Multiplex cytokine analyses in patients with rheumatoid arthritis require use of agents blocking heterophilic antibody activity

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Objectives: Heterophilic antibodies, such as rheumatoid factor (RF), are known to interfere with enzyme-linked immunosorbent assays (ELISAs). Treatment of rheumatoid arthritis (RA) with tumour necrosis factor (TNF)-α blockers is well established. The aims of this study were to develop a protocol for blocking the interaction of present heterophilic antibodies and to validate this procedure by evaluating the effect on correlations of cytokine levels to clinical response in RA patients treated with adalimumab.

Method: Fourteen patients with active RA were evaluated at baseline and 3 months after starting adalimumab treatment. Cytokines were analysed with a commercial 12-plex bead ELISA. To block interference by RF, a commercial blocker (HeteroBlock) was used. To determine the optimal concentration of HeteroBlock, patient sera were analysed with different concentrations of HeteroBlock. Subsequently, baseline and follow-up sera from the 14 patients were analysed and correlated with clinical outcome.

Results: Measured cytokine levels were reduced in the majority of samples when adding the blocker. The optimal concentration of HeteroBlock was 1600 μg/mL of serum. Sera with high RF levels were more prone to produce false positive values, although some RF-negative sera also demonstrated evidence of interference. HeteroBlock did not interfere with the analysis. In RA patients treated with adalimumab, changes in interleukin (IL)-6 levels between baseline and follow-up correlated with changes in erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) in sera with added HeteroBlock.

Conclusions: When analysing sera from patients with RA with multiplex bead ELISA, the assay should be evaluated for interference by heterophilic antibodies, and if present corrected with, for example, HeteroBlock.

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease that is associated with a number of abnormalities of the immune system, including circulating auto-antibodies such as anti-citrullinated peptide antibodies (ACPA) and rheumatoid factor (RF). RF binds the Fc portion of immunoglobulin (Ig)G and can belong to any isotype of immunoglobulin (e.g. IgM, IgA, IgG, IgD, IgE). IgM-RF (1), which is the most frequent isotype analysed in clinical practice, is present in approximately two-thirds of RA patients (2). RFs constitute a subset of heterophilic antibodies that are multispecific and can bind with weak affinity to the Fc or the Fab portion of immunoglobulins (3).

Previous studies have shown aberrant cytokine patterns in patients with RA compared to healthy controls (4–8). Cytokine profiling has previously been used for studying the prediagnostic phase (6, 7) of RA as well for prognostic purposes (9) in established RA. There have also been studies of changes in cytokine patterns in response to methotrexate (MTX) (4) and etanercept (8). However, in several of these studies, no evaluation of the interference of heterophilic antibodies has been performed (10, 11).

Analysing serum samples with bead-based enzyme-linked immunosorbent assay (bead ELISA) has the advantage that it enables analyses of several cytokines simultaneously using only a small volume of serum. It is well known that RFs can interfere with ELISA (12, 13). Previous studies using bead ELISA have shown interference by RFs and several different methods to deal with this problem have been proposed, yet without a consensus on how to solve it (14, 15).

Treatment with tumour necrosis factor (TNF) inhibitors has led to major clinical improvement in many cases of severe RA (16, 17). Adalimumab is a fully human monoclonal anti-TNF antibody that has been efficacious...
in clinical trials of MTX non-responders as well as in MTX-naïve patients (18, 19). Studies suggest that anti-inflammatory effects of TNF inhibitors may also reduce RA-associated vascular co-morbidity (20, 21), possibly due to reduced systemic endothelial activation (22).

The aims of this study were (a) to develop a protocol for blocking the interaction of present heterophilic antibodies when analysing sera from patients with RA using multiplex bead ELISA and (b) to validate this procedure by evaluating the effect on correlations of cytokine levels with clinical outcome in RA patients before and after treatment with adalimumab.

Method

These studies were approved by the regional research ethics committee in Lund, Sweden (no. 544/2004; 2004-10-28) and were performed in accordance with the Helsinki Declaration. The study of patients treated with adalimumab was also approved as a phase IV clinical trial by the Swedish Medical Products Agency, and monitored according to a standard protocol by an independent monitor. This study is registered with ClinicalTrials.gov, number NCT01270087. The results on the primary endpoint have been published (22). All participating patients gave their written informed consent.

Development of a protocol for blocking heterophilic antibody interference

To block interaction with heterophilic antibodies, HeteroBlock (Omega Biologicals, Bozeman, MT, USA), a mix of purified IgG from animal sources that has been shown to block activity of heterophilic antibodies in previous studies (14, 23, 24), was used. To determine the optimal concentration of HeteroBlock, four patients with RA and known high RF levels, two RF-positive patients with Sjögren’s syndrome, and one healthy control were analysed with different concentrations of HeteroBlock (0, 160, 1600, 3200 µg/mL serum) guided by previous results in the literature (14). HeteroBlock was added to the serum and samples were incubated for 30 min with shaking at 300 rpm. To evaluate the potential interference of HeteroBlock in the assay, the control serum was analysed with the same concentrations of HeteroBlock (0, 160, 1600, 3200 µg/mL serum). Additionally, a cytokine mix (Bio-Rad, Hercules, CA, USA) was added to the control serum at two different concentrations and then analysed with three different concentrations of HeteroBlock (160, 1600, 3200 µg/mL serum). After blocking and spiking with cytokines, samples were analysed using a custom-made bead ELISA 12-plex kit [IL-1β, IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12(p70)], IL-15, IL-17, interferon (IFN)-γ, TNF-α] from Bio-Rad on a Luminex 200 xMAP system (Luminex, Austin, TX, USA). The analysis was performed according to the protocol provided by the manufacturer. A standard curve with two additional dilution steps compared to standard was chosen to catch low values. All samples were analysed in duplicate, and mean values were used. Data analysis was performed using Bio-Plex Manager software version 6.1 (Bio-Rad).

Inclusion of patients for treatment with adalimumab and analysis of cytokines at baseline and after 3 months

Inclusion and exclusion criteria. Fourteen consecutive patients, seen at a single centre, who fulfilled the 1987 American College of Rheumatology (ACR) classification criteria for RA (25), and for whom treatment with adalimumab was indicated according to their rheumatologist, were included in an open-label trial. They had to have been non-responders to at least one disease-modifying anti-rheumatic drug (DMARD) and have active disease (≥ 6 swollen joints in the 28-joint index, and a CRP > 8 mg/L within the past 3 months). Details on further inclusion and exclusion criteria have been published previously (22).

Clinical evaluation

Patients were evaluated for RA disease activity at baseline and after 3 months, using standard measures: number of swollen joints, number of tender joints, RF, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), Health Assessment Questionnaire Disability Index (HAQ-DI), patient’s assessment of pain, and patient’s global assessment of disease activity.

Handling of samples

Fasting peripheral blood samples were obtained in a standardized fashion. After centrifugation (3000 rpm, 10 min), serum was removed and stored at −80°C until analysis. The mean storage time before analysis was 7.5 years. Baseline samples were thawed twice before the present analysis for pilot studies of the assay. Follow-up samples had not been thawed previously. Before analysis, samples were thawed and centrifuged at 14 300 rpm for 20 s to reduce debris.

Analyses of samples before and after treatment with adalimumab

Serum samples from the 14 patients included in the study were analysed using the 12-plex kit mentioned earlier. Samples were analysed both with and without HeteroBlock (1600 µg/mL serum). All samples were run in duplicate and mean values were used. The assay was performed according to the protocol provided by the manufacturer.
Statistical analyses

Statistical calculations were performed using SPSS for Mac, version 20 (SPSS Inc, Chicago, IL, USA). In the comparison of clinical parameters and cytokine levels at baseline with those observed after 3 months of treatment, the paired T test was used for parameters with a normal distribution, and the Wilcoxon signed rank test for skewed parameters. Spearman’s test was used to assess correlations between absolute values as well as between changes over time in cytokine levels and clinical parameters.

Results

Assay performance

The working range and limit of detection according to the manufacturer are presented in Supplementary Table 1 along with the lower limit of quantification (LLOQ) and the upper limit of quantification (ULOQ) measured in our study. The cytokines included in the statistical analyses of correlation to clinical outcome (IL-6, IL-7, and IL-8) were all in the declared working range of the assay by the manufacturer.

Figure 1. Measured cytokine levels in representative examples of serum from four RA patients and two Sjögren’s syndrome patients with different concentrations of HeteroBlock. Logarithmic scale unless otherwise indicated. *Linear scale due to different distribution.
Recovery

Baseline samples. Recovery was between 82% and 129% when concentrations were higher than background except in one case (IFN-γ 9%). In this case the value was below the declared working range of the assay and the fluorescence intensity value (FI-value) was very close to the blank (1.38 vs. 1.35); thus the result was probably due to inaccuracy of the assay in the lower spectrum (Supplementary Table 1).

Follow-up samples. In the analyses of the follow-up samples, recovery was between 69% and 139% when concentrations were higher than background (Supplementary Table 1).

Table 1. Baseline characteristics of the RA patients in the study.

| Characteristic                          | Value           |
|----------------------------------------|-----------------|
| n                                      | 14              |
| Gender (female/male)                   | 11/3            |
| Age at inclusion (years), mean (sd)    | 63.7 (8.9)      |
| Disease duration (years), median (IQR) | 9.0 (2.6–11.6) |
| RF positive, n (%)                     | 11 (78)         |
| RF > 60 IU/mL, n (%)                   | 6 (43)          |
| RF levels (IU/mL)*, median (IQR)       | 84 (37–296)     |
| Anti-CCP positive, n (%)               | 13 (93)         |
| MTX treatment at inclusion, n (%)      | 8 (73)          |

RA, Rheumatoid arthritis; RF, rheumatoid factor; anti-CCP, anti-cyclic citrullinated peptide antibody; MTX, methotrexate; sd, standard deviation; IQR, interquartile range.
* In RF-positive patients.

Figure 2. Control serum spiked with different concentrations of cytokines analysed with addition of different concentrations of HeteroBlock.
A substantial effect of HeteroBlock on measured cytokine concentrations was seen in almost all analytes except for IL-8 (Figure 1). The effect was most obvious between unblocked samples and samples blocked with HeteroBlock 1600 μg/mL of serum, but in several cases there was an additional effect when adding 1600 μg/mL. In general, no additional effect was seen when adding 3200 μg/mL of serum compared to 1600 μg/mL of serum (Figure 1). Therefore, 1600 μg/mL was chosen for the subsequent investigations of sera from RA patients treated with adalimumab.

The measured cytokine levels in the control sera were stable in unspiked samples and in those spiked with different cytokine concentrations, regardless of the concentration of added HeteroBlock (Figure 2).

RA clinical outcomes

The disease activity score based on 28 joint counts (DAS28) decreased from baseline to the 3-month evaluation (mean 5.6 vs 4.1; p = 0.007). A good or moderate European League Against Rheumatism (EULAR) response was seen in 8/14 patients. Disability measured by the HAQ, and inflammation, in particular when measured by CRP, were also reduced after 3 months (Table 2). Two patients achieved clinical remission according to the EULAR criteria (DAS28 < 2.6 at the follow-up at 3 months).

Data for RA patients at baseline and after 3 months with adalimumab treatment

Clinical baseline characteristics. Fourteen patients with active RA (Table 1) were started on treatment with adalimumab 40 mg subcutaneously every 2 weeks. Eight patients were on MTX at a mean dose 18.75 mg/week (range 10–25). The other six patients had previously been treated with MTX. Two of the patients had been treated with anti-TNF drugs in the past. One had stopped her only previous anti-TNF treatment just over 3 months before the start of the study. The other had received two previous anti-TNF treatments, where the last treatment was stopped more than 18 months before inclusion. Both had discontinued anti-TNF treatment due to adverse events. Four of the patients had extra-articular involvement in the form of rheumatoid nodules at inclusion, but no current vasculitis or other severe extra-articular manifestations were recorded. One patient had a history of systemic rheumatoid vasculitis.

Table 2. Clinical disease severity measures before starting adalimumab and after 3 months of treatment.

| Measure                        | Baseline | Follow-up at 3 months | p     |
|-------------------------------|----------|-----------------------|-------|
| DAS28                         | 5.6 (1.3)| 4.0 (1.4)             | 0.007 |
| Swollen joint count (out of 28) | 10.4 (5.4)| 3.1 (3.7)             | < 0.001 |
| Tender joint count (out of 28)  | 10.0 (8.6)| 5.5 (8.3)             | 0.07  |
| VAS global (mm)               | 55.4 (24.6)| 42.4 (29.3)           | 0.17  |
| VAS pain (mm)                 | 54.0 (33.1)| 35.5 (30.5)           | 0.10  |
| HAQ-DI                        | 1.48 (0.73)| 1.30 (0.81)           | 0.22  |
| CRP (mg/L)                    | 22 (9–39)| 8 (2–22)              | 0.05  |
| ESR (mm/h)                    | 30 (18–47)| 18 (9–31)             | 0.10  |

DAS28, Disease Activity Score based on 28-joint counts; VAS, visual analogue scale; HAQ-DI, Health Assessment Questionnaire Disability Index; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate. Values are given as mean (standard deviation) or median (interquartile range).
Table 3. Change in cytokine concentration between unblocked sera and sera blocked with HeteroBlock (1600 μg/mL serum) in patients with high vs. low RF levels.

| Cytokine | Median change (ng/L) | IQR | Range | Median change (ng/L) | IQR | Range |
|----------|---------------------|-----|-------|---------------------|-----|-------|
| IL-1β    | 14.1                | 0.6 to 253.2 | -0.5 to 865.5 | 0.3 | 0 to 6.2 | 0 to 30.4 |
| IL-2     | 191.3               | 8.5 to 1028.4 | 0 to 2821.0 | 3.3 | 0 to 78.9 | 0 to 163.9 |
| IL-4     | 3.1                 | 0 to 93.5 | 0 to 352.9 | 0 | 0 to 2.0 | 0 to 14.1 |
| IL-6     | 126.7               | -21.1 to 1211.6 | -24.0 to 3622.9 | 11.1 | 0 to 95.2 | -7.3 to 126.8 |
| IL-7     | 11.7                | -4.3 to 378.0 | -14.7 to 406.5 | 1.0 | -1.6 to 27.2 | -6.2 to 49.3 |
| IL-8     | 1.2                 | -0.9 to 4.4 | -1.0 to 7.6 | 0.7 | 0.2 to 2.5 | -2.5 to 2.7 |
| IL-10    | 4.1                 | 0 to 1391.7 | 0 to 2724.4 | 17.4 | 0 to 110.4 | 0 to 264.8 |
| IL-12(p70)| 30.9               | 13.3 to 3157.6 | 0 to 8858.6 | 42.8 | 4.1 to 118.3 | 0 to 387.9 |
| IL-15    | 59.4                | 0 to 302.6 | 0 to 818.8 | 0 | 0 to 16.5 | 0 to 25.1 |
| IL-17    | 0                   | -3.3 to 76.8 | -13.0 to 179.3 | 0 | 0 to 0 | 0 to 46.6 |
| IFN-γ    | 333.5               | 14.1 to 5344.6 | 0 to 17 403.7 | 12.7 | 0 to 210.8 | 0 to 1293.0 |
| TNF-α    | 165.4               | 0 to 2328.9 | 0 to 7808.5 | 10.0 | 0 to 102.7 | 0 to 557.5 |

RF, rheumatoid factor.

Table 4. Measured cytokine levels (ng/L) in patients treated with adalimumab. All samples blocked with HeteroBlock 1600 μg/mL of serum.

| Cytokine | Baseline | 3 months | IQR, Interquartile range; nd, not detected. |
|----------|----------|----------|-----------------------------------------------|
| IL-1b    | Median (IQR) | Min–max | Median (IQR) | Min–max |
|          | nd (nd–0.1) | 0.9–8.3  | nd (nd–0.1) | 0.9–11.9 |
| IL-2     | Median (IQR) | Min–max | Median (IQR) | Min–max |
|          | nd (nd–1.8) | 0.9–10.7 | nd (nd–nd) | 0.9–29.5 |
| IL-4     | Median (IQR) | Min–max | Median (IQR) | Min–max |
|          | nd (nd–nd) | nd–nd  | nd (nd–nd) | nd–nd  |
| IL-6     | Median (IQR) | Min–max | Median (IQR) | Min–max |
|          | 22.0 (14.3–91.0) | 5.7–269.3 | 5.7 (nd–67.1) | 5.7–301.0 |
| IL-7     | Median (IQR) | Min–max | Median (IQR) | Min–max |
|          | 12.3 (8.9–19.9) | 7.1–446.2 | 7.1 (9.9–15.6) | 7.1–546.2 |
| IL-8     | Median (IQR) | Min–max | Median (IQR) | Min–max |
|          | 12.0 (7.4–19.4) | 5.8–31.2 | 10.6 (7.6–15.7) | 6.2–33.5 |
| IL-10    | Median (IQR) | Min–max | Median (IQR) | Min–max |
|          | nd (nd–nd) | nd–20.1 | nd (nd–nd) | nd–67.9 |
| IL-12(p70)| Median (IQR) | Min–max | Median (IQR) | Min–max |
|          | nd (nd–14.2) | nd–591.0 | nd (nd–9.9) | nd–1145.5 |
| IL-15    | Median (IQR) | Min–max | Median (IQR) | Min–max |
|          | nd (nd–nd) | nd–12.0 | nd (nd–nd) | nd–52.6 |
| IL-17    | Median (IQR) | Min–max | Median (IQR) | Min–max |
|          | nd (nd–nd) | nd–43.8 | nd (nd–nd) | nd–218.1 |
| IFN-γ    | Median (IQR) | Min–max | Median (IQR) | Min–max |
|          | nd (nd–nd) | nd–73.4 | nd (nd–nd) | nd–nd |
| TNF-α    | Median (IQR) | Min–max | Median (IQR) | Min–max |
|          | nd (nd–nd) | nd–5168.7 | nd (nd–nd) | nd–8076.0 |

Cytokine levels before and after treatment with adalimumab

When analysing samples without a blocker, the measured cytokine levels were below the working range in 115 out of 336 cytokine results. When cytokine levels were measurable, addition of HeteroBlock reduced the measured levels of cytokines in the vast majority of samples and cytokines. The maximum difference was 17 403.7 ng/L vs. non detectable (IFN-γ). In the minority of results, where blocked values were higher than unblocked (33 out of 336 cytokine results), the difference between the results was low [maximum in absolute value 25.5 ng/L (9% of the unblocked value); maximum in relative value 350% (14.7 ng/L)]. Samples showing effects of blocking in one cytokine did so in all the others, with the exception of IL-8, if levels were measurable. The effect was most obvious in IFN-γ and TNF-α (Figure 3). Samples out of range, with values below the lowest point on the standard curve or below the blank, are presented as not detected.

IL-8 seemed to be unaffected by the added blocker. The range of values for IL-8, both blocked and unblocked, was 5.8–37.2 ng/L. Blocked samples differed from unblocked samples with a median difference of 1.2 ng/L (minimum 0.1 ng/L; maximum 14.6 ng/L) between unblocked and blocked values, in baseline and follow-up samples combined. The median percentage difference [1 – (blocked value/unblocked value)] was 12% (minimum 2%; maximum 43%).

Samples with higher IgM-RF levels (> 60 IU/mL) were more affected by adding HeteroBlock (Table 3), and samples showing extreme values unblocked all had high IgM-RF levels (> 296 IU/mL). Two patients with high IgM-RF levels showed interaction comparable with those with IgM-RF levels < 60 IU/mL. Out of three IgM-RF-negative samples, two showed a clear effect of blocking and one only a marginal effect (Supplementary Figures 1 and 2).
Table 5. Correlations between changes in serum cytokine levels over time, with and without blocking, and changes in disease activity parameters*.

| Change in CRP   | Change in ESR   | Change in DAS28 |
|-----------------|-----------------|-----------------|
| **With blocking** |                 |                 |
| Change in IL-6  | $r = 0.74$ (p = 0.002) | $r = 0.81$ (p < 0.001) | $r = 0.43$ (p = 0.12) |
| Change in IL-7  | $r = 0.14$ (p = 0.62)  | $r = 0.39$ (p = 0.17)  | $r = -0.21$ (p = 0.48)  |
| Change in IL-8  | $r = 0.12$ (p = 0.67)  | $r = 0.37$ (p = 0.20)  | $r = 0.05$ (p = 0.86)   |
| **Without blocking** |                  |                  |
| Change in IL-6  | $r = 0.51$ (p = 0.06)  | $r = 0.69$ (p = 0.006) | $r = 0.11$ (p = 0.71)   |
| Change in IL-7  | $r = 0.08$ (p = 0.78)  | $r = 0.42$ (p = 0.14)  | $r = -0.15$ (p = 0.61)  |
| Change in IL-8  | $r = 0.14$ (p = 0.65)  | $r = 0.46$ (p = 0.09)  | $r = -0.05$ (p = 0.86)  |

CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; DAS28, Disease Activity Score based on 28-joint counts.
*From baseline to 3 months after start of adalimumab.
†Heteroblock 1600 μg/mL of serum.
Spearman’s rank correlation.

After blocking, only IL-6, IL-7, and IL-8 had high enough measurable concentrations to perform the statistical analysis. Serum levels of IL-6 tended to decrease from baseline to the 3-month evaluation (p = 0.24), whereas there was no major change for IL-7 and IL-8 (Table 4).

When comparing baseline and follow-up samples for the individual patients, there was a difference in the ratio between blocked and unblocked values, but the titres of IgM-RF also differed between baseline and follow-up (data not shown).

Correlation between cytokines and clinical outcome

When analysing baseline samples with no blocking agent added, there was no significant correlation between IL-6 and CRP ($r = 0.19$, p = 0.51). By contrast, there was a significant correlation when using blocked samples ($r = 0.65$, p = 0.01). The same pattern was seen when analysing follow-up samples. The decrease in IL-6, in blocked analyses, from baseline to 3 months after adalimumab initiation correlated significantly with decreases in clinically relevant laboratory markers of inflammation (CRP and ESR), whereas there was no such pattern for IL-7 or IL-8 (Table 5). In unblocked analyses, the correlation with change in IL-6 reached statistical significance for change in ESR but not for change in CRP (Table 5).

Discussion

Bead ELISAs are being increasingly used for effective simultaneous multiplex cytokine assessments in investigations of autoimmune diseases. Interference by RF has been identified as a potential problem. We present here an attempt to reduce false highly positive values due to interaction of heterophilic antibodies, in a multiplex cytokine assay. Using this method in patients with RA treated with adalimumab, we have demonstrated that measured IL-6 levels had a stronger correlation with decreases in ESR and CRP than measured IL-6 levels in unblocked samples. In addition, there was only a correlation between levels of IL-6 and CRP in the analysis with HeteroBlock. As IL-6 is known to regulate the production of CRP (26), this suggests that the biological effect of IL-6 is better reflected by the analysis including HeteroBlock. This study illustrates the importance of being aware of the risk of interference with heterophilic antibodies when analysing sera from patients with RA. In this study, HeteroBlock reduced false positive values when added in sufficient concentrations. The optimal concentration found was higher than previous studies have shown to be effective (14, 23, 24). Possible reasons for the discrepancy are that there may have been patients in this study with higher titres of heterophilic antibodies, and that the detection/capture antibody pairs in the present assay were more prone to interfere with heterophilic antibodies. Although optimal blocking was observed at 1600 μg/mL of serum, the minimal effective concentration of HeteroBlock might be in the range between 160 and 1600 μg/mL of serum, as the effects of other concentrations were not explored further in this study. Even though cytokine levels were not detectable after blocking, the unblocked values differed between baseline and follow-up samples in the same patient. One possible explanation is that the production of RF and other immunoglobulins varies over time and that the binding capacity of heterophilic antibodies is influenced by other factors (27).

HeteroBlock did not interfere with the analysis itself to any major extent, as cytokine levels in serum from the healthy controls, both spiked and unspiked, were stable despite different concentrations of HeteroBlock. IL-8 stood out in this analysis as no extreme values were found and there was no effect when adding HeteroBlock. The reason for this may be that the capture and/or detection antibodies differed from the others used in the present kit and that they were less prone to be bound by heterophilic antibodies/RF (24). The concentration of IL-8 in this study
(5.8–33.5 ng/L) was in the normal range according to the reference values (< 2–62 ng/L) in our local clinical laboratory using the Immulite® 1000 immunoassay system (Siemens Medical Solutions Diagnostics, Los Angeles, CA) and according to Bio-Rad’s own reference (0.4–116 ng/L). We did not find any correlation between IL-8 and clinical baseline parameters or any significant reduction in IL-8 after treatment for 3 months, which contrasts to previous studies of patients treated with infliximab (11). Compared to the study by Klimiuk et al (11), the baseline IL-8 levels were substantially lower in our study, which also had a lower CRP threshold for inclusion. It is therefore possible that the patients studied by Klimiuk et al had a higher level of systemic inflammation and disease activity, which may explain the discrepancy, although other explanations related to methodology cannot be excluded.

When comparing the samples, the patients with the highest unblocked cytokine values were strongly positive for IgM-RF. However, not all patients who were strongly positive for IgM-RF produced high false positive values, suggesting variable affinities for RF and therefore variable interference with the assays. In addition, analyses of some samples from IgM-RF-negative (< 14 IE/mL) patients demonstrated evidence for interaction. A similar pattern has been reported previously in patients with RA (14). This is an important reminder to be cautious when analysing sera from all RA patients. Patients can be negative for IgM-RF but still have IgG-RF, IgA-RF, or IgM-RF levels under the cut-off for the RF analysis but with high avidity for certain immunoglobulins, or low avidity heterophilic antibodies that interfere in the analysis (24). The binding reactions of interacting antibodies are unpredictable and dependent on several different mechanisms (27); thus, as shown in our study, it is not necessarily the case that a serum strongly positive for IgM-RF will interact in a sandwich ELISA.

Several different ways of minimizing the effects of heterophilic antibodies have been proposed. One method is to precipitate the antibodies by adding PEG 6000 or Protein L (28). Another way is to add antibodies from mouse and other species to the sera and thus bind the heterophilic antibodies before the analysis. HeteroBlock, and several other commercial blockers such as heterophilic blocking reagent (HBR; Scantibodies Laboratories, Santee, CA, USA), immunoglobulin inhibiting reagent (IIR; Bioreclamation, Hicksville, NY, USA), and TRU Block (Ambsio, Abingdon, UK) are developments of this technique where animal (mouse) IgG is mixed with an ‘active’ blocking part, which is proprietary and not declared in detail. These methods have been described elsewhere in detail (15, 29). Another way of minimizing the risk of interference with heterophilic antibodies in an ELISA is to use truncated antibodies [Fab, F(ab')2, or F(ab')2] (30), or antibodies from species less prone to be bound by human heterophilic antibodies (24, 31).

Overall, our results are compatible with the results from Todd et al (14), except that we found a higher optimal concentration of HeteroBlock (1600 vs. 150 μg/L of serum).

Some limitations of this study are related to the small sample size. There was no analysis of cytokines with any other method as ‘gold standard’. Samples had been stored for a mean of 7.5 years before the analysis, which may have affected the concentration of the cytokines (32). However, the storage time was similar for baseline samples and follow-up samples. Furthermore, the baseline samples were thawed two times more than the follow-up samples. This may also affect cytokine concentrations, as shown by de Jager et al (32). However, this effect varied for different cytokines, and in that study, IL-6 and IL-10 were stable despite several freeze–thaw cycles. This suggests that our finding of correlations between IL-6 and CRP only in the analysis including HeteroBlock would not be affected by the additional two freeze–thaw cycles of the baseline samples.

Further studies should address the reproducibility of blocking, which has been reported to be a problem by other groups (6, 33). The strengths of this study include the use of high concentrations of blocker to determine optimal blocking of interference, and the careful characterization of a relevant patient sample.

Conclusions

When analysing sera from patients with RA using multiplex bead ELISA, the assay should be evaluated systematically for its interference with RF and other heterophilic antibodies. According to this study, sera with high IgM-RF levels seem more prone to interference, but also some IgM-RF-negative sera show considerable interference, probably due to other isoforms of RF, low titre IgM-RF with high avidity, or low avidity heterophilic antibodies. We therefore conclude that all sera from all RA patients should be treated as likely to interfere in multiplex bead ELISAs. As the reported effective concentration of HeteroBlock varies enormously (3–1600 μg/mL of serum) depending on the assays used, we suggest that the optimal HeteroBlock concentration should be defined (and reported) as the first step of every bead ELISA study in each laboratory if this blocker is used. The stronger correlation between measured clinically relevant laboratory markers of inflammation and IL-6 in analyses using HeteroBlock further underlines the importance of proper methodology that takes into account the interaction with heterophilic antibodies.

Acknowledgements

We thank Eugenia Cordero for her assistance with the laboratory analyses, and Käth Nilsson for her work on collecting samples and patient data.
PO and ET received funding for this work from Lund University and the Swedish Rheumatism Association; UB from the County of Skåne, the Swedish Research Council, and the Swedish Rheumatism Association; SJ from the Swedish Research Council, the Heart and Lung Foundation, and the Richard and Helen DeVos Foundation Cardiovascular Research Programme; and CT from the Swedish Research Council, Lund University, and the Swedish Rheumatism Association.

The clinical trial was that the basis for part of this study was funded by an unrestricted grant from AbbVie. Bio-Rad (Hercules, CA, USA) provided materials for the protocol development part of the study.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Supplementary Table S1. Assay performance
Supplementary Figure S1. Difference between measured concentrations of TNF-α in unblocked and blocked sera, by RF level.
Supplementary Figure S2. Difference between measured concentrations of IFN-γ in unblocked and blocked sera, by RF level.

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