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)

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Title:

Beware of antibodies to dietary proteins in “antigen-specific” immunoassays! Falsely positive anti-cytokine antibody tests due to reactivity with bovine serum albumin in rheumatoid arthritis (the Swedish TIRA project)

Running head: Erroneous results of antibody tests due to BSA

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Abstract

Objective. The aims were to evaluate (i) to what extent sera from healthy subjects and patients with rheumatoid arthritis (RA) contain antibodies to bovine serum albumin (BSA), and (ii) if anti-BSA antibodies interfere with results of enzyme-linked immunoassays (ELISAs) 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 ELISAs 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 levels and presence of antibodies were analyzed in relation to disease course and to the presence/absence of rheumatoid factor (RF).

Results. In RA patients, 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 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, pre-incubation 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 associate with RA disease activity or disease course, but may severely interfere with ELISAs containing BSA. The use of BSA as “blocking agent” or carrier protein in immunoassays should therefore be avoided.

Introduction

Enzyme-linked immunoassays (ELISAs) were developed in the late 1960s as an alternative to radiolabelling techniques for serological analyses and rapidly gained widespread use for antibody detection [1, 2]. 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 down-regulate or balance misdirected or excessive immune responses [3]. Therapeutic use of cytokines, *e.g.* beta interferon (IFN β) in multiple sclerosis, may induce formation of neutralizing anti-cytokine (auto-) antibodies leading to resistance to therapy [4], 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- (IL-) 6 receptor in rheumatoid arthritis (RA), and IL-1 targeting therapies in cryopyrin associated periodic fever syndromes [5–7]. Commercially available cytokines and other sensitive molecules are often provided in the presence of bovine serum albumin (BSA) as an inert carrier or stabilizer to preserve the biological functions and to allow prolonged storage. In ELISAs, it is commonplace to include BSA as a blocker in order to prevent non-specific 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 [3]. However, the most frequently used method of detection is different ELISA variants and, as pointed out by Bendtzen *et al*, there are several points that need to be addressed when developing an anti-cytokine ELISA [8, 9]; these precautions apply also for other immunosorbent assays. Apart from non-specific

binding to the antigen or to the plastic surface, immobilization of the antigen to the plastic surface may cause loss of its native three-dimensional structure and thereby exposure of neoepitopes. Furthermore, the presence of soluble cytokine receptors needs be taken into account when interpreting the results of anti-cytokine antibody assays [10].

In a previous study we described an inverse correlation between autoantibodies to TNF and disease activity in patients with systemic lupus erythematosus (SLE) [11]. Also, we reported decreased levels of autoantibodies to IL-1 α and TNF in RA patients, which also possibly associated with a worse disease course during three years [12]. The same cytokines were used to block anti-reactivity by pre-adsorption of the patient sera, whereas (very unfortunately) the non-relevant cytokine preparation used as control in the blocking experiments did not contain BSA! When we became aware of this, it urged 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 named ‘TIRA-1’ (Swedish acronym for “early interventions in rheumatoid arthritis”) [13]. The patients either fulfilled at least 4/7 of the 1987 American College of Rheumatology (ACR) classification criteria for RA, and the remainder presented with morning stiffness ≥ 60 min, symmetrical arthritis and small joint arthritis. Baseline sera from 189 TIRA-1 patients were available for the present study and characteristics of the patients are given in Table 1. Symptom duration was at least 6 weeks but less than 1 year, and 97% (184/189) fulfilled $\geq 4/7$ of the ACR criteria and 63% (119/189) were RF-positive as tested on a routine basis by means of latex particle agglutination. The patients were monitored regularly during three years regarding disease activity, pharmacotherapy and functional status. Disease activity was assessed by the 28-joint disease activity score (DAS28) [14]. The control population comprised 186 healthy blood donors (90 females, 96 males, mean age 41 years, range 20–66).

Antibody detection

Serum samples from patients and controls were taken at inclusion and were kept frozen (-72°C) until analysis. The ELISAs were performed with the antigen being either (1) carrier-free TNF (*E. coli*-derived; 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) TNF (*E. coli*-derived; R&D Systems, catalog no 210-TA) with BSA present as carrier protein and diluted with 0.2% HSA (Albuminativ) in PBS, identical to

the conditions in our previous studies [11, 12], or (3) BSA (Fraction V, endotoxin-tested, IgG-free; Serological proteins, Inc., Kanakee, IL, USA) diluted in PBS.

The procedures were performed as follows: 96-well microtitre plates (Immunolon 2 HB; Thermo Electron, Waltham, MA, USA) were coated overnight with either recombinant TNF 50 ng/mL or BSA 5 µg/mL in PBS. Residual binding sites were blocked by incubation step with 2% HSA/PBS for 2 h. Following 4 times of washing, serum samples diluted 1:100 in 2% HSA/PBS were added in triplicates and incubated for 2 hours in room temperature. Following 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 the addition of substrate (*p*-nitrophenyl phosphate; Sigma-Aldrich). Optical densities (ODs) were read at 405 nm (Multiskan Ascent with the 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, originated from TIRA-1 patients and controls with high ODs achieved in anti-TNF/BSA assay, were used in an inhibition assay. All sera were pre-incubated for 1 h in 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.

Statistics

Figures were prepared in GraphPad Prism (version 4.0; GraphPad Software Inc., 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).

Ethics

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

Results

Using a cut-off at 0.1 net OD, 25% (47/189) of TIRA-1 patients and 43% of the controls were anti-TNF/BSA positive; and the 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.

Likewise, 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 cut-off at 0.1 net OD. No significant difference in anti-BSA antibody levels were found between the groups ($p = 0.11$) 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 without correlation to anti-BSA (Spearman; $r = -0.087$, $p = 0.27$).

Figure 3 demonstrates the results of the inhibition assay including 12 sera each from patients and controls respectively, selected on the basis of elevated anti-TNF/BSA levels. Pre-incubation of these serum samples with BSA in PBS completely extinguished the anti-TNF/BSA signal, whereas pre-incubation with HSA not affected the results. No difference concerning this inhibition was seen between RA patients and controls.

Anti-BSA antibody levels were not significantly associated with rheumatoid factor (RF+ median 0.1095, IQR 0.484 *versus* RF– median 0.129, IQR 0.544; Mann-Whitney, $p = 0.97$) or anti-CCP antibody status (CCP+ median 0.526, IQR 0.621 *versus* CCP– median 0.064, IQR 0.5115; Mann-Whitney, $p = 0.27$). Anti-BSA levels did not correlate with the levels of rheumatoid factor (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 three

years as reflected by erythrocyte sedimentation rate, C-reactive protein (CRP), DAS28, physician's global assessment of disease activity (0–4) or the number of swollen joints.

Discussion

The first descriptions of antibodies against cytokines were made in the early 80s when the presence of antibodies to human interferon- α (IFN α) was reported in a patient with SLE [15] and in a patient suffering from *Varicella-zoster* infection [16]. Reports on the presence of autoantibodies to TNF, interleukins, interferons, growth and colony-stimulating factors (CSF) in autoimmune diseases, infections, graft-*versus*-host, malignancies and apparently healthy individuals followed [17–22]. Some studies revealed associations between levels of specific anti-cytokine antibodies and certain clinical manifestations. For instance, anti-IL-1 α has been suggested to prevent bone erosions in RA [23, 24], anti-IL-6 was found in patients with severe alcoholic cirrhosis [25], autoantibodies to granulocyte CSF were present in neutropenic SLE patients [26], and the appearance of anti-IFN γ was observed in relation to disease remission in Guillain-Barré syndrome [27]. Few of these studies have been confirmed by other research groups and far from all checked for cytokine-neutralizing capacity of the detected anti-cytokine antibody. In addition, the observations were made with several different assays, *e.g.* ELISAs, immunoblotting and radioimmunoassays (RIAs), all using purified cytokines as source of antigen. The presence, or absence, of BSA in the used cytokine preparations was rarely stated.

Bendtzen and co-workers have studied anti-cytokine antibodies in health and disease for many years [28]. Initially, they used immunoblotting and ELISAs [18] but abandoned these methods for radioiodinated-based methods due to lack of sensitivity [9]. Whether the anti-cytokine 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, and thus eventually resulting in various degrees of ‘cytokine deficiency’. However, when the presence of various naturally occurring anti-cytokine antibodies also became

apparent [21, 22, 29, 30], 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 it from proteolytic degradation [28]. Other potential *in-vivo* functions of anti-cytokine antibodies include roles as: (1) scavengers of residual cytokine in individuals treated with recombinant cytokine (*e.g.* IFN β and GM-CSF); (2) reservoir with balancing effects on the equilibrium between pro- and anti-inflammatory cytokines; and (3) Fc γ R-mediated transducers of cytokine–anti-cytokine complexes [reviewed in 3].

Our interest in anti-cytokine 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 β and TNF) could explain why SLE patients, in spite of high disease activity and elevated IL-6, often display a remarkably poor CRP response. In-house ELISAs were developed in accordance with the description by Elkarim *et al* [27] and the antigen specificities of our assays were validated as suggested by Bendtzen [8]. Contrary to our expectations, we found no evidence of raised autoantibody levels to CRP-inducing cytokines as compared to healthy controls. Neither did we find any correlations between CRP levels and any of the tested anti-cytokine autoantibodies [11]. However, anti-TNF antibody levels were significantly lower in patient sera from active disease as compared to remission samples, and anti-TNF levels were strongly inversely associated with disease activity in certain patients [11]. At first, the efforts to prove the antigen specificities of our different anti-cytokine ELISAs were apparently successful, since pre-incubation of the sera with “relevant” cytokine abolished antibody reactivity, whereas pre-incubation of “irrelevant” cytokine (consistently IFN γ) did not. In retrospect, however, we have become painfully aware of the fact that all of

the relevant cytokines used to coat microtitre plates and pre-adsorb 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 ELISAs have we used BSA as a blocker of non-specific antibody binding. On the contrary, we actively avoid this, well aware of the fact that anti-BSA antibodies are commonplace and that antibodies to dietary proteins may interfere with the results of antigen-specific immunoassays [31]. It is not known whether anti-BSA are ‘natural’ (innate) antibodies, *i.e.* occurring without specific immunisation, or if they are induced by MHC-restricted antigen-presentation. However, the latter seems probable, since BSA is a common dietary antigen. In the 1970–80:s Cunningham-Rundles published a series of papers on circulating immune complexes containing bovine milk antigens, especially in subjects with IgA-deficiency, and hypothesized that this could be explained to systemic immunization to dietary proteins due to lack of mucosal IgA-mediated immune exclusion [32]. However, also in populations-based samples, the occurrence of IgG antibodies to BSA is a common finding although levels seem to decline with age [33, 34]. In human disease, anti-BSA has attracted most interest regarding insulin dependent diabetes mellitus (IDDM), where increased levels of anti-BSA antibodies as well as cross reactivity between a BSA peptide sequence and a pancreatic β -cell surface protein has been suggested [35, 36] and the potential role of BSA and other cow’s milk proteins in the development of IDDM is still a matter of considerable interest [37, 38]. In a study by Mogues *et al*, a sensitive quantitative anti-BSA antibody assay was developed, and a proper cut-off level was carefully defined [39]. By this method, they found IgG-class anti-BSA antibodies in sera of slightly above 50% among lung cancer patients undergoing surgery. The levels and frequency of anti-BSA antibodies in healthy blood donors were similar. In patients exposed to BSA in an intra-thoracic surgical sealant after pulmectomy, the antibody levels increased steeply and the frequency of positive

anti-BSA tests rose to 96% [39]. In diseases characterized by a compromised gastrointestinal barrier, such as Crohn's and celiac disease, levels of anti-BSA antibodies seem to be increased [40, 41], and in SLE, anti-BSA levels have both been found to be elevated and to correlate to an increased risk of cardiovascular disease [42]. Instead of measuring different levels of circulating anti-TNF antibodies in SLE during flare and remission [11], 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 worthwhile further studies.

Rosenau & Schur recently reported that the serum levels of anti-TNF autoantibodies in RA did not correlate with disease activity (CRP, erythrocyte sedimentation rate), but concluded that anti-TNF autoantibodies may reduce disease activity, since RA patients with high serum levels did not develop joint erosions [43]. In a meeting abstract 2004, we presented a similar conclusion regarding disease-modifying properties of anti-TNF and anti-IL1 α autoantibodies in RA since patients with subnormal levels (as compared to healthy controls) showed a tendency to higher disease activity over time as compared to patients with normal antibody levels [12]. Rosenau & Schur used buffer containing 2% BSA to block non-specific antibody binding to TNF-coated microtitre plates, but in contrast to us they also took the precaution to dilute serum samples with the same 'blocking buffer' [43]. 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 meddle with the results of immunoassays. This may be due to reaction with secondary detection antibodies [44], but also due to interaction with soluble immune complexes in the patient serum, or by reaction with serum antibodies immobilized on the microtitre plate. Furthermore, even when taking the precaution of neutralizing serum antibodies with the "blocking agent" (such as BSA), rheumatoid factor of any isotype may interact both with the "iatrogenic" immune

complexes in serum and with antibodies bound to the microtitre plate and 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 RA patients.

The results of the present study clearly demonstrate that our previously published results on anti-cytokine antibodies in SLE and RA patients [11, 12] were seriously confounded by the presence of BSA. It is highly likely that similar erroneous results have been reported by others unknowing 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” are 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, the present investigation yielded three important observations: (i) First, we confirm that antibodies to BSA are common and equally frequent in sera from early RA patients and healthy individuals. (ii) Secondly, anti-BSA antibody levels did not show any significant relation to either disease activity, serologic features (*i.e.* anti-CCP/rheumatoid factor status) or disease progression during three years. This finding contrasts to the proposed possibility of a molecular mimicry mechanism in RA involving BSA [45]. (iii) Finally, the convincing direct correlation between anti-TNF/BSA and anti-BSA (Figure 2), as well as the results from the inhibition assay (Figure 3), strongly supports the notion that BSA can meddle with the results of “antigen-specific” ELISAs. Due to the frequent occurrence of anti-BSA antibodies, BSA should be avoided in ELISAs.

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Figure legends

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 ($p = 0.11$); line indicate mean value.

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 and 12 controls using BSA-containing TNF as antigen. Pre-incubation with BSA or HSA was performed. *** represents $p < 0.0001$.

Table 1. Baseline characteristics of the 189 patients in the TIRA-1 cohort. Data in brackets are given as standard deviations or percent (were indicated). DMARD, disease-modifying anti-rheumatic drugs.

Number of patients	189
Females	129 (68%)
Males	60 (32%)
Age (years), mean	54.9 (16.5)
Fulfilled ACR criteria (number), mean	4.6 (0.7)
Erythrocyte sedimentation rate (mm/h), mean	36 (22.9)
C-reactive protein (mg/L), mean	27.7 (24.9)
Disease activity score (DAS28), mean	5.4 (1.1)
Cyclic citrullinated peptide antibody positive (IgG)	123 (65%)
Rheumatoid factor positive (IgM)	119 (63%)
Patients prescribed any DMARD	102 (54%)
Patients prescribed oral corticosteroids	76 (40%)

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