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Which antigen to use in the detection of rheumatoid factors? Comparison of patients with rheumatoid arthritis and subjects with 'false positive' rheumatoid factor reactions

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SUMMARY

In the search for differences between rheumatoid factors (RF) in patients with rheumatoid arthritis (RA) and in non-rheumatoid subjects, the reactivity of IgM- and IgA-class RF with rabbit IgG (rIgG) and Fc fragments of rabbit and human IgG (rFc, hFc) was studied by enzyme immunoassay. From a community-based cohort ($n=7124$) representing the adult population of Finland, RA patients ($n=130$), other subjects positive in the Waaler–Rose (WaRo, sensitized sheep cell agglutination) test ($n=137$), and controls matched for age, sex and living area were selected for further study. In RA sera there was a good correlation between the results in the WaRo test and in the IgM-RF ELISA (rIgG, rFc and hFc). A considerable number of 'false positive' sera, though positive in the WaRo test and the rIgG-ELISA, were negative in the rFc-ELISA. Twenty-two per cent of the false positive sera reacted with either hFc or rFc, both types being equally common. IgA-RF reacted more frequently with hFc than with rFc in both the RA and the 'false positive' sera. In some sera, IgM- and IgA-RFs reacted differently with human and rabbit Fc, e.g. IgM-RF reacted only with human Fc, and IgA-RF reacted with both hFc and rFc, thus suggesting different regulation of their formation.

Keywords rheumatoid factor rheumatoid arthritis enzyme immunoassay

INTRODUCTION

Rheumatoid factors (RF), autoantibodies directed to antigenic determinants on the Fc fragment of human and also other mammalian IgG molecules, are believed to play an important role in maintaining the inflammatory events leading to tissue damage in rheumatoid arthritis (RA) (Roitt *et al.*, 1982). Still, at the population level, only about one-third, if that, of positive RF reactions are connected to RA (Aho *et al.*, 1985), rendering the majority of RF-positive persons 'false positives.' Some have connective tissue diseases other than RA, chronic liver diseases, lymphoproliferative diseases or infections (Fong, Carson & Vaughan, 1985; Carson *et al.*, 1987), but the majority have no obvious disease linked to the production of RF. Since in most instances the appearance of RF seems to precede the onset of clinical RA by many years (Aho et al., 1985b; Walker *et al.*, 1986; del Puente *et al.*, 1988), a small proportion of the healthy false positives will later develop RA. To determine the role of RF in RA, it would be important to ascertain whether the RA–RF differs from the 'false positive' RF. Such differences would prove useful in confirming the diagnosis of early RA and possibly even in tracing the 'false positives' that tend to develop RA later.

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RF in RA are considered to be more polyclonal and of higher titre (with the exception of M components) and to react more frequently with animal IgG than the RF occuring in other disorders (Carson, 1981). Traditionally, IgM-RF are measured with agglutination techniques employing as antigen either rabbit IgG (rIgG), as in the Waaler–Rose (WaRo) (sensitized sheep cell agglutination) test, or human IgG (hIgG), as in the latex test. The WaRo test is considered to be more specific for RA than the latex-fixation test, which has led to the assumption that rIgG as antigen would be more specific for RA than hIgG.

Numerous immunoassays have been developed to specifically detect RF of IgM, IgG, and IgA classes. Pepsin digestion of the serum makes it possible to detect IgG-RF without interference from IgM-RF (Wernick *et al.*, 1981), but its suitability in IgA-RF assays has been less well established (Teitsson *et al.*, 1984; Tuomi *et al.*, 1988). RIgG, hIgG, or their Fc fragments have served as antigens in various assays, but since there are no comprehensive studies comparing the reactivity of RF with these antigens, it is difficult to compare the results from different tests. Most studies dealing with the species-specificity of RF have centred either on comparing the reactivities of different RF subpopulations existing in a single serum, or on comparing the RF in RA and some other diseases linked to RF production (Normansell, 1972; Pope & McDuffy, 1981; Hay *et al.*, 1983).

Information about the molecular characteristics of RF of

non-RA people at the community level is scarce. In the present study the IgM- and IgA-RF of RA patients and non-rheumatoid subjects from a large epidemiologic cross-sectional survey were studied with respect to their reactivity with Fc fragments of human and rabbit IgG.

MATERIALS AND METHODS

Sera

Between 1978 and 1980 a representative sample of the over 30-year-old population of Finland was collected in order to investigate major health problems, with musculoskeletal diseases being one of the main targets (Mini-Finland Health Survey of the Social Insurance Institution of Finland) (Aho *et al.*, 1989). Of the 8000 subjects invited, 7217 participated in the screening phase of the study, and underwent a health interview and physical and laboratory examinations. The WaRo test was performed as part of the screening phase for 7124 subjects. The re-examination, including a clinical examination by a physician, was done for the subjects with symptoms or findings suggestive of the diseases under study.

Peripheral arthritis was verified on the basis of medical history, symptoms, and clinical examination. RA was defined as a peripheral arthritis not fulfilling the criteria for other forms of specific arthritis, such as psoriatic arthropathy or ankylosing spondylitis with peripheral joint involvement. At the clinical examination all patients defined as having RA had evidence of active inflammation or deformities in at least two limb joints. Other subjects positive in the WaRo test had no arthritis and are referred to as 'false positive' cases (Aho *et al.*, 1989). All those positive in the WaRo test (RA and 'false positive' cases) and those with seronegative RA were selected for further study (292 cases). Controls matched for age, sex, and living area were picked from the same survey. Sera from 267 cases (130 RA and 137 'false-positive') were available for this study. Of the matched controls from the Mini-Finland Health Survey, 271 were used as controls in the IgM-RF assays and IgA-RF assay with whole rabbit IgG as antigen; 103 healthy blood donor samples obtained from Dr Jukka Koistinen (Finnish Red Cross Blood Transfusion Service, Helsinki) served as controls in the other IgA-RF assays.

Conventional agglutination tests

The WaRo test was performed on microtitre plates with U-shaped wells. Sheep red blood cells were sensitized with one-third of the minimum agglutinating dose of rabbit amboceptor. Sera were tested after absorbing the natural agglutinins. The RF titre was read following incubation overnight at 4°C. Titres $\geqslant 32$ were regarded as positive.

The latex slide agglutination test with hIgG coupled to latex particles was performed on heat-inactivated serum according to the manufacterer's instructions (Behringwerke, Marburg, FRG). The titre used as the limit for positivity (1/10) equals 40 IU of RA serum, according to the manufacturer.

ELISA

Polystyrene microtitre plates (Immunoplate I, Nunc, Roskilde, Denmark) were coated with purified Fc fragments of hIgG or rIgG (Jackson Immunoresearch Laboratories, West Grove, PA) at a concentration of 2·5 μg/ml in 50 mM carbonate buffer, pH 9·6, for 3 h at room temperature, followed by overnight incubation of the plates at 4°C. According to the manufacturer, no contaminating proteins or fragments are observed at a minimum protein concentration of 20 mg/ml when tested by immuno-electrophoresis against anti-whole serum, anti-immunoglobulin class-specific, or anti-fragment-specific antisera. Control wells were coated in the same way with human serum albumin (HSA). The residual protein binding sites were blocked with 1% HSA in carbonate buffer. The volumes used were 100 μl/well, and all incubations were at room temperature unless otherwise indicated.

For assaying IgA-RF, the sera were tested both with and without prior pepsin digestion. The sera were treated with pepsin for 18 h according to Wernick *et al.* (1981), and diluted 1:20 in the Tris buffer used for the termination of the enzymatic reaction and supplemented with 0·05% Tween 20, 0·2% HSA, and 4% polyethylene glycol 6000 (PEG). The untreated sera were diluted 1:20 in phosphate-buffered saline (PBS) with the same supplements (PBS–T–HSA–PEG). For IgM-RF assay, the sera were diluted 1:100 in PBS–T–HSA.

A positive control serum was included in each plate, diluted 1:20, 1:100 and 1:500 (IgA-RF), or 1:100, 1:500, and 1:1000 (IgM-RF).

The wells were incubated with test serum dilution for 1 h (IgA-RF) or 2 h (IgM-RF). After washing with PBS–T, optimally diluted enzyme–antibody conjugates were added for 1 h in PBS–T–HSA–PEG (IgA-RF) or 2 h in PBS–T–HSA (IgM-RF) (alkaline phosphatase conjugated $F(ab')_2$ fragment of rabbit anti-human IgA, α-chain specific, 1:3000; alkaline phosphatase conjugated $F(ab')_2$ fragment of anti-human IgM, Fc5μ-specific, 1:2000; Jackson Immunoresearch Laboratories, West Grove, PA). After washing, *P*-nitrophenyl-phosphate (1 mg/ml in 50 mM carbonate buffer, containing 1 mM $MgCl_2$, pH 9·8) was added for 30 min. The reaction was stopped by the addition of 50 μl/well of 3 M NaOH. The colour formed was read in a Titertek Multiskan spectrophotometer (Eflab, Helsinki, Finland). The results were expressed as OD values (OD_{405} nm) from which the binding to control antigen (HSA) was subtracted.

The sera had earlier been tested for RF isotypes using whole rabbit IgG (1 μg/ml) as antigen, as presented in part previously (Aho *et al.*, 1988). The assay was otherwise as described above, except for 1:800 dilution of the anti-α-chain-conjugate and 1:2500 dilution (PBS–T–HSA–PEG) of a μ-chain specific rabbit IgG antibody (Dakopatts, Glostrup, Denmark) conjugated to alkaline phosphatase. The results for IgM- and IgA-RFs are given here for comparison.

Statistical analysis

Significances were calculated by the χ^2 test unless otherwise indicated.

RESULTS

IGM-RF

The results in the ELISA with whole rIgG as antigen (rIgG-ELISA) paralleled those in the WaRo test. Some 'false positive' cases were negative in the rIgG-ELISA (Table 1), but they were mainly borderline cases in the WaRo test, giving a titre of 32. All WaRo-positive RA cases except one were also positive in the rIgG-ELISA.

Table 1. IgM-RF positivity with different test antigens in patients with rheumatoid arthritis (RA) and non-rheumatoid subjects with positive Waaler-Rose (WaRo) reaction ('false positives')

	Number of positives					
			RA			
	False positives ($n=137$)		WaRo$^+$ ($n=37$)		WaRo$^-$ ($n=93$)	
Test	*n*	%	*n*	%	*n*	%
ELISA						
rIgG	117	85	36	97	14	15
rFc	66	48	32	86	7	8
hFc	64	47	31	84	11	12
hFc or rFc	80	58	34	92	11	12
hFc & rFc	50	37	30	81	7	8
only hFc	14	10	1	3	4	4
only rFc	16	12	3	8	0	
Latex (hIgG)	86	63	32	86	10	11

Limit for positivity is set so that 95% of controls give negative results.

The RA sera reacted equally well with rIgG and its Fc fragment (rFc) (Table 1; Spearman correlation coefficient 0·90). The frequency of positive cases was also similar in the ELISA using human Fc fragment (hFc) and in the latex test (Table 1). The RA sera reacted almost equally with human and rabbit Fc (Fig. 1, Table 1).

Significantly fewer false positive sera reacted with rFc than rIgG ($P<0·001$, Table 1). Even some cases with strongly positive results in the rIgG-ELISA were negative in the rFc-ELISA (Fig. 2). Similarly, fewer of the false positive sera were positive in the hFc-ELISA than in the latex test (Table 1; $P<0·01$). When the false positive sera were grouped according to the WaRo titre, their rFc-ELISA OD values were nearly evenly distributed, and there was only a slight correlation with the WaRo titres (Fig. 3). There was great variability among the non-rheumatoid sera in their reactivities with human and rabbit Fc. Twenty-two per cent of the false positives were reactive with either hFc or rFc only, each specificity occurring with equal frequency (Fig. 1, Table 1). The whole false-positive group contained 66 men and 71 women. However, of the 14 subjects who were positive in the IgM-RF-ELISA with human Fc only (Table 1), 12 were men and only two were women ($P<0·01$). Of the 16 subjects reacting only with rabbit Fc, seven were men and nine were women.

The OD levels in rFc- and hFc-ELISAs ranged as high in the false-positive group as in RA, though the means for positive cases were somewhat higher in RA patients (rFc RA $0·779\pm0·458$ and false positives $0·652\pm0·438$; hFc, RA $0·716\pm0·452$ and false positives $0·587\pm0·455$; not statistically significant).

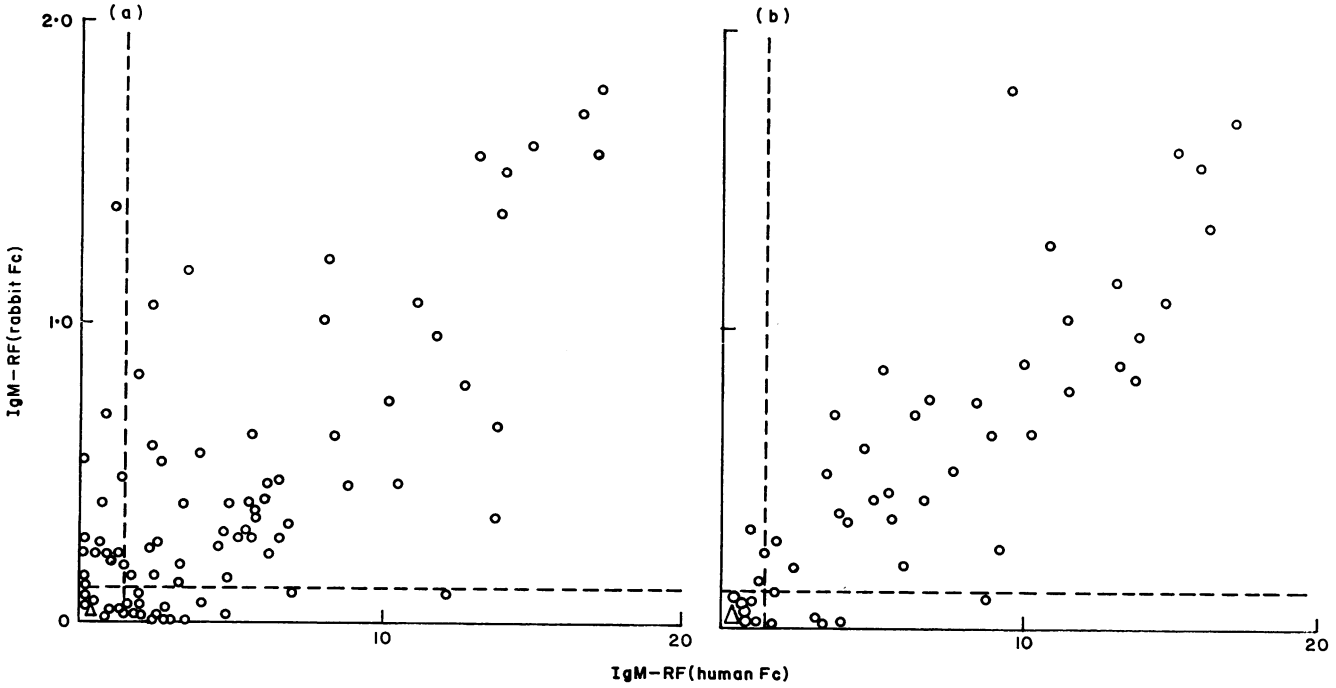

Fig. 1. Comparison of IgM-RF results as measured by ELISA with Fc fragments of rabbit and human IgG as antigen in 'false positive' (a) and RA subjects (b) (o) = 1, (O) = 10, and (△) = 50 subjects. Broken lines depict the limits for positivity, exceeded by 5% of the controls. The variance of OD difference (IgM-Rf(hFc) and IgM-RF(rFc)) was greater in the false-positive group ($P<0·001$).

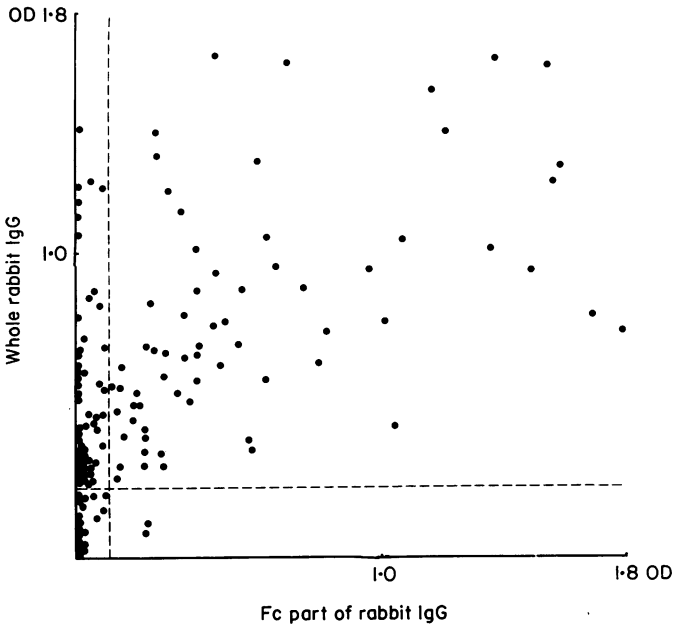

Fig. 2. Distribution of IgM-RF results of 'false-positive' subjects as measured by ELISA with whole rabbit IgG and Fc fragments of rabbit IgG as antigen. Broken lines depict the limits for positivity, exceeded by 5% of the controls. Spearman correlation coefficient 0·55.

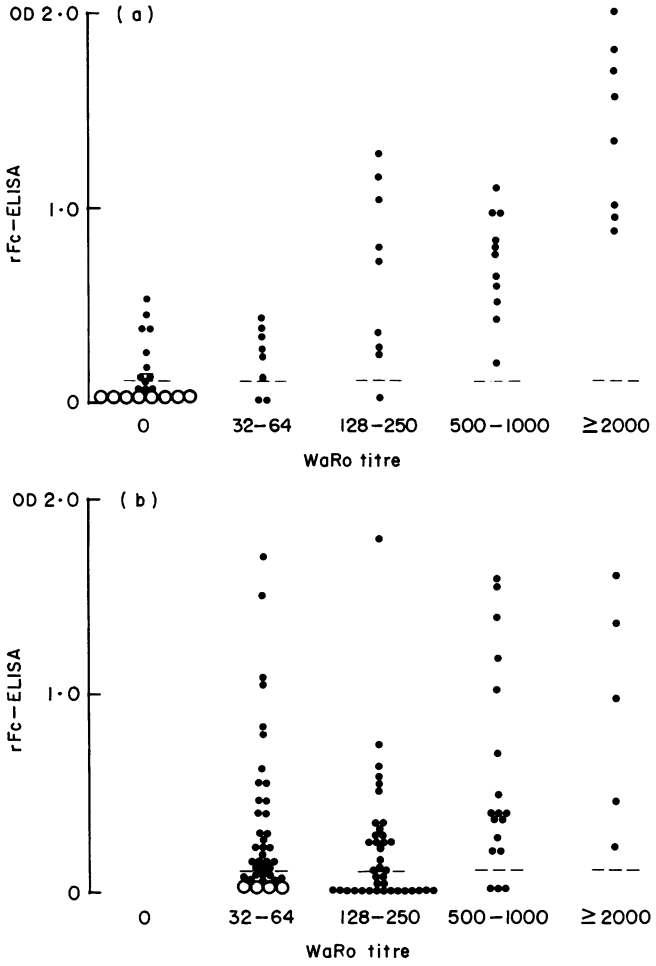

Fig. 3. Distribution of IgM-RF-ELISA results (with Fc fragments of rabbit IgG as antigen) according to the Waaler-Rose (WaRo) titre in RA patients (a) and 'false positive' subjects (b) (●)=1 and (○)=10. Broken lines depict the limit for positivity, exceeded by 5% of the controls. Statistically significant difference between RA and 'false positive' groups is reached only in the middle titre group (128–250) ($P<0·01$, calculated by median test).

IgA-RF

The IgA-RF levels were fairly similar, irrespective of pepsin treatment (Spearman correlation coefficient, 0·84 for hFc and 0·90 for rFc). Although omission of pepsin digestion increased the number of positive results in the IgA-RF(hFc) assay in the 'false-positive' sera and WaRo-negative RA sera (Table 2), this increased frequency of positivity was marginal: all the sera that were positive only when tested without pepsin digestion just barely exceeded the limit for positivity (data not shown).

Both RA and false-positive sera reacted more frequently with hFc than with rFc. The RA patients differed from the false-positive subjects in having a higher percentage of positivity with both hFc and rFc assays and higher levels of hFc-reactive IgA-RF (Table 2, Fig. 4).

Some sera had IgM-RF reacting only with either hFc or rFc, and IgA-RF reacting with both hFc and rFc, and *vice versa* (Table 3).

DISCUSSION

Derived from a large cross-sectional survey of Finnish adult population, the study subjects represent both the average RA patients and the RF-positive population in general. As the screening test for RF, the WaRo test had been performed on all participants. The fact that only one-third of cases who were diagnosed as having RA were positive in the WaRo test is in concordance with other reports from population-based surveys, where only 19–25% of individuals in the population who meet the American Rheumatism Association criteria for RA have RF (Lichtenstein & Pincus, 1988; Isomäki & Kaarela, 1988). Of hospital patients and outpatients, 70–90% are seropositive, with the same rIgG-ELISA that was used in this study (Tuomi *et al.*, 1988). The difference could be due to a greater number of cases with benign or self-limiting disease in the cross-sectional population surveys (Isomäki & Kaarela, 1988).

The latex test and RF-ELISA were performed only on those selected for further study. As a result, the study material may be biased towards reactivity with rIgG; it can only be speculated how using a human-specific test for screening would have influenced the results. However, there were some WaRo-positive and latex-negative sera reacting better with hFc than rFc, and some latex-positive control (i.e. WaRo-negative) sera reacting equally with hFc and rFc, whereas some others reacted solely with hFc. Though these were only odd findings, it seems that conclusions about the reactivity of RF with gamma globulins or their fragments from different species cannot be drawn directly from the results of the classical agglutination tests, but a more precise assay system is needed.

The Fc-specific IgM-RF tests gave markedly fewer 'false-positive' values than the tests using whole IgG molecules. Since the sera had earlier been examined for class-specific RF using ELISA with whole rIgG as antigen, these results could be used

Table 2. IgA-RF positivity in ELISA with rIgG and Fc fragments of rabbit and human IgG (rFc and hFc) as antigens in 'false positive' subjects and patients with rheumatoid arthritis. Limit for positive is set so that 95% of controls give negative results

	Number of positives											
	'False positives' ($n=137$)				RA patients ($n=130$)							
					WaRo$^+$ ($n=37$)				WaRo$^-$($n=93$)			
	Pepsin-				Pepsin-				Pepsin-			
	Treated		Non-treated		Treated		Non-treated		Treated		Non-treated	
	n	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
rIgG	55	40	ND		30	81	ND		10	11	ND	
rFc	23	17	20	15	22	59	23	62	9	10	11	12
hFc	30	22	47	34	29	78	30	81	17	18	30	32
only hFc	13	9	29	21	8	22	8	22	11	12	21	23
only rFc	6	4	2	1	1	3	1	3	4	4	2	2

ND = not done.

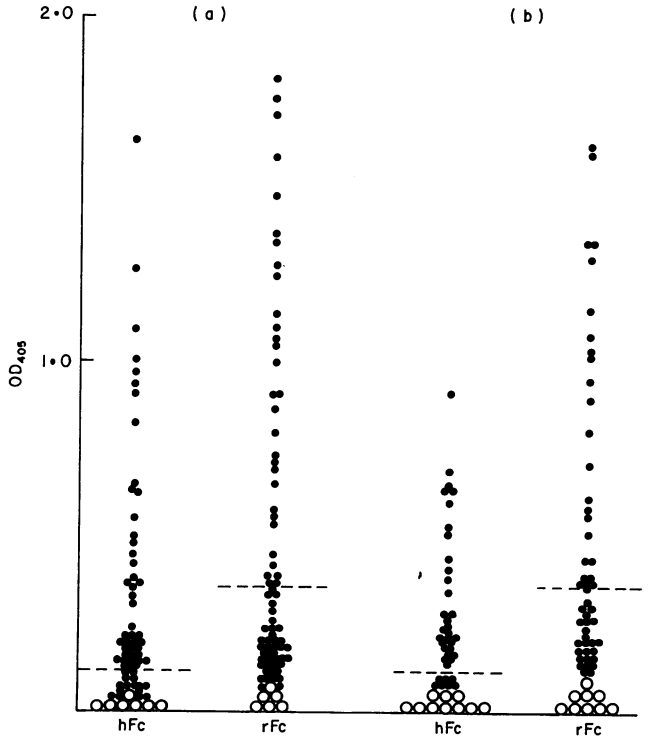

Fig. 4. IgA-RF results of RA patients and 'false positive' subjects as measured by ELISA with Fc fragments of human and rabbit IgG as antigens (without pepsin digestion of the sera). (●) = 1 and (○) = 10 subjects. Broken lines depict the limits for positivity, exceeded by 5% of the controls. The difference between the OD levels of IgM-RF(hFc)-positive persons in RA and 'false-positive' groups is statistically significant ($P<0{\cdot}01$, calculated by Student's *t*-test of the logarithms of positive OD values).

Table 3. Reactivity of IgM- and IgA-RFs of four 'false positive' sera with Fc fragments of human and rabbit (hFc and rFc) IgG as measured with ELISA

	IgM-RF		IgA-RF	
Case number	hFc	rFc	hFc	rFc
14407	1·720*	1·790*	0·921*	0·066
18606	0·394*	0·059	0·199*	1·358*
36681	0·697*	0·098	0·694*	1·309*
64659	0·124	1·392*	0·529*	0·393*

Results are expressed in OD_{405} values (range 0·0–2·0).

* Positive cases exceeding the values of 95% of the controls: hFc IgM-RF, 0·145; rFc IgM-RF 0·113; hFc IgA-RF 0·116; and rFc IgA-RF 0·360.

to verify that the discrepancy was not caused merely by a difference between the agglutination and ELISA tests, but was in fact due to a real difference in the antibody specificity. One explanation to this higher frequency of positivity with rIgG than rFc in the 'false positive' group could be antibodies combining with the Fab portions of IgG molecules, which have been detected in a variety of conditions, among them rheumatic diseases and old age (Wolfe, Abruzzo & Heimer, 1984).

With few exceptions, the RA sera were reactive with both hFc and rFc, which is in concordance with earlier data (Carson, 1981). The 'false-positive' group was less uniform: many sera reacted only with either hFc or rFc, and even in those sera reacting with both the correlation between RF levels was poor. No evidence for preferential human reactivity was found, equal numbers of sera reacting with only hFc or rFc. Since the antibodies reacting only with hFc may be anti-allotype IgG resulting from transplacental immunization (Fong, Carson & Vaughan, 1985), the sex distribution of subjects with only human-reactive RF was checked. It is interesting that they were

predominantly men, excluding the possibility of transplacental immunization. There is no evident explanation for this male preponderance, and its significance remains obscure.

By splitting immunoglobulins into $F(ab')_2$ and Fc fragments, pepsin ruins the polymeric structure and multivalency of IgM-RF. Since IgA-RF may be predominantly polymeric (Elkon *et al.*, 1982; Schrohenloher, Koopman & Alarcon, 1986), the effect of pepsin digestion on its reactivity was ascertained. Teitsson *et al.* (1984) mention that pepsin digestion had no effect on their IgA-RF standard, which included three high-reactive sera. The actual OD levels in the IgA-RF assay with and without pepsin digestion correlated well also in this study, although fewer of the sera treated with pepsin exceeded the limit used for positivity. The background was higher without pepsin, and the discrepant positive cases were mainly of very low level. The fact that IgA-RF can be measured practically equally with and without previous pepsin digestion implies that multivalency is not essential for the reactivity of the antibody, at least not in the present assay.

Irrespective of pepsin digestion, the IgA-RF of both RA and 'false-positive' sera reacted more frequently with hFc than rFc, as Dunne *et al.* (1979) have earlier reported for RA and Sjögren syndrome sera. In the IgA-RF(hFc) assay the RA sera differed from the 'false positives' in having higher levels and a higher frequency of positive cases. These findings favour the use of hFc in IgA-RF detection. However, should we want a corresponding assay for IgG-RF, an enzyme–antibody conjugate highly specific for the Fd part of hIgG would be needed to avoid reactivity with the hFc used as coating antigen.

Little is known of the mechanisms regulating RF isotype switching and production. While the occurrence of IgG- and IgA-RF in RA is usually restricted to IgM-RF-containing patients, in some other diseases IgA-RF can exist as the sole detectable antiglobulin (Saulsbury, 1986; Sinico *et al.*, 1986). As to anti-DNA antibodies, it has been reported that a private idiotype of IgG anti-DNA was not expressed on IgM anti-DNA of the same lupus erythematosus patient (Suenaga & Abdou, 1988). In the present study, human and rabbit reactivities of IgM- and IgA-RF did not coincide: there were subjects with only human-reactive IgM-RF and both human and rabbit reactive IgA-RF, and *vice versa*; these findings would support the view that separate pathways regulate IgM- and IgA-RF production.

When hFc is used instead of rFc, IgA-RF are detected more frequently (the sensitivity of the IgA-RF assay is increased). When Fc fragment is used instead of whole IgG molecules, less false positive IgM-RF are detected (the specificity of the IgM-RF assay is increased).

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