

Reaction of antibodies to rheumatoid arthritis nuclear antigen with a synthetic peptide corresponding to part of Epstein-Barr nuclear antigen 1

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SUMMARY Antibodies to rheumatoid arthritis nuclear antigen (RANA) are detected by immunodiffusion (ID) and immunofluorescence (IF), though reports of the identity of the antigen(s) have been conflicting. In this study it is shown conclusively that ID and IF anti-RANA react with epitopes on Epstein-Barr nuclear antigen 1 (EBNA-1) and that the major epitope detected by immunofluorescence is represented by a synthetic peptide, P62, corresponding to part of EBNA-1. In an enzyme linked immunosorbent assay (ELISA) anti-P62 antibodies in 35 rheumatoid arthritis sera were threefold higher than those of 35 age and sex matched controls, with the highest levels occurring in young patients with active joint disease.

Key words: Epstein-Barr virus.

Antibodies to rheumatoid arthritis nuclear antigen (RANA) were originally detected as precipitin reaction with Epstein-Barr virus (EBV) transformed cell lines in sera from 61% of patients with rheumatoid arthritis (RA) and 6% of controls.¹ The demonstration that the antigen was detectable only in cell lines which had been transformed by the EBV² led to speculation that EBV was a candidate pathogen in RA. This concept was not supported by seroepidemiological studies, which showed that the prevalence of EBV infection, as defined by the presence of antibodies to EB viral capsid antigen (VCA), was the same in RA and the normal population both in adults,³⁻⁴ and in children.⁵⁻⁶

More recently, evidence has accumulated suggesting that in patients with RA the regulation of EBV infection is impaired. This has been shown by defective T cell control of EBV infected B cells in vitro,⁷⁻¹⁰ increased numbers of copies of EBV genome in RA B cells,¹⁰⁻¹¹ and by raised titres of antibodies to EBV antigens in RA. The regulatory defects are not restricted to EBV, however, nor has

there been general agreement on the serological evidence. Although some studies have found raised titres of anti-EBVCA in patients with RA,¹²⁻¹³ others have found them to be normal.¹⁴⁻¹⁵ In contrast with these discrepant findings, there is general agreement that anti-RANA antibodies are more frequent in RA and present in titres 2-16-fold higher than in controls.^{1-4, 12-13} This in turn implies that anti-RANA antibodies may represent a specific immune response to one EBV encoded antigen in RA.

The apparent selectivity of the immune response to RANA in RA has led to interest in the nature of the antigen itself. When first described its distinction from EBNA was explored in detail by Alspaugh *et al.*² Furthermore, anti-RANA has been detected in sera negative for anti-EBNA both in RA²⁻⁴ and in infectious mononucleosis.¹⁶ The distinction between RANA and EBNA was later complicated by the description of at least two antigens, EBNA-1 and EBNA-2, which were recognised by anti-EBNA positive sera.¹⁷ It was then suggested that anti-RANA antibodies reacted with the EBNA-1 polypeptide by immunoblotting.¹⁸⁻¹⁹ An explanation for differences between RANA and EBNA was that their respective antibodies reacted with different

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epitopes on the same polypeptide. These data were challenged by Sculley and his colleagues,^{20, 21} who confirmed that RA sera reacted with EBNA-1 but that the greatest difference between RA and normal sera was in a reaction with a higher molecular weight (94 kilodalton) polypeptide that they identified as EBNA-2,²¹ which correlated with antibodies to RANA by immunofluorescence. Based on this evidence they suggested that EBNA-2 was the target for anti-RANA antibodies.

A recent report described the use of several synthetic peptides corresponding to sequences in EBNA-1 in enzyme linked immunosorbent assays (ELISA) for measuring anti-EBNA antibodies.²² Several peptides reacted strongly with RA sera, but the highest levels of binding and the greatest difference between RA and normal sera were found with a peptide, P62, containing 20 amino acids, corresponding to part of the internal repeat sequence of EBNA-1. In that study, however, the patients and controls were not age matched, nor was there any attempt to define whether the antibodies reacting with P62 had anti-RANA or anti-EBNA specificities, or both.

The purpose of this study was to use P62 to characterise antibodies reacting with RANA and EBNA-1 by immunofluorescence, Western blot, immunodiffusion, and ELISA in patients with RA and healthy controls. Because each of the antibody systems is operationally defined we have chosen the following nomenclature for each antibody system: anti-EBNA detected by anticomplementary immunofluorescence is termed ACIF anti-EBNA, and anti-EBNA-1 refers to antibodies detected by Western blot. Anti-RANA by indirect immunofluorescence is termed IF anti-RANA, and by immunodiffusion ID anti-RANA. The term anti-P62 refers to specific antibodies detected by ELISA. We have also examined antibodies to P62 in a group of patients with RA and compared the results with those for age and sex matched controls to see whether, like anti-RANA, high titres of these antibodies are found in RA.

Patients and methods

PATIENTS AND SERA

Serum from 35 patients with classical or definite RA,²³ comprising 30 women and five men (mean age 49.6 years), and 35 healthy controls (F:M 30:5, mean age 49.9 years) were used in a controlled study for measuring antibodies to P62 by ELISA. Also tested were 30 younger patients with systemic lupus erythematosus (F:M 28:2, mean age 38.4) as disease controls.

Three reference sera were used for the detection

of anti-RANA antibodies by immunofluorescence and immunodiffusion, and anti-P62 antibodies by ELISA. Serum No 1 was that used in our previous study⁴ and was used in the immunodiffusion experiments. Serum No 2, strongly positive for anti-RANA and anti-P62, was used as the standard serum for anti-P62 ELISA and for the affinity purification of anti-RANA antibodies. Serum No 3,²⁴ from a healthy donor, was used because this had been previously characterised as containing only antibodies to EBNA-1 by immunoblotting.

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR ANTI-P62

The antigen, P62, with the amino acid sequence GAGGGAGGAGAGGGAGGAGA conjugated to a single cysteine residue was used exactly as previously described²² with serum dilutions of 1:20 and 1:320 except that results were expressed in relation to a standard curve of serial dilutions of serum No 2 in order to standardise the assay and to improve measurement of antibody in serum. Because column fractions often gave results falling above or below the standard curve these results were expressed as optical density units. In each case such fractions were assayed on the same ELISA plate.

AFFINITY PURIFICATION OF ANTI-P62 AND ANTI-RANA ANTIBODIES

Affinity columns were prepared by coupling 1 mg of P62/ml of gel to cyanogen bromide activated Sepharose (Pharmacia, Hounslow, Middlesex, UK) followed by blocking with 2 M glycine pH 8.0 according to the manufacturer's instructions. Control columns, conjugated with glycine or alanine were prepared in the same way but the initial conjugation with P62 was omitted. Poly-L-lysine coupled to Sepharose was purchased commercially (Pharmacia). Because 1:20 was the optimal dilution for detecting IF anti-RANA, all column fractions were recovered at a dilution equivalent to 1:20 by taking 5 ml fractions from an original applied volume of 250 µl of serum. Each serum (250 µl), followed by 100 µl of phosphate buffered saline (PBS), was run into 1 ml affinity columns and left for one hour at 20°C in the gel. The flow through was collected by running 5 ml of PBS through the column. After a further wash with 200 ml of PBS, bound antibody was eluted with 5 ml eluting buffers: 0.1 M glycine, 0.5 M NaCl pH 2.5; 1 M guanidine HCl and/or 3 M guanidine HCl. Each column fraction was dialysed separately with 1 litre of PBS over 48 hours with three changes of buffer and then tested by immunofluorescence for IF anti-RANA using the modified method described by Alspaugh *et al.*²⁵ by ACIF for

anti-EBNA antibodies,²⁴ by ELISA for anti-P62, and by immunoblotting for anti-EBNA-1.

Because anti-RANA antibodies on immunodiffusion were found to react optimally with a serum dilution of 1:4 a similar method to that above, but with an 8 ml affinity column containing 16 mg of P62, was used to purify antibody from 2 ml of serum No 2. The original serum, an 8 ml flow through, and 8 ml 3 M guanidine eluate, each corresponding to a serum dilution of 1:4, were tested on immunodiffusion with WI-L2 extract and P3HR-1 as previously described.⁴ Also tested on immunodiffusion were P62 (unconjugated), P62 conjugated to human serum albumin (HSA) with a molar ratio of approximately 10:1, and P62 conjugated to keyhole limpet haemocyanin (KLH) (ratio 2:1). The conjugates²² were tested at concentrations of 200, 20, and 2 µg/ml of P62.

IMMUNOBLOTTING OF EBNA-1

For immunoblotting 5 µl extracts from WI-L2, P3HR-1, and Raji containing approximately 30 mg/ml of protein, and 5 µl of the conjugates each containing 10 µg/ml of P62 were loaded into the wells of a 5–15% gradient gel prepared as previously described.²⁶ For immunoblotting of precipitins the immunodiffusion gel in Fig. 1 was used. After washing for five days in PBS the precipitins were cut

out in 2 mm×5 mm×2 mm slices. The four opposite the well containing WI-L2 extract and the four opposite that containing P3HR-1 were each amalgamated as WI-L2 and P3HR-1 precipitins respectively. Slices from two areas of gel, each 2 mm×10 mm×2 mm, immediately above and below the P3HR-1 wells in which no precipitins were seen, were amalgamated as the control. The precipitins were prepared by boiling them twice with 50 µl concentrated starting buffer and quickly applying them to a polyacrylamide gel before the agarose reset. For the immunoblotting of multiple fractions on one antigen the cassette described by Williams *et al* was used.²⁶ The transfer to nitrocellulose, incubation with antibody, diluted 1:100, and development with a peroxidase based ELISA method was also as reported by Williams *et al*.²⁶

Results

REACTION OF ANTI-RANA ANTIBODIES ON IMMUNODIFFUSION WITH EBNA-1

When tested by immunodiffusion serum No 1 (anti-RANA) and serum No 3 (anti-EBNA-1) reacted both with WI-L2 and P3HR-1 with a line of complete immunological identity between the two cell extracts (Fig. 1). In other experiments (not shown) serum No 3 precipitin gave a line of complete identity with serum No 1 to a titre of 1/8. Because serum No 3 lacked antibodies to EBNA-2 and because P3HR-1 does not contain EBNA-2 we concluded that the RANA precipitin did not contain EBNA-2. The presence of EBNA-1 in the precipitins was confirmed by immunoblotting of the precipitins together with EBV transformed cell lines in which the EBNA-1 polypeptide has a known variable molecular weight: 80 kilodaltons in WI-L2, 78 kilodaltons in P3HR-1, and a lower molecular weight polypeptide in Raji (72 kilodaltons) (Fig. 2). There was only a very weak reaction with EBNA-1 in gel cut from an area in which there was no visible precipitin. There was also a reaction with HSA conjugated to P62, migrating as a diffuse band between 80 and 100 kilodaltons with some higher molecular weight bands. This confirmed that P62 was immunoreactive on blotting and that there were on average 10 P62 peptides on each HSA molecule, accounting for its increased molecular weight.

REACTION OF ANTI-RANA ANTIBODIES WITH P62 AND EBNA-1

On indirect immunofluorescence P3HR-1 was found to give the characteristic discrete speckled staining pattern when tested with anti-RANA reference sera (Fig. 3). Serum No 2 was purified by affinity

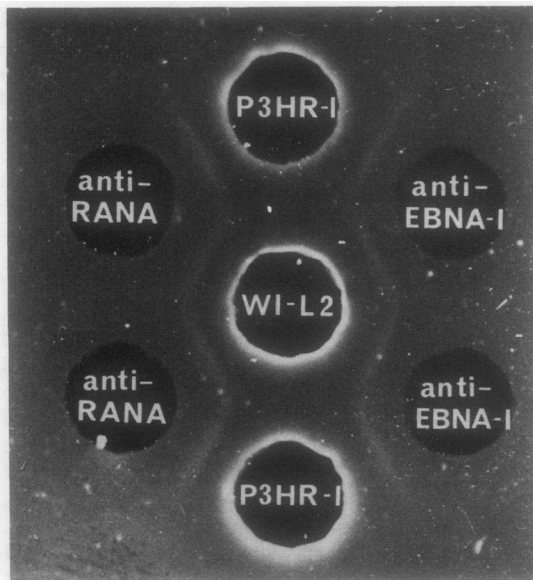


Fig. 1 Immunodiffusion experiment showing RANA in both WI-L2 extract and P3HR-1 reacting with an anti-RANA reference serum (No 1, 1:4) and a serum containing only antibodies to EBNA-1 (No 3, 1:2).

chromatography, and bound antibody eluted with three different buffers (Table 1). Relatively low concentrations were found in the acid and 1 M guanidine eluates, most of the antibody being recovered from the 3 M guanidine eluate. This showed that in this serum most of the antibody was of high affinity and its antigen specificity was not

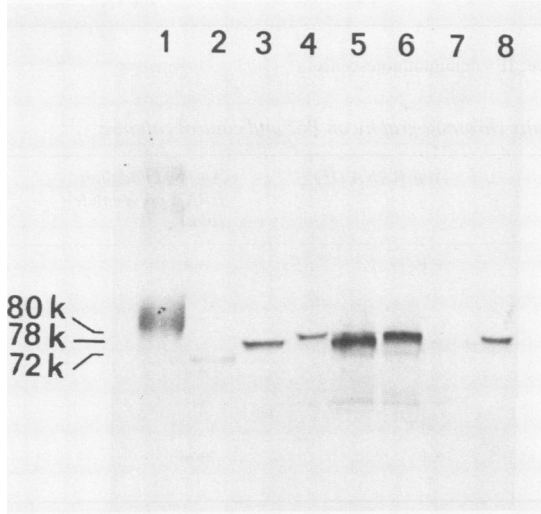


Fig. 2 Western blot with serum No 2 (1:100) as a probe. Reactivity with bands in the P62 human serum albumin conjugate (lane 1), EBNA-1 in Raji cell extract (lane 2), P3HR-1 (lane 3), WI-L2 (lanes 4 and 8), and with precipitins cut from the immunodiffusion gel in Fig. 1 (lanes 5 and 6). Lane 5: P3HR-1 precipitin, lane 6: WI-L2 precipitin, lane 7: control area of gel.

denatured by 3 M guanidine. When tested for anti-EBNA by ACIF the original serum and the flow through were positive for anti-EBNA. The acid eluate was weakly positive, but the guanidine eluate was negative. In contrast, anti-RANA, also strongly positive in the original serum, was recovered from the 3 M guanidine eluate and was not detectable in the flow through. All fractions except the 1 M guanidine eluate reacted with the 80 kilodalton EBNA polypeptide by Western blot; the strongest reaction (judged by the intensity of staining of the band on the nitrocellulose) was with the 3 M guanidine eluate.

Because 3 M guanidine recovered most of the antibody bound to the P62 column this buffer was used to elute antibody from specificity control columns. When serum No 2 was used on four different columns, anti-P62, as expected, bound to the P62 column. Surprisingly, some anti-P62 antibody, but not IF anti-RANA, also bound to the glycine conjugated column, suggesting that a proportion of anti-P62 antibodies bound to glycine residues alone. There was no binding to columns coupled to alanine or to poly-L-lysine (Table 2).

These experiments led us to adopt the following protocol for examining further sera. Each serum was passed first through the glycine conjugated column to absorb antiglycine antibodies, then through the P62 column, and the flow through collected. The two columns were disconnected and each eluted separately with 3 M guanidine (Table 3). In each case anti-EBNA was detected in the flow through but not in the eluate, whereas anti-RANA activity was abolished in the flow through and recovered in

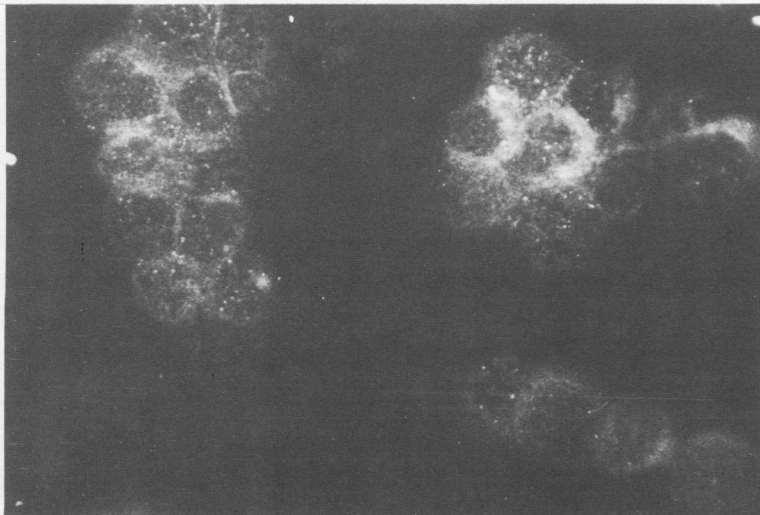


Fig. 3 Immunofluorescence showing characteristic RANA staining pattern on P3HR-1 (serum No 2 at 1:20).

Table 1 Reactivity of fractions purified from serum No 2 by affinity chromatography on a P62 column

	Anti-P62 ELISA (OD units) [†]	Anti-EBNA (ACIF) [†]	Anti-RANA (IF) [†]	Anti-EBNA-1 (immunoblot)
Serum (1:20)	1500	+++	++	++
Flow through*	38	++	-	+
Acid eluate	235	+	-	+
1 M guanidine eluate	20	-	-	-
3 M guanidine eluate	1500	-	++	+++

*All column fractions equivalent to a serum dilution of 1:20.

[†]OD=optical density; ACIF=anticomplementary, immunofluorescence; IF=immunofluorescence.

Table 2 Reactivity of fractions of serum No 2 separated by affinity chromatography on P62 and control columns

	ELISA (OD)	Anti-RANA (IF)	Anti-80 kilodalton EBNA polypeptide (blot)
Serum (1:20)	1400	++	++
P62: Flow through	215	-	+
Eluate	1290	+	+++
Glycine: Flow through	1227	++	+
Eluate	1223	-	+++
Alanine: Flow through	1352	++	++
Eluate	71	-	-
Lysine: Flow through	1338	++	++
Eluate	63	-	-

Table 3 Reactivity of fractions of sera passed sequentially through a column coupled to glycine (polyglycine) followed by a P62 column

	Serum	Flow through	Polyglycine eluate	P62 eluate
Serum No 2 (1:20)				
ELISA (OD)	1500	90	1290	340
Anti-RANA (IF)	1/160	-	-	1/40
Anti-EBNA (ACIF)	++	+	-	-
RA 2				
ELISA	653	58	49	783
Anti-RANA (IF)	+	-	-	+
Anti-EBNA (ACIF)	+	+	-	-
RA 3				
ELISA	667	70	41	175
Anti-RANA (IF)	ANA +++*	ANA ++	-	+
Anti-EBNA (ACIF)	+	+	-	-
RA 4				
ELISA	689	50	27	288
Anti-RANA (IF)	ANA +++*	ANA ++	-	+
Anti-EBNA (ACIF)	+	+	-	-
RA 5				
ELISA	817	134	606	780
Anti-RANA (IF)	++	-	-	++
Anti-EBNA (ACIF)	-+	-	-	-
RA 6				
ELISA	1500	71	985	1010
Anti-RANA	++	-	+	++
Anti-EBNA (ACIF)	-+	-	-	-

*Specific RANA staining obscured by antinuclear antibodies (ANA) in RA 3 and 4.

[†]Although these sera were negative for anti-EBNA by ACIF both contained antibodies to EBVCA and to EBNA-1 by immunoblotting.

the eluate (Fig. 4). Two sera, RA 3 and 4, were deliberately selected for the presence of high titre antinuclear antibodies (ANA), one homogeneous and one speckled, which obscured any specific RANA staining. The ANA were recovered in the flow through, whereas the eluates, which had been completely cleared of ANA staining, showed the characteristic RANA staining pattern. Two further sera, RA 5 and 6, were tested because they were IF anti-RANA positive but ACIF anti-EBNA negative.

It is of interest that in one of these the eluate from the glycine column contained anti-RANA activity.

Affinity chromatography was also used to characterise anti-RANA antibodies detected by immunodiffusion. Because this assay requires higher concentrations of serum to produce a precipitin, a serum dilution of 1:4 and column fractions equivalent to a serum dilution of 1:4 were used. ID anti-RANA was detected in the serum and the flow through but not in the eluates from either the glycine or the P62

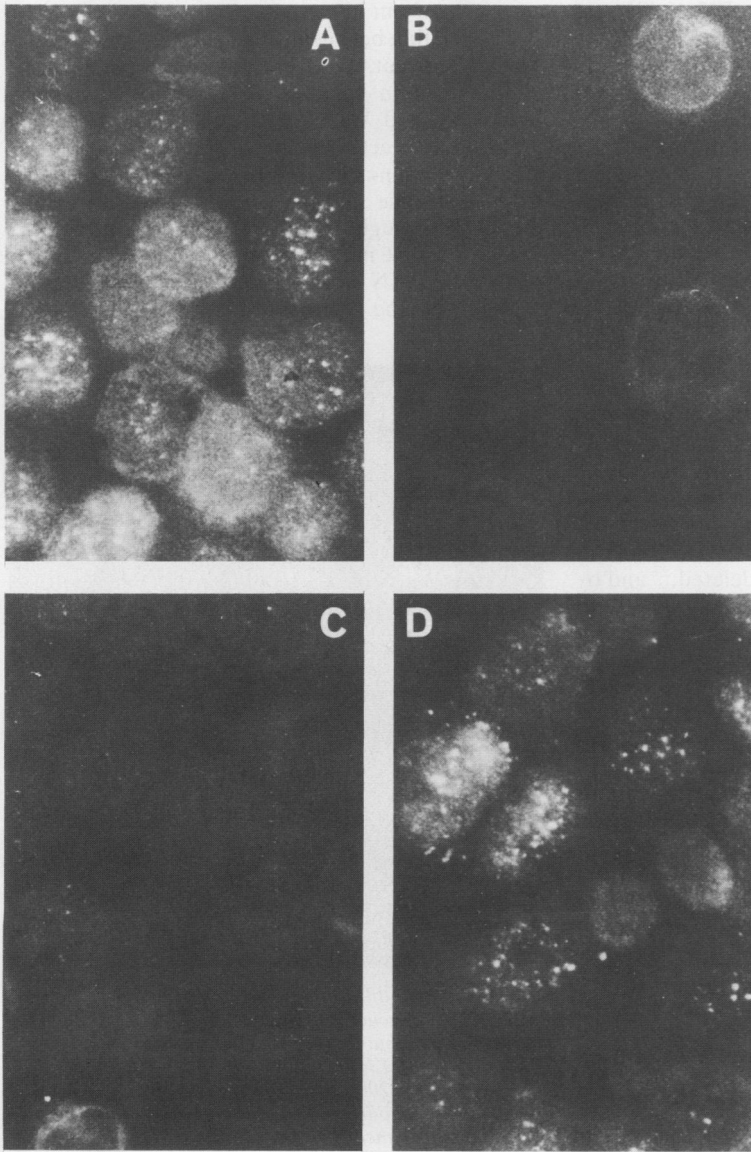


Fig. 4 Anti-RANA immunofluorescence of fractions from affinity chromatography with P62. (A) Serum No 2 1:20 on WI-L2 cells; (B) control with conjugate alone; (C) flow through showing depletion of anti-RANA activity; and (D) eluate from the P62 column showing anti-RANA staining.

column. There was depletion of anti-RANA activity in the flow through from the columns, however, in that the titre fell from 1/128 to 1/16. In addition, we attempted to purify anti-RANA antibodies by elution from the P62 column without preabsorbing with glycine, and again no precipitins were seen in the eluate. We also tested P62, P62 conjugated to KLH, and P62 conjugated to HSA as antigens on immunodiffusion. At concentrations of 2, 20, and 200 µg/ml neither P62 alone nor the HSA or KLH conjugates formed precipitins with three anti-RANA sera (Nos 1, 2, and 3) or with the eluate recovered from the P62 column (Fig. 5).

By ELISA, serum No 2 bound to P62 at dilutions greater than 1:30 000 and was used as the standard serum. With screening dilutions of 1:20 the RA sera were found to contain anti-P62 antibodies in titres twofold higher than those of the age and sex matched controls (not shown), and at a screening dilution of 1:320, the titres were 3.5 times higher (Fig. 6). Of the 35 patients with RA tested, very high levels (greater than five standard deviations above normal) were found in four. All four were young (aged less than 35), DR4 positive, and had active joint disease. Only one was receiving second line antirheumatic treatment.

Discussion

This study has conclusively shown that both IF and ID anti-RANA antibodies react with the EBNA-1. The recent suggestion that anti-RANA antibodies react with EBNA-2 was based on correlative data, and is contradicted by our findings that both ID and IF RANA are detectable in P3HR-1, a cell line in which the EBNA-2 gene has been deleted,²⁷ and by the finding that a serum lacking EBNA-2 antibodies reacted with RANA both by immunofluorescence and immunodiffusion. The reaction with EBNA-1 was confirmed by immunoblotting RANA precipitins. A similar experiment was also reported by Billings *et al* with the same result, though they did not illustrate their findings in a figure.¹⁸

Our data suggest that P62 contains the major epitope recognised by IF anti-RANA antibodies. Antibodies were depleted by a single passage through the P62 column and then recovered from the eluate in six RA sera. This suggests that the short amino acid sequence contains most if not all of the epitopes recognised by IF anti-RANA. This may be because P62 contains all possible epitopes on the IR3 region of the EBNA-1 peptide: it has two glycine triplets, two glycine doublets, and three single glycine residues each separated from each other by single alanine residues.

In contrast with IF anti-RANA, ACIF anti-EBNA did not appear to react with P62, though it is

possible that treatment with 3 M guanidine, even after dialysis back into saline, affected the complement fixing abilities of the antibody. This possibility is supported by the recovery of some ACIF anti-EBNA reactivity in the acid eluate. Nevertheless, the recovery of ACIF anti-EBNA in the flow through suggests that antibodies detected by this technique have a more diffuse reactivity, including epitopes on EBNA-2.¹⁷ These findings also explain discrepancies between anti-RANA and anti-EBNA which have been observed in sera from patients with RA (including two sera in this study), from those with Burkitt's lymphoma,² and in those from patients recovering from infectious mononucleosis.¹⁶

The relation between ID anti-RANA and P62 was not so clear cut. We were not able to show a precipitin reaction between affinity purified anti-P62 antibodies and WI-L2 extract and there were no precipitins detected between P62 (alone or conjugated to proteins such as KLH or albumin) and anti-RANA positive reference sera. The fall in titre in the flow through, however, supports our earlier suggestion that more than one antigen may play a part in the RANA precipitin reaction, and that one of these may be represented by P62.^{4, 19} The exact

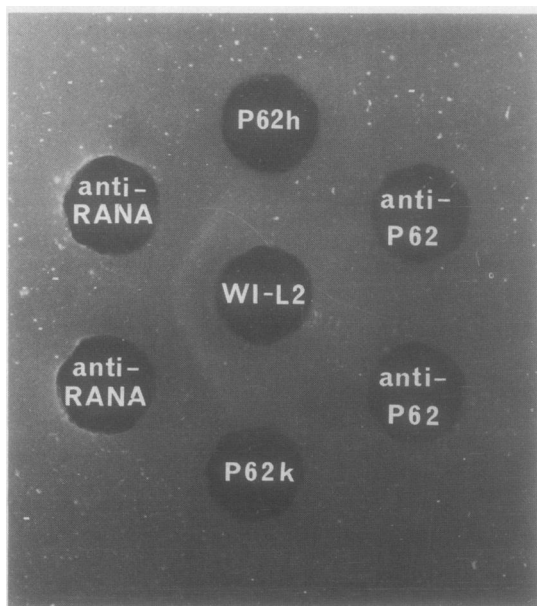


Fig. 5 Immunodiffusion showing no reaction between an anti-RANA reference serum (No 1) and P62 conjugated to human serum albumin (HSA) (P62h) or to keyhole limpet haemocyanin (KLH) (P62k), each containing P62 at a concentration of 200 µg/ml. There is no reaction between anti-P62 affinity purified from serum No 2 with WI-L2 extract or with either of the conjugates.

specificity of ID anti-RANA antibodies remains unknown, though we can be certain that major epitopes lie on EBNA-1. Our findings also challenge the assumption that ID anti-RANA and IF anti-RANA are the same.

We have previously suggested that the higher titres of ID anti-RANA antibodies in RA, and the finding of anti-RANA antibodies in sera from patients free of EBV infection, could be due to cross reactive autoantibodies. In this study cross reactions were found between antibodies binding to a glycine

conjugated column (antiglycine antibodies), anti-P62, and anti-EBNA-1 by immunoblotting. Specificity was proved by showing no binding to columns conjugated with alanine or lysine. Such a cross reaction is perhaps not surprising when it is considered that 13 of the 20 amino acids which comprise P62 are glycine residues. It is possible that such a cross reaction could account for the range of host proteins also reactive with IgM anti-EBNA-1 from infectious mononucleosis sera.²⁸ Although we demonstrated a cross reaction between antiglycine

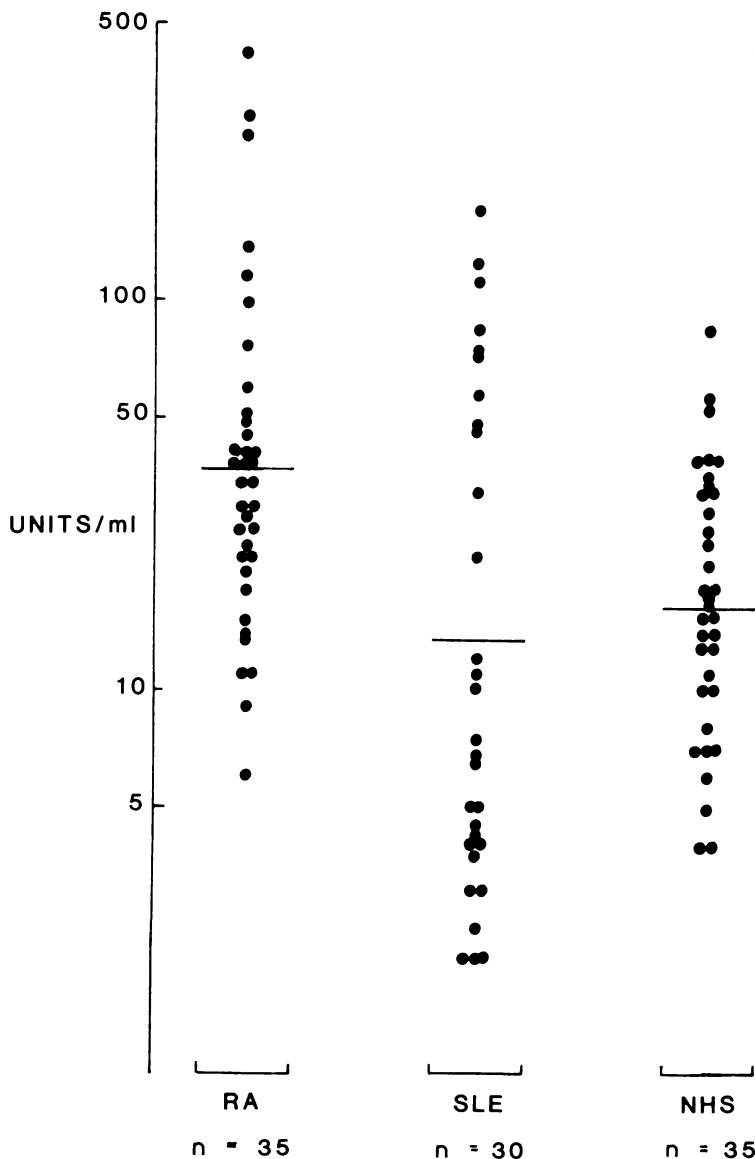


Fig. 6 Antibodies to P62 by ELISA in RA, systemic lupus erythematosus (SLE), and normal controls (NHS). The horizontal bars represent the geometric means.

and IF anti-RANA in only one out of six RA sera tested, it is possible that antiglycine antibodies could increase the titres of IF anti-RANA and anti-P62 in RA sera. This was supported by the finding that sera from the three patients with the highest levels of anti-P62 antibodies (serum No 2, RA 5, and RA 6) also contained the largest proportion of their antibody binding to the glycine conjugated column.

Repeat sequences containing glycine are commonly found in structural proteins, a particularly striking example being cytokeratin, which contains several glycine triplets alternating with serine.²⁹ It is possible that IgG autoantibodies to keratin, which have been reported in RA,³⁰ may be cross reactive with P62. A more intriguing possibility is suggested by a recent study which demonstrated a host protein in the cytoplasm of cells in the lining layer of rheumatoid synovial membranes which reacted with a monoclonal antibody to EBNA-1.³¹ If the same antigen were recognised by anti-P62 antibodies from rheumatoid sera it could, for the first time, demonstrate mechanisms whereby anti-RANA could be related to an important antigen within the synovial membrane.

As anti-P62 by ELISA and IF anti-RANA are detecting the same antibodies it is perhaps disappointing that in our study RA sera contained anti-P62 antibodies in titres only two to three times greater than those of controls. This seems to be a trivial increase in titre compared with the increase reported for IF anti-RANA by Ng *et al.*,³² who showed such a difference between RA and normal sera (97% positive v 16%) that their claim that anti-RANA was a 'marker' antibody for RA seemed to be justified. More recent studies using immunofluorescence have not found such differences, however; in most cases titre differences of two to four dilutions have been reported,^{22 33 34} and these are much more in accord with our findings and those of Rhodes *et al* using a P62 ELISA.²² We suggest that ELISA merits further investigation as it is objective, quickly performed, and not susceptible to the errors of interpretation which can occur when attempting to assess end points of dilutions on immunofluorescence. Our survey is small, but even with the limited numbers that we have examined so far we have found sera with very high levels of anti-P62 antibody. The possibility that these represent a subset of RA which may be of clinical or immunogenetic significance is presently under investigation.

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