggested to us that additional reactivity with non-histone proteins could be playing a part. This was supported by the reactions seen on the Western blot. Although blotting of histones was seen with one of the RFs tested, the reaction was faint and was much less striking than that seen with three polypeptides of molecular weight 48 000, 94 000, and 200 000. These antigens were clearly not histones, as the molecular weight was too high (the largest histone H1 migrates in SDS PAGE at mol. wt 31 000) and they were present in an isotonic saline extract and not in the high salt (histone-enriched) extract. The relative lack of reactivity with histones on blotting could be due to their separation from DNA, which will occur after gel electrophoresis. This interpretation is supported by previous observations¹⁻⁷ that both DNA and histones are required for antigenicity, and by our findings that inhibition with DNA-cellulose-histone was complete and only partial with free histones.

Thus it appears that RFs bind to two different sets of antigens depending on the type of assay used: histones by immunofluorescence on rat liver and the higher molecular weight polypeptides on blotting.

The blotting activity was completely inhibited by absorption with DNA-cellulose-histone and could be recovered from the eluate, suggesting that this dual reactivity was not due to different populations of RFs in our preparations. One explanation is that a major component of the immunofluorescent reaction is not due to histones but to the soluble polypeptides detected on blotting. This could explain the granular staining pattern on Hep₂ cells, which is quite unlike the more homogeneous pattern seen with antihistone antibodies. The fact that the staining disappeared after elution with acid is not evidence against this interpretation, because a number of nuclear antigens are destroyed or eluted by this treatment.¹¹ It is possible that histones cross react with these polypeptides by sharing antigenic determinants. Alternatively it is conceivable that the binding of the RFs to histones which was shown on the column by inhibition in solution, and to the histone reconstituted immunofluorescent slides was a non-specific interaction due to their highly basic charge, as suggested by Johnson.¹² This interpretation is supported by our finding of partial inhibition of binding to nuclei by protamine, another basic protein. However, our studies were conducted in conditions deliberately designed to minimise such interactions by performing all assays at a physiological pH in normal saline, and unlike Johnson we did not see inhibition by monomeric IgG on blotting or immunofluorescence, indicating a higher avidity of RFs for nuclear antigens than free IgG, though it appeared to be lower than that for aggregated IgG.

A recent study has suggested²² that monoclonal rheumatoid factors prepared from a MRI/lpr mouse reacted with a range of nuclear antigens, as determined by direct binding and inhibition assays. In our study we have extended the range of nuclear antigens recognised by human rheumatoid factors to include three soluble polypeptides which appeared to react with the population of RFs that also reacted with histones and IgG. So far we have not been able to show any relationship between these antigens and RANA or any other of the many antigens to which autoantibodies are found in RA. However, we suggest that the polypeptides recognised by RFs on the Western blot merit further investigation, and that the immunopathogenic significance of RFs may not be related solely to their anti-Fc activity.

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