

# Beware of Antibodies to Dietary Proteins in “Antigen-specific” Immunoassays! Falsely Positive Anticytokine Antibody Tests Due to Reactivity with Bovine Serum Albumin in Rheumatoid Arthritis (The Swedish TIRA Project)

CHRISTOPHER SJÖWALL, ALF KASTBOM, GUNNEL ALMROTH, JONAS WETTERÖ, and THOMAS SKOGH

**ABSTRACT. Objective.** To evaluate (1) to what extent sera from healthy subjects and patients with rheumatoid arthritis (RA) contain antibodies to bovine serum albumin (BSA); and (2) if anti-BSA antibodies interfere with results of enzyme-linked immunoassays (ELISA) containing BSA.

**Methods.** The ELISA used was a previously developed in-house assay of autoantibodies to tumor necrosis factor (TNF). Anti-TNF and anti-BSA antibodies were analyzed by ELISA in 189 patients with early RA and 186 healthy blood donors. TNF preparations containing either BSA or human serum albumin (HSA) as carrier proteins were used as antigens in the anti-TNF assay. The presence and levels of antibodies were analyzed in relation to disease course and to the presence/absence of rheumatoid factor (RF).

**Results.** In patients with RA, anti-TNF/BSA levels strongly correlated with anti-BSA levels ( $r = 0.81$ ,  $p < 0.001$ ), whereas anti-TNF/HSA did not ( $r = -0.09$ ). Neither the presence nor the levels of anti-BSA in RA patients were associated with disease progression, and antibody levels were not significantly altered compared to controls ( $p = 0.11$ ). IgG reactivity with TNF/HSA was negligible. In paired sera, preincubation with BSA abolished the anti-TNF/BSA reactivity. There were no indications of RF interference with anti-BSA or anti-TNF reactivity.

**Conclusion.** Antibodies to BSA are common in patients with RA as well as in healthy individuals. Their presence does not seem to be associated with RA disease activity or disease course, but may severely interfere with ELISA containing BSA. The use of BSA as a “blocking agent” or carrier protein in immunoassays should therefore be avoided. (J Rheumatol First Release Nov 1 2010; doi:10.3899/jrheum.100690)

*Key Indexing Terms:*

ANTICYTOKINE ANTIBODIES

ENZYME-LINKED IMMUNOASSAY

BOVINE SERUM ALBUMIN

RHEUMATOID ARTHRITIS

TUMOR NECROSIS FACTOR

Enzyme-linked immunoassays (ELISA) were developed in the late 1960s as an alternative to radiolabeling techniques for serological analyses and rapidly gained widespread use for antibody detection<sup>1,2</sup>. In the field of immune-mediated

diseases, the presence of autoantibodies to cytokines has attracted much debate and interest. Theoretically, neutralizing autoantibodies against cytokines may counteract physiological regulation of inflammatory responses, but possibly also serve as a physiological means to downregulate or balance misdirected or excessive immune responses<sup>3</sup>. Therapeutic use of cytokines, e.g., beta-interferon (IFN- $\beta$ ) in multiple sclerosis, may induce formation of neutralizing anticytokine (auto-) antibodies leading to resistance to therapy<sup>4</sup>, and possibly deficient cytokine homeostasis. In other instances, therapy targeting cytokines or cytokine receptors can efficiently halt the progression of autoimmune or autoinflammatory diseases, as illustrated by antibodies to tumor necrosis factor (TNF) or interleukin 6 (IL-6) receptor in rheumatoid arthritis (RA), and IL-1-targeting therapies in cryopyrin-associated periodic fever syndromes<sup>5,6,7</sup>. Commercially available cytokines and other sensitive molecules are often provided in the presence of bovine serum

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albumin (BSA) as an inert carrier or stabilizer to preserve the biological functions and allow prolonged storage. In ELISA, it is commonplace to include BSA as a blocker in order to prevent nonspecific binding.

A large number of studies have addressed the occurrence and possible roles of autoantibodies to cytokines in different autoimmune conditions, providing evidence of their existence and describing possible associations with clinical outcomes<sup>3</sup>. However, the most frequently used method of detection is different ELISA variants and, as pointed out by Bendtzen, *et al*, several issues need to be addressed when developing an anticytokine ELISA<sup>8,9</sup>, or other immunosorbent assays. Apart from nonspecific binding to the antigen or to the plastic surface, immobilization of the antigen to the plastic surface may cause loss of its native 3-dimensional structure and thereby exposure of neopeptides. Further, the presence of soluble cytokine receptors needs to be taken into account when interpreting the results of anticytokine antibody assays<sup>10</sup>.

In a previous study we described an inverse correlation between autoantibodies to TNF and disease activity in patients with systemic lupus erythematosus (SLE)<sup>11</sup>. Also, we reported decreased levels of autoantibodies to IL-1 $\alpha$  and TNF in patients with RA, which was also possibly associated with a worse disease course during 3 years of observation<sup>12</sup>. The same cytokines were used to block antireactivity by preadsorption of the patient sera, whereas (very unfortunately) the nonrelevant cytokine preparation used as control in the blocking experiments did not contain BSA! When we became aware of this, it prompted us to perform a systematic review of our previous results.

## MATERIALS AND METHODS

**Subjects.** Between January 1996 and March 1998, 320 patients with early RA were enrolled in a prospective observational cohort study in southeastern Sweden, the TIRA-1 study (Swedish acronym for “early interventions in rheumatoid arthritis”)<sup>13</sup>. Patients fulfilled at least 4/7 of the 1987 American College of Rheumatology (ACR) classification criteria for RA<sup>14</sup>, or they presented with morning stiffness  $\geq$  60 min, symmetrical arthritis, and small-joint arthritis. Baseline sera samples from 189 TIRA-1 patients were available for the present study; characteristics of patients are given in Table 1. Symptom duration was at least 6 weeks but less than 1 year; 97% (184/189) fulfilled  $\geq$  4/7 of the ACR criteria and 63% (119/189) were rheumatoid factor-positive as tested on a routine basis by agglutination. The patients were monitored regularly over a period of 3 years regarding disease activity, pharmacotherapy, and functional status. Disease activity was assessed by the 28-joint Disease Activity Score (DAS28)<sup>15</sup>. The control population comprised 186 healthy blood donors (96 men, 90 women, mean age 41 yrs, range 20–66 yrs).

**Antibody detection.** Serum samples from patients and controls were taken at inclusion and were kept frozen ( $-72^{\circ}\text{C}$ ) until analysis. The ELISA were performed with the antigen being either (1) *E. coli*-derived carrier-free TNF (R&D Systems, Minneapolis, MN, USA; catalog no. 210-TA/CF) diluted with 0.2% HSA (Albuminativ, Octapharma, Lachen, Switzerland) in phosphate-buffered saline (PBS; pH 7.4); or (2) *E. coli*-derived TNF (R&D Systems; catalog no. 210-TA) with BSA as carrier protein and diluted with 0.2% HSA (Albuminativ) in PBS, identical to the conditions in our previous studies<sup>11,12</sup>; or (3) endotoxin-tested

Table 1. Baseline characteristics of 189 patients with RA in the TIRA-1 cohort.

Characteristic	
Female, n (%)	129 (68)
Male, n (%)	60 (32)
Age, yrs, mean (SD)	54.9 (16.5)
No. of fulfilled ACR criteria, mean (SD)	4.6 (0.7)
Erythrocyte sedimentation rate, mm/h, mean (SD)	36 (22.9)
C-reactive protein, mg/l, mean (SD)	27.7 (24.9)
Disease Activity Score (DAS28), mean (SD)	5.4 (1.1)
IgG Cyclic citrullinated peptide antibody-positive, n (%)	123 (65)
Rheumatoid factor-positive, n (%)	119 (63)
Patients prescribed any DMARD, n (%)	102 (54)
Patients prescribed oral corticosteroids, n (%)	76 (40)

DMARD: disease-modifying antirheumatic drugs.

Fraction V IgG-free BSA (Serological Proteins Inc., Kankakee, IL, USA) diluted in PBS.

Procedures were performed as follows: 96-well microtiter plates (Immunolon 2 HB; Thermo Electron, Waltham, MA, USA) were coated overnight with either recombinant TNF 50 ng/ml or BSA 5  $\mu\text{g/ml}$  in PBS. Residual binding sites were blocked by incubation step with 2% HSA/PBS for 2 h. After washing 4 times, serum samples diluted 1:100 in 2% HSA/PBS were added in triplicates and incubated 2 h at room temperature. After thorough washing, a biotinylated goat anti-human IgG antibody (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:2000 in PBS-Tween was added for 60 min. Avidin-biotin-alkaline phosphatase complex (ABC-AP; Vector Laboratories, Burlingame, CA, USA) was added to the wells 30 min prior to washing, followed by addition of substrate (p-nitrophenyl phosphate; Sigma-Aldrich). Optical densities (OD) were read at 405 nm (Multiskan Ascent with version 2.6 Ascent Software; Thermo Labsystems Oy, Helsinki, Finland) and net OD values were calculated by subtracting the corresponding values from wells coated with HSA only.

**Inhibition assay.** Altogether, 24 sera originating from TIRA-1 patients and controls with high OD achieved in anti-TNF/BSA assays were used in an inhibition assay. All sera were preincubated 1 h at room temperature with PBS containing either 2% BSA or HSA. Each sample was then diluted to a final concentration of 1:100, applied to plates coated with TNF/BSA, and treated as described above.

All patients gave written informed consent and the local ethics committee in Linköping approved the study protocol.

**Statistics.** Data were prepared in GraphPad Prism (version 4.0; GraphPad Software, San Diego, CA, USA). Correlation analyses were performed using Spearman's rank correlation (GraphPad) and differences between groups were calculated with the Mann-Whitney U test (GraphPad).

## RESULTS

Using a cutoff at 0.1 net OD, 25% (47/189) of TIRA-1 patients and 43% of the controls were anti-TNF/BSA-positive; anti-TNF/BSA antibody levels were not significantly different between the groups ( $p = 0.24$ ). Data revealed no significant difference in antibody reactivity between women and men.

Similarly, as illustrated in Figure 1, antibodies to BSA were common in both groups; 50% (95/189) of RA patients and 62% (116/186) of controls were anti-BSA-positive using a cutoff at 0.1 net OD. No significant differences in anti-BSA antibody levels were found between the groups

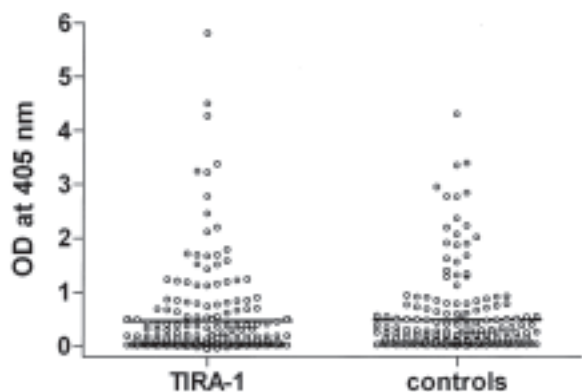


Figure 1. Anti-BSA antibody analyses by ELISA demonstrated with optical density (OD) in 189 RA patients in the TIRA-1 cohort and 186 controls. No significant difference in anti-BSA levels between the groups was found ( $p = 0.11$ ); horizontal line shows mean value.

( $p = 0.11$ ) and no difference in antibody reactivity between women and men was found. However, as shown in Figure 2, we found a solid correlation between anti-TNF/BSA and anti-BSA levels (Spearman  $r = 0.811$ ,  $p < 0.001$ ). Very low levels of anti-TNF/HSA were found in individual RA sera, but with no correlation to anti-BSA (Spearman  $r = -0.087$ ,  $p = 0.27$ ).

Figure 3 shows the results of the inhibition assay including 12 sera each from patients and controls, selected on the basis of elevated anti-TNF/BSA levels. Preincubation of these serum samples with BSA in PBS completely extinguished the anti-TNF/BSA signal, whereas preincubation with HSA did not affect the results. No difference in inhibition was seen between RA patients and controls.

Anti-BSA antibody levels were not significantly associated with RF (RF-positive, median 0.1095, IQR 0.484, vs RF-negative median 0.129, IQR 0.544; Mann-Whitney  $p = 0.97$ ) or anti-cyclic citrullinated peptide (anti-CCP) antibody status (CCP-positive median 0.526, IQR 0.621, vs

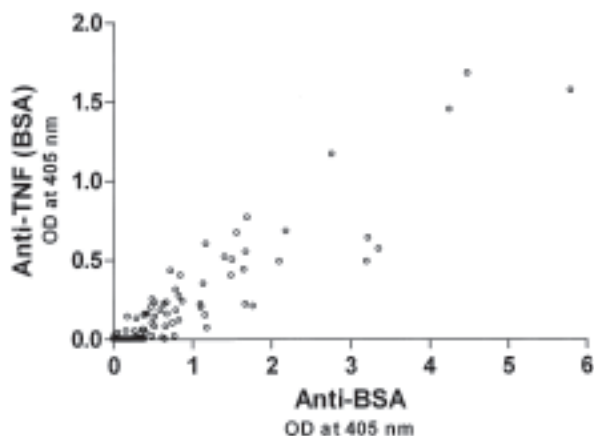


Figure 2. Optical densities of anti-TNF/BSA and anti-BSA antibody levels demonstrated in RA patients in the TIRA-1 cohort. Spearman correlation coefficient = 0.811,  $p < 0.001$ .

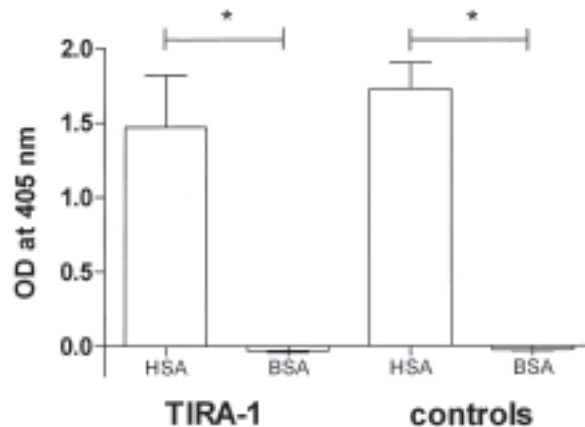


Figure 3. Median OD values with standard deviations in paired sera from 12 TIRA-1 patients with RA and 12 controls using BSA-containing TNF as antigen. Preincubation with BSA or HSA was performed. \* $p < 0.0001$ .

CCP-negative median 0.064, IQR 0.5115; Mann-Whitney  $p = 0.27$ ). Anti-BSA levels did not correlate with the levels of RF (IgM or IgA) or anti-CCP (IgG or IgA). Neither the presence nor the levels of anti-BSA in RA patients were associated with disease progression during 3 years of observation as indicated by erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), DAS28, physician's global assessment of disease activity (0–4 scale), or the number of swollen joints.

## DISCUSSION

The first descriptions of antibodies against cytokines were given in the early 1980s when the presence of antibodies to human IFN- $\alpha$  was reported in a patient with SLE<sup>16</sup> and in a patient with Varicella-zoster infection<sup>17</sup>. Reports on the presence of autoantibodies to TNF, interleukins, interferons, growth and colony-stimulating factors (CSF) in autoimmune diseases, infections, graft-versus-host condition, malignancies, and apparently healthy individuals followed<sup>18-23</sup>. Some studies revealed associations between levels of specific anticytokine antibodies and certain clinical manifestations. For instance, anti-IL-1 $\alpha$  has been suggested to prevent bone erosions in RA<sup>24,25</sup>, anti-IL-6 was found in patients with severe alcoholic cirrhosis<sup>26</sup>, autoantibodies to granulocyte CSF were present in neutropenic patients with SLE<sup>27</sup>, and the appearance of anti-IFN- $\gamma$  was observed in relation to disease remission in Guillain-Barré syndrome<sup>28</sup>. Few of these studies have been confirmed by other research groups and many remain unconfirmed for cytokine-neutralizing capacity of the detected anticytokine antibody. In addition, the observations were made with several different assays, e.g., ELISA, immunoblotting, and radioimmunoassays, all using purified cytokines as the antigen source. The presence or absence of BSA in the cytokine preparations used was rarely stated.

Bendtsen and coworkers have studied anticytokine anti-

bodies in health and disease for many years<sup>29</sup>. Initially, they used immunoblotting and ELISA<sup>19</sup>, but, due to lack of sensitivity, they replaced these methods with radioimmunoassay<sup>9</sup>.

Whether the anticytokine antibodies are friends, foes, or just innocent bystanders has long been a subject of debate. Therapy-induced antibodies to cytokines were generally thought to inhibit cytokine functions, thus eventually resulting in various degrees of "cytokine deficiency." However, when the presence of various naturally occurring anticytokine antibodies also became apparent<sup>22,23,30,31</sup>, it was proposed that some of these antibodies could serve as specific carriers to deliver cytokines to target cells. Hypothetically, this physiological carrier function could also delay cytokine elimination from the circulation and protect from proteolytic degradation<sup>29</sup>. Other potential *in vivo* functions of anticytokine antibodies include roles as (1) scavengers of residual cytokine in individuals treated with recombinant cytokine (e.g., IFN- $\beta$  and granulocyte-macrophage-CSF); (2) a reservoir with balancing effects on the equilibrium between pro- and antiinflammatory cytokines; and (3) Fc $\gamma$ R-mediated transducers of cytokine-anticytokine complexes, as reviewed<sup>3</sup>.

Our interest in anticytokine antibodies arose in relation to SLE, a disease characterized by deviating cytokine patterns and multiple autoantibodies to antigens in cell nuclei, cytoplasm and cytoplasmic organelles, cell-surface antigens, and circulating plasma proteins. We hypothesized that neutralizing antibodies to cytokines essential for the induction of CRP (i.e., IL-6, IL-1 $\beta$ , and TNF) could explain why patients with SLE, in spite of high disease activity and elevated IL-6, often display a remarkably poor CRP response. In-house ELISA were developed in accord with the description by Elkarim, *et al*<sup>28</sup> and the antigen specificities of our assays were validated as suggested by Bendtzen, *et al*<sup>8</sup>. Contrary to our expectations, we found no evidence of raised autoantibody levels to CRP-inducing cytokines as compared to healthy controls. Nor did we find any correlations between CRP levels and any of the anticytokine autoantibodies tested<sup>11</sup>. However, anti-TNF antibody levels were significantly lower in sera from patients with active disease as compared to remission samples, and anti-TNF levels were strongly inversely associated with disease activity in certain patients<sup>11</sup>. At first, the efforts to prove the antigen specificities of our different anticytokine ELISA were apparently successful, since preincubation of the sera with "relevant" cytokines abolished antibody reactivity, whereas preincubation of "irrelevant" cytokines (consistently IFN- $\gamma$ ) did not. In retrospect, however, we have become painfully aware that all of the relevant cytokines used to coat microtiter plates and preadsorb serum samples actually contained considerable amounts of BSA as carrier protein, whereas the "irrelevant" cytokine had HSA as carrier protein!

In none of our previous or subsequent in-house ELISA

have we used BSA as a blocker of nonspecific antibody binding. On the contrary, we actively avoid this, well aware that anti-BSA antibodies are commonplace and that antibodies to dietary proteins may interfere with the results of antigen-specific immunoassays<sup>32</sup>. It is not known whether anti-BSA are "natural" (innate) antibodies, i.e., occurring without specific immunization, or if they are induced by MHC-restricted antigen presentation. However, the latter seems probable, since BSA is a common dietary antigen. Beginning in the 1970s, Cunningham-Rundles and colleagues published a series of reports on circulating immune complexes containing bovine milk antigens, especially in subjects with IgA deficiency, and hypothesized that this could be explained by systemic immunization to dietary proteins due to lack of mucosal IgA-mediated immune exclusion<sup>33</sup>. However, again in population-based samples, the occurrence of IgG antibodies to BSA is a common finding, although concentrations seem to decline with age<sup>34,35</sup>. In human disease, anti-BSA has attracted interest regarding insulin-dependent diabetes mellitus (IDDM). Increased levels of anti-BSA antibodies as well as cross-reactivity between a BSA peptide sequence and a pancreatic  $\beta$ -cell surface protein have been suggested<sup>36,37</sup>, and the potential role of BSA and other cow's milk proteins in the development of IDDM is still a matter of considerable interest<sup>38,39</sup>. In a study by Mogue, *et al*, a sensitive quantitative anti-BSA antibody assay was developed, and the cutoff level was carefully defined<sup>40</sup>. By this method, they found IgG-class anti-BSA antibodies in slightly over 50% of patients with lung cancer undergoing surgery. The levels and frequency of anti-BSA antibodies in healthy blood donors were similar. In patients exposed to BSA in an intrathoracic surgical sealant after pulmectomy, the antibody levels increased steeply and the frequency of positive anti-BSA tests rose to 96%<sup>40</sup>. In diseases characterized by a compromised gastrointestinal barrier, such as Crohn's and celiac disease, levels of anti-BSA antibodies seem to be increased<sup>41,42</sup>; and in SLE, anti-BSA levels have been found to be elevated and also to correlate with an increased risk of cardiovascular disease<sup>43</sup>. Instead of measuring different levels of circulating anti-TNF antibodies in SLE during flare and remission<sup>11</sup>, our results could merely have reflected fluctuations in the levels of anti-BSA antibodies. What pathophysiological implications this may have, if any, may possibly be worth further studies.

Rosenau and Schur recently reported that the serum concentrations of anti-TNF autoantibodies in RA did not correlate with disease activity (CRP, ESR), but concluded that anti-TNF autoantibodies may reduce disease activity, since RA patients with high serum levels did not develop joint erosions<sup>44</sup>. We presented in abstract format<sup>12</sup> a similar conclusion regarding disease-modifying properties of anti-TNF and anti-IL-1 $\alpha$  autoantibodies in RA: patients with subnormal levels (compared to healthy controls) showed a tenden-

cy to higher disease activity over time compared to patients with normal antibody levels. Rosenau and Schur used buffer containing 2% BSA to block nonspecific antibody binding to TNF-coated microtiter plates, but in contrast to our procedure they also took the precaution of diluting serum samples with the same “blocking buffer”<sup>44</sup>. However, apart from interference by antibodies to dietary antigens (such as BSA and other milk proteins commonly used as blockers of non-specific reactions), it is well known that rheumatoid factors can interfere with the results of immunoassays. This may be due to reaction with secondary detection antibodies<sup>45</sup>, but also to interaction with soluble immune complexes in the patient serum, or by reaction with serum antibodies immobilized on the microtiter plate. Further, even when taking the precaution of neutralizing serum antibodies with the “blocking agent” (such as BSA), rheumatoid factor of any isotype may interact with both the “iatrogenic” immune complexes in serum and with antibodies bound to the microtiter plate, thereby producing erroneous results. However, although RF may hypothetically obscure the results of any ELISA, this did not appear to be a major risk in our study, since we found no differences regarding anti-BSA antibody results comparing seropositive and seronegative patients with RA.

The results of this study clearly demonstrate that our previously published results on anticytokine antibodies in patients with SLE and with RA<sup>11,12</sup> were seriously confounded by the presence of BSA. It is highly likely that similar erroneous results have been reported by others unaware of the presence of antibodies to “blocking agents.” Thus, at least with respect to RA, we are convinced that all of what we previously reported regarding “anti-TNF antibodies” is in fact attributable to anti-BSA antibodies. Data on the relation between ingested dietary proteins and anti-BSA antibody levels would certainly have added significantly to the study, but unfortunately this information is lacking.

To conclude, our investigation yielded 3 important observations. (1) We confirm that antibodies to BSA are common and are equally frequent in sera from patients with early RA and healthy individuals. (2) Anti-BSA antibody concentrations did not show any significant relation to disease activity, serologic features (i.e. anti-cyclic citrullinated peptide or RF status), or disease progression during 3 years of observation. This finding contrasts with the proposed possibility of a molecular mimicry mechanism in RA involving BSA<sup>46</sup>. (3) Finally, the convincing direct correlation between anti-TNF/BSA and anti-BSA (Figure 2), and the results from the inhibition assay (Figure 3) strongly support the notion that BSA can interfere with the results of “antigen-specific” ELISA. Due to the frequent occurrence of anti-BSA antibodies, BSA should be avoided in ELISA.

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