Utility of Bovine Serum Albumin Antibodies as a Biomarker for Arthritis in Children

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We studied the significance of antibodies to bovine serum albumin (BSA) as a biomarker for juvenile idiopathic arthritis (JIA). Sera from 30 children newly diagnosed with polyarticular JIA and sera from 30 healthy children matched for sex and age were tested for IgA, IgG and IgM by ELISA. BSA was used as a target to characterize changes in levels of interacting immunoglobulins. Initial results obtained before removal of antibodies that interacted with BSA suggested that JIA patients had increased levels of IgA in their sera. The serum IgA level was significantly ($p<0.0001$) higher in JIA children (mean±standard deviation (SD)=2.07±0.089 mg/mL) than in healthy children (1.69±0.109 mg/mL), while there was no significant difference in the levels of serum IgG and IgM between JIA children and healthy children; mean±SD of IgG serum level was 7.31±0.43 mg/mL and 7.39±0.52 mg/mL in JIA and healthy children ($p=0.234$), respectively, and IgM serum level was 1.22±0.24 mg/mL and 1.27±0.28 mg/mL in JIA and healthy children ($p=0.238$), respectively. However, the mean±SD of IgA level in JIA sera after purification from antibodies that interacted with BSA was 1.71±0.096 mg/mL, indicating the responsibility of anti-BSA IgA antibodies for increased IgA level in JIA patients. In conclusion, a humoral immune response against this antigen might serve as a diagnostic tool for detection of high-risk patients. Thus, positive BSA titers assist in discriminating children with JIA from healthy children.

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Introduction

Understanding of the immunologic defects that contribute to the development of autoimmunity will provide an insight into the pathogenesis of the autoimmune process. Polyclonal B cell activation is one of immunological abnormalities commonly found in rheumatoid arthritis (RA) patients. B-lymphocytes play a major pathogenetic role by the generation of autoantibodies, such as rheumatoid factors and antinuclear antibodies.[1] "Juvenile idiopathic arthritis (JIA)," a term for the most prevalent form of arthritis in children, is applied to a family of illnesses characterized by chronic inflammation and hypertrophy of the synovial membranes. Others[2,3] have proposed that the pathogenesis of rheumatoid disease in adults and children involves complex interactions between innate and adaptive immunity. Polyarticular juvenile arthritis is a distinct clinical subtype characterized by inflammation and synovial proliferation in multiple joints (four or more); including the small joints of the hands.[4] This subtype of JIA may be severe, because it involves multiple joints and progresses rapidly over time.

The diagnosis of JIA was made based on standard criteria.[5] Non-inflammatory forms of musculoskeletal pain, overuse syndromes and mechanical forms of musculoskeletal pain were diagnosed based on history, physical findings, and, where appropriate, laboratory and/or radiographic findings as described by Sherry and Malleson.[6] An increase in circulating IgA concentrations was reported among RA patients and RA complications were associated with a significant increase in serum IgA concentration.[7,8] RA patients with serum IgA level beyond the normal range were more likely to be positive for the autoantibodies.[9] Bovine serum albumin (BSA) is one of the most widely studied proteins; its structure is well known and its antigenic characteristics have been described in several papers.[10,11]

The present study was therefore designed to delineate heterophile antibody interference in our ELISA detection and to propose strategies for resolving the problem. We attempted to determine using ELISA whether antibodies that interact with BSA provide any diagnostic value as a risk factor for arthritis in children. In addition,

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the sensitivities of immunoassays for antibodies to JIA may be increased by including antigens such as BSA.

Materials and Methods

Materials

The serum samples used in the present study were collected from children newly diagnosed with polyarticular JIA and healthy children without food allergy to cow milk or beef. We excluded JIA children if they had been treated with corticosteroids or methotrexate, or if they had received therapeutic doses of nonsteroidal anti-inflammatory drugs for more than 3 weeks before the study. Patients with active disease presented with proliferative synovitis of multiple joints and erythrocyte sedimentation rates ranging from 35 to 100 mm/h. Patients were characterized by inflammation and synovial proliferation in multiple joints (four or five). Only 30 patients (19 boys and 11 girls) with JIA (Group A) fulfilled these conditions for admission to this study; they were aged from 10-12 years with the mean of 10.9 years. We selected 30 healthy children as a control group (Group B) from Chatby hospital matching to each patient by sex and age (within 1 year difference). All sera were collected within 4 months and stored in small aliquots at -80°C until tested under code. We halved each serum sample to analyze with and without BSA pretreatment.

Informed parental and patient consent was obtained in every case and the use of blood for scientific studies was approved by the local Ethical Committee.

Anti-human IgA (G, M) antiserum (raised in rabbit), human IgA (G, M), rabbit anti-human IgA (G, M) conjugated to horseradish peroxidase (HRP), and tetramethylbenzidine were purchased from Sigma (Sigma-Aldrich Company Ltd., Gillingham, UK) and all other chemicals were supplied from BDH (VWR International Ltd., Leicestershire, UK).

Human immunoglobulin measurement by ELISA

Coating antibody [anti-human IgA (IgG, IgM) antiserum] was diluted 1 in 1000 in 1× coating buffer (0.02 M Tris-HCl, 1.5 M NaCl pH 9.0) and 100 μL was added to each of the wells of a microtiter plate.[14,15] After overnight incubation at 4°C the plate was washed 4 times with PBST20 (0.1% (w/v) [Tween 20 in 1× PBS (phosphate buffered saline; 0.25 M NaCl, 0.0268 M KCl, 0.081 M Na_2HPO_4 and 0.0146 M KH_2PO_4)]. Sites unoccupied by antibody were blocked by addition of 5% (w/v) Marvel (dried skimmed milk) in PBS for 1 h at room temperature followed by washing 6 times with PBST20. The human serum samples were initially diluted 1 in 2000 in 1× PBS, and 2 fold serial dilutions subsequently performed on the plate. Diluted samples were allowed to bind to the first antibody and the plate was then washed 6 times in PBST20.

Rabbit anti-human IgA (IgG, IgM) conjugated to HRP (second antibody) was diluted 1 in 1000 in 1× PBS, 100 μL was added to each well of the microtiter plate, incubated at room temperature for 1 h and then washed 6 times in PBST20. The amount of bound second antibody was determined by adding 200 μL of the substrate solution (tetramethylbenzidine 6 mg/mL in 0.1 M sodium acetate buffer pH 6.0) to each well. After incubation, in the dark at room temperature for 20 min, the reaction was stopped by adding 50 μL of 10% (w/v) H_2SO_4 to each well. The optical density of each sample was read with an ELISA plate reader with a 450-nm filter. A standard curve was constructed by plotting absorbance against concentration for the standard solutions and the concentration of immunoglobulin (mg/mL) in the samples was determined.

Human immunoglobulin A measurement by turbidimetric assay

Human serum samples were titrated previously against antisera to obtain the optimum optical densities (precipitation).[16] Briefly, 8 μL of human serum samples were diluted with 492 μL of 1× PBS and, in addition, 25 mL of anti-IgA antiserum (developed in rabbit) with 475 μL of 1× PBS. After dilution, the antiserum and serum were mixed and incubated for 1 h at room temperature. At the same time, the standard human IgA was titrated by adding equal volumes of antiserum, mixed well and incubated for 1 h at room temperature. The degree of precipitation was quantified by measuring the optical density at 600 nm. The concentration of IgA (mg/mL) was calculated from the standard dilution series.

Purification of JIA sera from the effect of antibodies that interact with BSA

Bovine serum albumin, 56 μL, at a concentration of 50 mg/mL solution in 1× PBS, pH 7.2 were mixed with 200 μL of human serum samples (diluted 1 in 10) to minimize further crossreactivity to BSA. The absorption was carried out for 1h at 37°C, followed overnight at 4°C. The JIA sera were clarified by centrifugation at 10,000×g for 20 min at 4°C before testing.[17,18] The absorption of JIA sera with BSA completely removed the positive reaction of BSA antibodies, and then the concentration of IgA present in each of these samples was determined by ELISA as described above.

Statistical analysis

The measurements were summarized as mean±standard deviation (SD). The effects of BSA treatment on IgA in the Groups A and B were analyzed by paired t-test, and the difference in the IgG and IgM levels between the Groups A and B were analyzed by t-test. Necessary calculations were performed by SPSS for windows (Statistical Package for the Social Sciences, Salem, OR).

Results

ELISA measurements having been carried out without BSA pretreatment indicated that serum IgA level (mean±SD) was significantly ($p<0.0001$) higher in JIA affected children (2.07±0.089 mg/

mL) than in healthy children (1.69±0.109 mg/mL), while no significant difference was observed between JIA affected children and healthy children in the serum IgG level (7.31±0.43 mg/mL *vs* 7.39±0.52 mg/mL; *p*=0.234) or IgM level (1.22±0.24 mg/mL *vs* 1.27±0.28 mg/mL; *p*=0.238). We therefore further measured IgA level by ELISA in serum with BSA pretreatment. As shown in Figure 1, BSA pretreatment significantly (*p*<0.0001) decreased the serum IgA level in JIA affected children (2.07±0.089 mg/mL *vs* 1.71±0.096 mg/mL), while the change with BSA pretreatment observed in healthy children was negligible (1.69±0.109 mg/mL *vs* 1.68±0.092 mg/mL) although the difference was statistically significant (*p*=0.039). These results were validated by measuring the concentration of serum IgA in five selected JIA sera using a turbidimetric assay (Table 1).
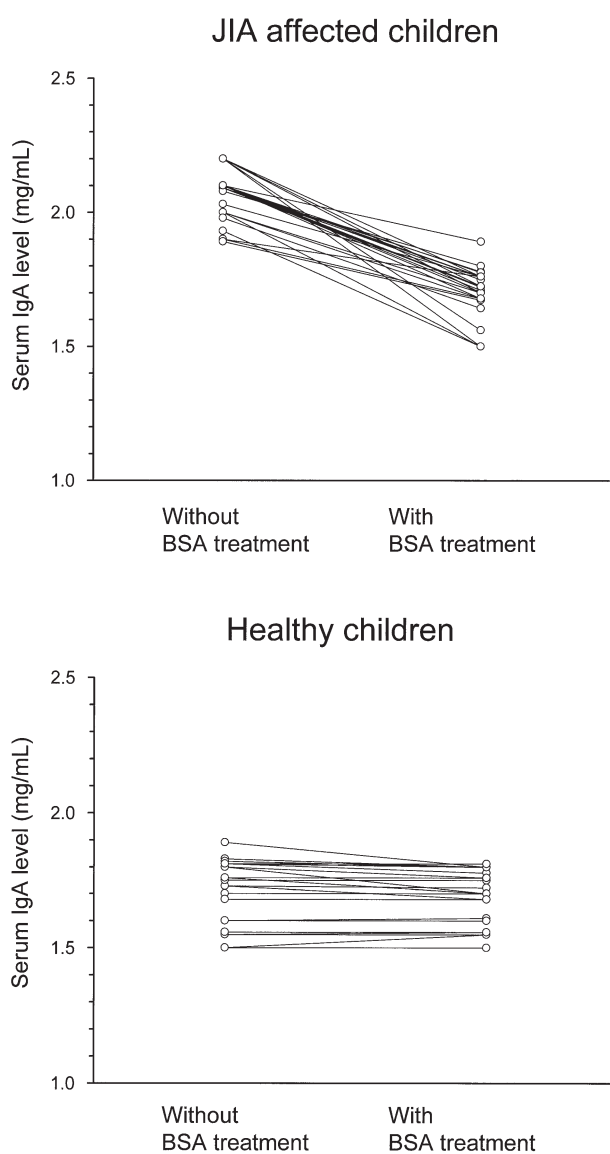
Table 1. Effects of BSA pretreatment on JIA sera evaluated by serum IgA level (mg/mL) measured with turbidimetric assay

JIA affected children	Without BSA pretreatment	With BSA pretreatment	% Decrease in IgA concentration
A	2.10	1.68	20.0
B	1.89	1.67	11.6
C	2.20	1.50	31.8
D	2.10	1.72	18.1
E	2.00	1.50	25.0
Mean±SD	2.06±0.117	1.61±0.106	

The present study did not indicate any statistical significant variations by sex (data not shown).

Discussion

Our results question the role of BSA as a cross-reacting antigen with IgA in the etiology of JIA. Many autoimmune disorders have been strongly associated with a high number of immune abnormalities; both humoral and cellular, occur either transiently or permanently in rheumatoid arthritis.[1,8,19,20] Despite this abundance of reports, most researchers consider that whether the role of immunological factors is primarily pathogenetic, co-causative, secondary or simply chronologically associated to pathogenetic events has not been definitively answered.[21,22] This problem has directed research towards other possible immunological factors likely to be present in rheumatoid arthritis, in the attempt to elucidate further the complex immuno-pathogenetic interactions of the disease: our finding of antibody interaction in JIA sera, leading to inaccuracies in IgA estimation by ELISA, represents a move in this direction.

In order to overcome this problem of antibody interaction with BSA, the JIA human sera were pre-incubated with BSA to eliminate this interaction. Interestingly, the levels of these antibodies decline after purification; hence, this proves that it is not reliable to use the human JIA sera directly without previous purification. Thus, this sort of interaction led to over-estimation of immunoglobulin levels in JIA sera, which in turn produces unreliable results. Therefore, it was necessary to examine with other non-immunochemical assay, such as Turbidimetric assay, to find out what sort of complex can be seen due to this interaction. Results suggested that other antibodies interact and co-precipitate with the antigen-antibody complex heavy chains. The fact that different antibodies recognize different epitopes together with the fact that the antibody molecules are dimeric means that antibodies found in JIA sera are able to form a cross-linked structure when mixed with antiserum. Hence, an antibody in antiserum binds to antibodies in JIA sera is not indicative that the bound antibody is the antigen. These concluded that there arose an anti-BSA antibody interaction during the analysis of immunoglobulin A.

Immunopathological findings indicated that the immune response to BSA in the knee joints could induce a rheumatoid arthritis like chronic synovitis in dogs.[23] Defects within one component of the

JIA affected children

Healthy children

Figure 1. Effects of BSA pretreatment on serum IgA level in JIA affected children (upper panel) and healthy children (lower panel). Each pair of open circles connected by line depicts the serum IgA levels for the same individual.

immune system may alter the way a pathogen induces an immune response and lead to an inflammatory response directed at self-antigens. On the basis of these findings, it is interesting to speculate that IgA antibodies to BSA were significantly increased at onset of polyarticular JIA, suggesting a possible role for IgA in the pathogenesis of the vascular complications of juvenile rheumatoid arthritis.

The presence of these anti-BSA-IgA antibodies in JIA may reflect the increase in production of autoantibodies and then, lead to humoral immune abnormalities. This is best explained by suggesting that there is an interaction producing spurious immuno-precipitation as well as a circulating immunoglobulin being capable of binding other autologous immunoglobulins which may well interact with other immune factors, thus participating in vivo in the complex immuno-pathological events which occur in JIA.

In conclusion, this study indicates the risk factor of antibodies reacting with human immunoglobulins in sera from JIA patients. However, the time course for the development of antibodies before onset of clinical JIA, which might be most sensitive or specific for predicting future development of the disease activity, is unknown. The high prevalence of elevated anti-BSA IgA antibodies levels in JIA children enhances the clinical utility of this immune marker due to autoantibodies generation.

Finally, these preliminary investigations will need to be followed up longitudinally with a larger group of polyarticular JIA patients, and individual response profiles will need to be developed for specific agents (e.g. nonsteroidal anti-inflammatory drugs, methotrexate, etc). Despite these limits, these data conclusively demonstrate the power of using BSA test towards the prediction and diagnosis of complex diseases such as polyarticular JIA.

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