Rheumatoid arthritis (RA) is a heterogeneous disease with regard to disease expression and outcome. Several factors have been shown to be helpful in identifying in an early phase patients at risk for a poor prognosis (1). These prognostic factors include rheumatoid factor (RF), HLA-DR4 and HLA-DR2, sex, age and high initial disease activity, as assessed by number of active joints, erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP) levels (2, 3). RF has long been used to distinguish 'seropositive' from 'seronegative' RA, the latter generally showing a milder course. The definition of seronegative RA is still a matter of debate (4—6), which may in part be related to selection of patients (i.e. exclusion of other forms of seronegative polyarthritis) and the sensitivity of the assays.

Since the development of enzyme-linked immunoassays (ELISA) for detection and quantitation of the different RF-isotypes, divergent conclusions on the prognostic significance of these RF-isotypes have been reported (7-9). Methodologic aspects of the assays, patient selection, and different analytical approaches may have contributed to these differences (10). In our prospective study of patients with early RA (11), we analysed the prognostic value of IgM-RF, IgA-RF and IgG-RF at entry, and their potential significance as process variables during follow-up. Special attention was given to the question of whether RF-isotype measurements may influence the conclusions, the assays were evaluated on the point of peptic digestion and IgM-RF interference.

Patients and methods

Consecutive patients with classical or definite RA according to the 1958 ARA criteria, with joint symptoms for less than one year, who had not previously received second-line anti-rheumatic drugs (DMARDs), were invited to participate in a prospective follow-up study. Data from 149 patients, with a follow-up of at least 3 years, were analysed. All but 3 patients fulfilled also the 1987 ARA-criteria for Rheumatoid Arthritis (22). According to the protocol, at monthly visits, 52 peripheral joints were examined for tenderness and soft tissue swelling, and blood samples were collected for laboratory measurements. Radiographs of hands and feet were obtained at study entry and after 3 years, and joint damage (erosions and joint space narrowing) was assessed according to Sharp with some modifications (12, 13). The maximum total score of this method is 448. The progression of radiological damage was determined as the difference between the total score at entry and after 3 years. Pelvic radiographs were made at entry and after 3 years to evaluate the occurrence of sacroiliitis. ESR, CRP, and IgM-RF were measured in the monthly samples of all 149 patients. IgA-RF and IgG-RF were measured in samples of all patients at entry and every 6 months during follow-up. In addition, in the first 20 patients of the cohort measurements of IgA- and IgG-RF were performed monthly. For comparison between CRP, ESR, and IgM-RF with radiological progression during the 3 years follow-up, time-integrated values of serial measurements of CRP, ESR, and IgM-RF were calculated (area under the curve). Patients were treated with non-steroidal anti-inflammatory drugs and second-line antirheumatic
drugs (DMARDs) as clinically indicated. Additional treatment with low-dose corticosteroids was allowed when necessary. Clinical remission was defined according to the ARA criteria for remission (14).

Laboratory measurements

CRP was measured by ELISA (15), ESR according to Westergren.

HLA-DR typing was performed by the two colour fluorescence method (16).

IgM-RF, IgA-RF, and IgG-RF were measured by ELISA:

Antigens: Human IgG was isolated from a pool of normal sera by chromatography on DEAE-52 cellulose (Whatman). Rabbit IgG was purchased from Nordic (Tilburg, The Netherlands). Heat-aggregated human and rabbit IgG were prepared by heating 10 mg/ml IgG for 30 minutes at 63°C.

Antisera: For detection of IgM- and IgA-RF, peroxidase labelled F(ab’)2 fragments of goat anti-human IgM (μ-chain specific) and anti-human IgA (α-chain specific) were used (Cappel, Organon Teknika, Turnhout, Belgium). Mouse monoclonal antibodies against human IgG (IgG-Fd specific, clone HP6045) (17), obtained from Calbiochem (San Diego, California USA), were used for detection of IgG-RF in combination with rabbit anti-mouse IgG conjugated to HRPO (Dako, Glostrup, Denmark).

RF standard preparations: the WHO International reference preparation for rheumatoid factor (WHO 64/1), containing 500 IU/ml rheumatoid factor (18), and the Netherlands Reference Serum Preparation (NRSP) (19) were used in this study.

IgM-RF assay: Flat-bottomed 96-well microtitre plates (HyCult, Uden, The Netherlands) were coated by incubating each well with 40 μg/ml heat-aggregated human IgG in 0.1 M carbonate, pH 9.6, at 37°C for one hour. The plates were washed with washing buffer +2% BSA (starting at 1:100) were run overnight at 4°C, the plates were washed and incubated for 30 minutes at 63°C.

IgM-RF assay: according to the assay for IgM-RF, except for the use of the goat F(ab’)2 anti-human IgA conjugate.

IgG-RF assay: the method differed from the IgM-RF assay at the following points. Rabbit IgG was used as antigen. Prior to the assay, serum samples were digested with pepsin (Sigma) as described by Wernick (20).

Serial dilutions of treated serum samples were analysed starting at a dilution of 1:30. Bound IgG-RF was detected with mouse monoclonal anti-human IgG-Fd (HP6045), incubation for one hour at 37°C, followed by rabbit anti-mouse IgG-HRPO for 30 minutes at 37°C.

Results of IgA-RF and IgG-RF measurements were expressed in IU/ml assuming a concentration of 500 IU/ml for all RF-isotypes in the WHO international reference preparation.

Cut-off points. Normal controls were recruited among apparently healthy members of the medical and nursing staff of the hospital (n = 50, 24 male, 26 female, age 22–61 yr, median 34.5 yr). Cut-off points were assessed requiring >97% of the normal control sera having negative results. Consequently, values of IgM-RF ≥10 IU/ml, IgA-RF ≥10 IU/ml, and IgG-RF ≥25 IU/ml were considered positive. Intra- and inter-assay variations were <10% for all assays.

Data of control experiments on some technical aspects of the RF-assays

1. Comparison of aggregated human and rabbit IgG as antigen in the IgM-RF and IgA-RF assays: no significant differences were found in absolute levels (r = 0.950, n = 113), nor in the discrimination between positive or negative results for either IgM-RF or IgA-RF (data not shown).

2. Influence of pepsin-digestion on IgG-antibody activity.

Anti-tetanus antibodies: post tetanus-revaccination sera (n = 32) were tested in an ELISA using tetanus toxoid (RIVM Bilthoven, Netherlands) coated plates (21). Anti-DNA antibodies were measured in SLE-sera (n = 25) by ELISA with calf thymus DNA (Sigma, St Louis, USA) as the antigen (21). IgG-RF was measured in sera from RA patients (n = 61). All sera were tested before and after pepsin-digestion (20) using the HP6045 anti-IgG-Fd monoclonal antibody. In addition, anti-tetanus and anti-DNA antibodies were measured in untreated sera with goat anti-human IgG-Fc conjugate (Kallestadt, Austin, Texas USA).

Both untreated and pepsin-digested sera of RA patients were tested in the IgG-RF assay. No IgM-RF could be detected after pepsin treatment using the anti-human μ-chain conjugate as detecting antiserum, indicating that the Fc-parts of IgM-RF had been eliminated. In most samples lower IgG-RF levels were measured after pepsin pretreatment. The strongest reduction in concentration was seen in those samples containing a distinct amount of IgM-RF (>20 IU/ml), whereas only a slight or no reduction was found in samples with low (<20 IU/ml) IgM-RF levels. For comparison, the influence of pepsin digestion on other IgG-antibodies was assessed: both IgG anti-tetanus and...
M. A. van Leeuwen

Fig. 1. Results for IgG-RF, anti-tetanus (TET), and anti-DNA (DNA) antibodies before and after pepsin digestion: The antibody activity preserved after pepsin digestion is expressed as % of the antibody activity measured in untreated sera. △: samples containing >20 IU/ml IgM-RF; □: samples containing <20 IU/ml IgM-RF.

Anti-DNA antibodies were evaluated. The results for IgG-RF, anti-tetanus, and anti-DNA are shown in figure 1. The antibody activity preserved after pepsin digestion is expressed as the percentage of antibody activity measured in untreated sera. Antibody levels measured with the anti-IgG-Fc conjugate and the anti-IgG-Fd mAb before pepsin treatment were comparable (data not shown). The results of these control experiments indicate that for IgG-RF measurement pretreatment of sera with pepsin is necessary to eliminate the interference of IgM-RF, and that the pretreatment according to Wernick did not significantly interfere with the IgG antibody activity.

3. Evaluation of mutual interference of RF-isotypes in the respective RF ELISA's.

Because different RF-isotypes are often present in one serum sample, competition in binding to the solid phase antigen may occur, leading to 'false' low levels of the specific RF-isotype measured. Special attention was given to the interference of IgM-RF.

IgA- and IgG-RF concentrations were measured in RA-sera before and after the addition of an excess of IgM-RF to 7 different sera. For these experiments, RA sera (n = 7) were selected with low IgM-RF levels (<20 IU/ml), but IgA- and IgG-RF concentrations in a wide range. For the addition of IgM-RF, both a serum with a high concentration of polyclonal IgM-RF (500 IU/ml) but low IgA- and IgG-RF concentrations and a purified monoclonal IgM-RF preparation (500 IU/ml) were used. The monoclonal IgM-RF was isolated from plasma of a patient with IgM-cryoglobulinaemia. The results are shown in Table I and indicate that IgA and IgG-RF measurements were not significantly influenced by the presence of IgM-RF.

Statistics

Correlations were determined by Spearman's rank correlation. Multivariable regression analysis was performed using radiological progression during 3 years as dependent variable and initial CRP level, initial radiological score (X-score), RF-isotypes, HLA-DR4 and HLA-DR2, sex, and age as independent variables (2). Log-transformation of radiological scores, RF concentrations, and CRP level was performed to correct for skewness. Chi-square test was used for comparison between groups.

Results

Characteristics of the patients at study entry are shown in Table II.

None of the patients developed clinical or radiological signs of a seronegative spondylarthropathy or sacroiliitis, nor any other identifiable rheumatic disorder other than RA during follow-up. In addition, no patient did show psoriatic skin- or nail lesions, chronic inflammatory bowel disease or uveitis, and none of the patients had a first degree family member with a seronegative spondylarthropathy. During follow-up, 133 patients were treated with DMARDs, mainly hydroxy-
chloroquine, gold, and sulphasalazine, either as the single drug used during follow-up or in succession. Ten patients were treated with methotrexate or azathioprine. Thirteen patients had additional low-dose corticosteroids at any time during follow-up.

Prognostic significance of RF-isotypes at entry for radiological progression

The percentages of patients with positive results for the respective RF-isotypes and for combinations of RF-isotypes at study entry are shown in Table III. The results are given for three subgroups of patients: 1. no radiological damage during the first 3 years, 2. without damage at entry, but with development of radiological damage during follow-up and 3. with radiological damage at entry. The percentages of patients with positive results for the different RF-isotypes were significantly higher in group 2 and 3, compared to group 1, whereas the differences between group 2 and 3 were not significant. The majority of patients had positive results for both IgM-RF and IgA-RF; only 5 patients were IgM-RF positive/IgA-RF negative and 3 patients were IgM-RF negative/IgA-RF positive (with IgA-RF levels of 10, 10, and 30 IU/ml) with 1 patient in group 2 and 2 patients in group 3. IgG-RF was positive in 7 IgM-RF negative patients (with IgG-RF levels of 25–78 IU/ml), 3 patients in group 1, 2 in group 2 and 2 in group 3.

The concentrations of IgM-RF, IgA-RF and IgG-RF at entry were each significantly correlated (P<0.001) to the radiological progression during the following 3 years (figure 2) with a correlation coefficient of 0.483 for IgM-RF, 0.453 for IgA-RF and 0.375 for IgG-RF.

To assess the prognostic significance of the RF-isotypes compared to other well known prognostic factors multiple regression analysis was performed with radiological progression during three years as dependent variable. The variability of the extent of radiological progression appeared to be explained for 35% (R = 0.590) by the combination of initial CRP-level (as measure of initial disease activity), initial X-score, HLA-DR4, HLA-DR2, sex, and age, if analysed without RF. If the initial IgM-RF level (as the only RF-isotype) was added to these independent variables in the analysis, the explained variance was 46% (R = 0.683) and the progression of radiological damage after three years appeared to be significantly related to the initial IgM-RF level, initial CRP level, initial X-score (all P < 0.001), absence of HLA-DR2 (P < 0.002), and younger age (P < 0.05). No significant contribution appeared to exist for HLA-DR4 and sex. Finally, if the initial IgA-RF and IgG-RF levels were added to the independent variables, the explained variance of radiological progression did not improve (47%, R = 0.687), with no significant contribution of IgG-RF and IgA-RF. If these analyses were performed with the initial RF-isotypes as ‘positive’ or ‘negative’ (instead of absolute concentrations), the combination of IgM-RF and IgA-RF performed slightly better than IgM-RF alone. The results of these multiple regression analyses show that the initial CRP level, initial X-score, absence of HLA-DR2, and initial IgM-RF level appear to be the major prognostic factors for the extent of radiological progression during the first 3 years, and that the initial IgA-RF and IgG-RF levels yield no additional information to this prognosis.

For the subgroup of patients without erosions at entry (n = 65), the occurrence of IgM-RF appeared to discriminate patients who became erosive during the next three years from those who did not develop erosions. However, no significant differences were found between the relative risks calculated with the results of IgM-RF alone, or with the results of both IgM-RF and IgA-RF, or with the results of all three RF-isotypes. To evaluate the contribution of the three RF-isotypes to the prediction of the extent of radiological progression in the subgroup of patients without erosions at entry, multiple regression analysis was performed with the same independent variables. No differences in the explained variance of the extent of radiological progression were found if the analysis was performed with
RF levels during three years follow-up

Seroconversion for IgM-RF (149 patients, monthly measurements):

28 patients (20%) were negative for IgM-RF at entry (Table III); 7 of these 28 patients were IgM-RF positive (≥ 10 IU/ml) at some time during follow-up (1–9 monthly values per patient, in 4 patients in a continuous period of at least 6 months). However, these IgM-RF concentrations were only slightly beyond the cut-off level (mostly < 15 IU/ml and occasionally between 15 and 20 IU/ml). Twenty-one patients were thus persistently IgM-RF negative (< 10 IU/ml). From the 121 patients who were positive for IgM-RF at entry, 92 patients were persistently positive during follow-up, and 29 patients were negative at some time, 22 of them for a period of at least 6 months. This indicates that in most patients the changes are trend-like and not incidental. The highest IgM-RF levels in these 29 patients were 11–800 IU/ml (median 50). The conversion from IgM-RF negative to positive never occurred within the first 6 months after presentation, whereas the conversion from positive to negative occurred 1–20 months (median 12 months) after presentation. Thus, based on monthly measurements during the first 3 years of the disease and a cut-off level of 10 IU/ml, 62% of the 149 patients were persistently IgM-RF positive, 14% persistently IgM-RF negative and 24% showed a seroconversion during follow-up (4% negative to “marginal” positive and 20% from positive to negative). If only the half-yearly results of IgM-RF were used, 66% of the 149 patients were classified as persistently IgM-RF positive, 16% persistently negative and 18% as patients with seroconversions (3% negative to positive and 15% positive to negative).

IgA-RF and IgG-RF: at entry 30 patients (20% of 149) were IgA-RF negative and 41 patients (27%) IgG-RF negative (Table III). Using the 6-monthly results of the 149 patients, in 4 patients (2.5% of 149) a conversion from negative to positive was found for IgA-RF and in 5 patients (3%) for IgG-RF, whereas conversion from positive to negative was found in 14 patients (9%) for IgA-RF and in 20 patients (13%) for IgG-RF. The patients showing conversions from IgA-RF or IgG-RF negative to positive were persistently IgM-RF positive with the exception of one patient who

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Fig. 2. Relation between RF-isotype concentrations at entry and radiological progression during 3 years of follow-up. a. IgM-RF; b. IgA-RF; c. IgG-RF; X-progr = radiological progression.
showed a conversion from negative to positive for IgM-RF as well. A conversion from IgA-RF or IgG-RF positive to negative was always accompanied by a significant decrease or seroconversion for IgM-RF. Like IgM-RF, conversions from IgA or IgG-RF negative to positive seems to be rather exceptional, while the opposite occurs more frequently.

Validation of the use of the results for 6 monthly measurements of IgA-RF and IgG-RF for the estimation of seroconversions was performed by analysis of a subgroup of 20 patients with monthly measurements. In all patients showing a seroconversion from IgA-RF or IgG-RF positive to negative, the negative period lasted for at least 6 months. Conversion from IgA-RF negative to positive was not found in this subgroup, but conversion from IgG-RF negative to positive lasted also for at least 6 months in all cases. It may be concluded that changes in IgA-RF and IgG-RF have a trendlike pattern and are not incidental.

Relation between IgM-RF status and clinical remission

Thirty-five of the 149 patients had at least 1 period of clinical remission according to the ARA-criteria. Eight of these 35 patients were persistently IgM-RF negative. Of the 27 patients, who were IgM-RF-positive at entry, 6 patients became negative during clinical remission, 7 patients the IgM-RF concentration was significantly decreased (>50%), and in 14 patients the IgM-RF concentrations remained in the same range during the periods of clinical remission.

RF-isotype concentrations in relation to clinical and laboratory parameters of disease activity

Intra-individual correlations between IgM-RF concentrations and both CRP levels and number of swollen joints were calculated (121 IgM-RF positive patients). The mean and 95% CI (obtained after Z-transformation) are shown in Table IV (indicating that in the majority of the patients IgM-RF levels fluctuate in concordance with disease activity as measured by CRP and swollen joints. In addition, the intra-individual correlations between both IgA-RF and IgG-RF, and CRP and swollen joints were calculated for the 20 patients with monthly results for the three RF-isotypes (Table IV). IgM-RF appears to perform better than IgA-RF and IgG-RF in their intra-individual relations with disease activity parameters.

Course of IgM-RF levels during follow-up in relation to radiological progression

To evaluate the global correlation between IgM-RF levels and radiological progression, time-integrated values of serially measured IgM-RF levels were calculated for the 121 patients with positive results at entry. For comparison with other process variables, the correlation between time-integrated values of CRP and time-integrated values of the number of swollen and painful joints with radiological progression were calculated as well. The results are shown in Table V. Although a significant correlation between time-integrated values of IgM-RF and radiological progression was found, the cumulative disease activity as assessed by CRP, ESR and swollen joints during follow-up is more predictive for the extent of radiological progression.

Discussion

The prognostic significance of RF-isotypes for radiological progression in RA has been investigated in several studies (7–9, 23) with contradictory conclusions about the significance of especially IgA-RF. We analysed the prognostic significance of RF-isotypes in a multiple regression analysis to assess their significance in relation to other factors. In combination with initial IgM-RF, HLA-DR4 and HLA-DR2, initial CRP (as measure of initial disease activity), and initial X-score, no contribution of IgA-RF or IgG-RF could be demonstrated to the prediction of the extent of radiological progression during the first 3 years of disease. This was found both for the whole patient group and for the subgroup of patients without radiological damage at entry. The prognostic significance of IgA-RF has mainly been reported as the result of an analysis of IgM-RF and IgA-RF separately and in different patient groups. A prognostic significance of IgA-

<table>
<thead>
<tr>
<th>CRP correlation coefficients</th>
<th>nr of swollen joints correlation coefficients</th>
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<tbody>
<tr>
<td>mean 95% CI</td>
<td>mean 95% CI</td>
</tr>
<tr>
<td>IgM-RF (n = 121)</td>
<td>0.453 0.429-0.477</td>
</tr>
<tr>
<td>IgM-RF (n = 20)</td>
<td>0.395 0.329-0.457</td>
</tr>
<tr>
<td>IgA-RF (n = 20)</td>
<td>0.187 0.124-0.289</td>
</tr>
<tr>
<td>IgG-RF (n = 20)</td>
<td>0.284 0.192-0.333</td>
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</table>

Table V. Correlations between time-integrated values of IgM-RF, CRP, ESR, nr of swollen joints, nr of tender joints, and radiological progression after 3 years follow-up (n = 121)

<table>
<thead>
<tr>
<th>X-progression</th>
<th>IgM-RF*</th>
<th>CRP*</th>
<th>ESR*</th>
<th>nr swollen joints*</th>
<th>nr tender joints*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.293†</td>
<td>0.608†</td>
<td>0.413†</td>
<td>0.536†</td>
<td>0.105</td>
</tr>
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</table>

* time-integrated values; † P<0.001; ‡ P<0.01

Prognostic significance of RF-isotypes in RA
RF has been reported in patients with early synovitis (7) and in patients with slightly longer standing disease (9). Our study was performed in a well defined patient group with early definite or classical RA according to the 1958 ARA criteria, who had not previously received DMARDS or corticosteroids. In addition no patient developed an arthropathy other than RA during a follow-up of at least three years. The stage of the disease seems to be of importance in the interpretation of the actual RF level and the classification as RF positive or negative, as seroconversion during the course of the disease is a well known phenomenon and may be related to changes in disease activity and/or DMARD treatment. Criteria for seronegative RA have been proposed to include a follow-up of at least 3 years to exclude other forms of arthritis and at least 3 negative RF tests (4). This prospective follow-up study with monthly observations permitted detailed analysis of seroconversions for IgM-RF during the first years of the disease, with an initial measurement before drug treatment was instituted. Only a few patients converted from IgM-RF negative to positive, and their levels never exceeded borderline values. These borderline values should be interpreted in view of the fact that RF levels obtained with these sensitive quantitative assays are continuous variables and the cut-off level for the discrimination between positive/negative is defined statistically using results in a normal population. The low frequency of conversion from negative to (marginal) positive is in agreement with the results of a community based follow-up study, showing that > 90% of patients with RF-positive recent-onset arthritis already had a positive RF-test in their pre-illness sera (24). Conversion from IgM-RF negative to positive was found more frequently. Similar results for seroconversions were found for IgG-RF and IgA-RF. Although based on less frequent observations, this conclusion on seroconversions of IgA-RF and IgG-RF seems to be warranted in view of the comparison between monthly and 6-monthly IgM-RF measurements and the trendlike changes according to the monthly measurements of IgA-RF and IgG-RF in a subgroup of patients. Studies in patients with longer standing RA may thus have a greater risk to classify patients wrongly as RF negative.

The occurrence of IgA-RF or IgG-RF at presentation without simultaneous occurrence of IgM-RF appeared to be exceptional (23, 25). Measurement of IgA and IgG-RF in addition to IgM-RF will thus hardly increase the number of RF-positive cases in early RA. We found slight differences in the prognostic significance of IgA-RF using the RF-isotype as absolute concentrations or as positive/negative in the multivariable regression analysis. This is not surprising, as only a few patients did have discrepant results in IgM-RF and IgA-RF positivity. For the absolute concentrations, the lack of contribution of IgA-RF may be explained by the finding that negative results for IgA-RF in IgM-RF positive patients did only occur in the lower range of IgM-RF values.

The association of HLA-DR4 with a more progressive course of RA has been reported in several studies in hospital referred patients (3, 26). The relation between HLA-DR4 and RF-positivity in RA is less conclusive, which may in part be the result of poor definition of seronegative RA (4–6, 27, 28). In our study, no significant contribution of HLA-DR4 to the explained variance of radiological progression was found if analysed in combination with IgM-RF and the other prognostic factors studied.

In contrast to HLA-DR4, HLA-DR2 appeared to contribute significantly to the explained variance of radiological progression, as a prognostic favourable factor. This is in agreement with earlier reports showing a negative correlation between HLA-DR2 and the severity of erosions (29).

In the majority of our patients a significant intra-individual correlation was found between IgM-RF levels and measures of disease activity (such as ESR, CRP, and swollen joints), and time-integrated IgM-RF values appeared to be related to radiological progression. Compared to the acute phase response and swollen joints however, IgM-RF seems to be a less suitable process variable with regard to radiological progression.

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References


