Antibodies reacting with human immunoglobulin in sera from autoimmune thyroid disease patients as a risk factor for false positive results in IgA assessment

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Abstract

Autoimmune thyroid diseases (ATD) are organ-specific autoimmune disorders namely, Hashimoto's thyroiditis and Graves' disease, characterized by the production of antibodies against the thyroid peroxidase and thyroid-stimulating hormone receptor in the thyroid gland. The purpose of this study was to optimize the ELISA tests for quantifying serum immunoglobulins in ATD patients by minimizing the interference of anti-goat immunoglobulin and to evaluate the prevalence effect on the measurement of the humoral immune response. Anti-immunoglobulin antibodies were defined using goat immunoglobulins as a target to characterize distinct changes in patterns of immunoglobulin levels in ATD patients. Concentrations of serum immunoglobulin A, G and M in thirty five patients with ATD with positive anti-thyroid antibodies and thirty five matched normal healthy adult individuals were tested by ELISA. The effect of anti-goat immunoglobulin antibodies leads to an overestimation of serum IgA level in ATD and is different for each serum sample tested. Initial results obtained before purification from the interfering anti-goat immunoglobulin antibodies suggested that ATD patients had increased levels of IgA in their sera. It was found that normal individuals had mean IgA, IgG and IgM levels of 2.75 mg/ml, 9.67 mg/ml and 1.81 mg/ml, respectively while individuals suffering from ATD had mean levels of 5.01 mg/ml, 10.17 mg/ml and 1.92 mg/ml ($p<0.0004$, $p<0.20$ and $p<0.34$). However, the mean level of IgA in ATD sera treated from anti-goat immunoglobulins was determined to be 2.81 mg/ml. Therefore, there was no significant difference in IgA level in patients with ATD compared to normal individuals ($p<0.63$) after removal of anti-goat immunoglobulin antibodies. Visualization of IgA by immunoblotting confirms that anti-goat immunoglobulins antibodies, which were unrelated to antigen, were co-precipitated with the antigen-antibody complex. A circulating immunoglobulin reacting with other immunoglobulins is thus present at increased levels in adult subjects with ATD and may well play a part in the complex immunopathogenetic interactions.

Key words: *autoimmune thyroid diseases, goat-immunoglobulin, IgA, IgG, IgM.*

(Centr Eur J Immunol 2008; 33 (4): 208-212)

Introduction

Immunological self-tolerance can be defined as a meta-stable state in which the immune system does not react destructively against self-molecules, cells or tissues. Lack or loss of self-tolerance is likely to result in autoimmune responses, cellular and tissue damage, and eventually the clinical onset of autoimmune disease [1].

Over the past four decades, the list of diseases associated with autoantibodies against tissues, cells, or specific autoantigens has grown enormously [2]. The two main disorders that comprise autoimmune thyroid disease are

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Hashimoto's thyroiditis and Graves' disease. The former is the most common cause of hypothyroidism, whereas the latter is a major cause of hyperthyroidism. Hashimoto thyroiditis was reported to be an autoimmune disease more than forty years ago and, together with Graves disease, has served as a model disease for the dissecting of disease pathogenesis. Hashimoto's thyroiditis and Graves' disease are also the most common organ-specific autoimmune diseases affecting humans [3-5].

A better understanding of the autoantigens involved and the development of autoantigen-specific antibody or T cell assays may help to cover the way for future clinical applications. Similarly, numerous questions remain to be answered regarding the immune responses to infection and the resulting induction of autoimmunity. The identification of individual autoantigens is a key not only to uncovering the etiology and pathogenesis of an autoimmune disease, but also to improving the autoantibody assays used to diagnose or verify a disease. Improved biochemical and molecular methods have allowed a rapid dissection of autoantigens associated with specific autoimmune diseases. Standardization of autoantibody assays is critical to their use in the clinic to predict, diagnose, and treat this very diverse group of disorders. Since T cell–based tests have yet to be developed and standardized, it is likely that many of the estimated 5% of individuals in the US with one or another autoimmune condition will benefit from the use of reliable autoantibody tests to predict disease [6].

Activation of the immune response is a major feature of many disease processes. Immune responses can be protective, as in infectious diseases, or destructive, as in autoimmune inflammatory diseases, or both. The immune response usually involves activation of both T and B cells, the latter producing antibodies that can be detected in the sera and can be used to guide the clinical management of certain diseases.

The present study was therefore designed to investigate the presence of anti-immunoglobulin antibodies in ATD patients and to optimize the ELISA tests for quantifying serum immunoglobulins. Anti-immunoglobulin antibodies were defined using goat immunoglobulins as a target to determine the effect of anti-immunoglobulin antibodies on the measurement of the humoral immune response in patients with ATD in an attempt to clarify some immunopathogenetic relationship aspects between the titer of the autoantibodies and the severity of the disease.

Materials and Methods

Anti-human IgA-I8760, IgG-I8635, IgM-I9385 whole antiserum produced in rabbit, human IgA-I1010, IgG-I4506, IgM-I8260 and tetramethylbenzidine were purchased from Sigma (Sigma-Aldrich Company Ltd, Gillingham, UK). Rabbit anti-human IgA-CB9119, IgG-CB0948, IgM-CB9105, affinity purified (less than 1% cross reactivity was observed against other human heavy or light chain isotypes) conjugated to horseradish peroxidase (HRP) were purchased from Cortex Biochem (Fitzgerald Industries International, USA). All other chemicals were supplied from BDH (VWR International Ltd, Leicestershire, UK).

Subjects

Sera were collected from 35 healthy volunteers (group 1) and 35 consecutive patients with ATD (group 2) with positive antiperoxidase and/or antithyroglobulin antibodies matched for age and sex (median age, 35 years [range, 25 to 49 years]). Twenty patients had Hashimoto's thyroiditis and 15 had Graves' disease. The diagnosis of ATD was made according to established criteria. None of the above patients had a history of systemic autoimmune disorders. The above subjects were first examined for the presence of antinuclear antibodies in their sera. Antinuclear antibodies positive subjects were further evaluated by a complete history and physical examination, blood tests, urine tests as well as immunological studies for other more specific autoantibodies. Medical history, physical examination and routine laboratory investigations were completely normal in all unaffected subjects. They did not use any medication prior to this study. All sera were collected within four months and stored in small aliquots at -80°C until tested under code. Informed patient consent was obtained in every case and the use of blood for scientific studies was approved by the local Ethical Committee.

Sample preparation for Immunoblotting

Autoimmune thyroid diseases serum samples (4 µl) were mixed with 20 µl of 2× Laemmli sample buffer, water (20 µl), 1 M iodoacetamide (8 µl) and then incubated at 95°C for 3 min. A fraction of this mixture (20 µl) was electrophoresed overnight on a discontinuous 10% polyacrylamide gel containing 0.1% (w/v) SDS at a constant voltage of 45 V at room temperature [7].

Immunoblotting of gel

After electrophoresis, the protein was electro-blotted onto a sheet of nitrocellulose (Millipore HAHY 00010) at 500 mA for 1 h [8, 9]. The nitrocellulose was blocked by incubation with 5% (w/v) Marvel (dried skimmed milk) in 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) for 1 h, washed three times with PBST (PBS containing 0.1% (w/v) Tween 80; 10 min per wash). The filter was then incubated with a 1:200 dilution of rabbit anti-human IgA serum in PBSM (PBS containing 0.1% (w/v) Marvel) for 1 h, followed by washing three times in PBST. The rabbit immunoglobulin was detected by incubation in a 1:1000

dilution of donkey anti-rabbit immunoglobulin conjugated to horseradish peroxidase in PBSM. The peroxidase was visualized by staining with 100 ml of a solution containing 0.5 mg/ml of diaminobenzidine in 25 mM phosphate buffer pH 7.4, 0.03% (w/v) $CoCl_2$, 0.03% (w/v) ammonium phosphate to which 5 µl of 100 Vol. H_2O_2 was added immediately prior to staining [10].

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 [11, 12]. 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.

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.

Purification of ATD infected sera from the effect of antibodies that interact with goat immunoglobulins

Goat immunoglobulins were isolated previously from goat serum by affinity chromatography using the appropriate sepharose-bound antibody. The final purified antibody preparation contains only antigen-specific active antibody plus a small amount of denatured antibody resulting from elution procedure (Tago, 4100 series Ab). Goat immunoglobulins, 200 µl, at a concentration of 10 mg/l in PBS, pH 7.2 were mixed with 200 µl of human serum samples (diluted 1 in 10) to minimize further cross-reactivity to goat immunoglobulins. The absorption was carried out for 1h at 37°C, followed overnight at 4°C. The human sera were clarified by centrifugation at 10 000 × g for 20 min at 4°C before testing [13-15]. The absorption of human sera with goat immunoglobulins completely removed the positive reaction of anti-immunoglobulin antibodies, and then the concentration of immunoglobulin present in each of these samples was determined by ELISA as described above.

Statistical analysis

After tabulating the data, the arithmetic mean for each group was calculated. The variation or variability in each group was represented by the standard deviation (SD). The means of the groups were compared to see if the differences were significant. Student's *t*-test was used to assess the significance of the difference between groups. Necessary calculations were performed by SPSS for windows (Statistical Package for the Social Sciences, Salem, OR).

Results

The probability that immunoglobulins from ATD sera bind and co-precipitates with goat immunoglobulins more than do immunoglobulins from unaffected individuals was investigated. It is not possible to differentiate between IgA and other immunoglobulin heavy chains using polyacrylamide gel electrophoresis therefore, immunoblotting technique with anti-human IgA was carried out to determine if this increased interaction included IgA. Visual examination of Figure 1 shows that the band intensities in ATD sera purified from anti-goat immunoglobulin antibodies was lower than that seen in ATD sera without pre-treatment.

Therefore, the absorption of ATD sera with goat immunoglobulins was carried out to eliminate the positive reaction of these sera and/or investigate whether this would affect the IgA level as measured by ELISA. Results (Figure 2) demonstrated that pretreatment of ATD sera with goat immunoglobulins prior to ELISA affected IgA

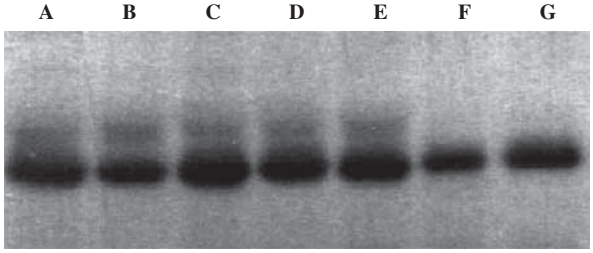

Fig. 1. Visualization of IgA by immunoblotting after denaturing polyacrylamide gel electrophoresis. IgA heavy chain was detected by immunoblotting. Lanes A, B, C, D & E contained non-purified ATD serum samples and lanes F & G contained purified ATD serum samples

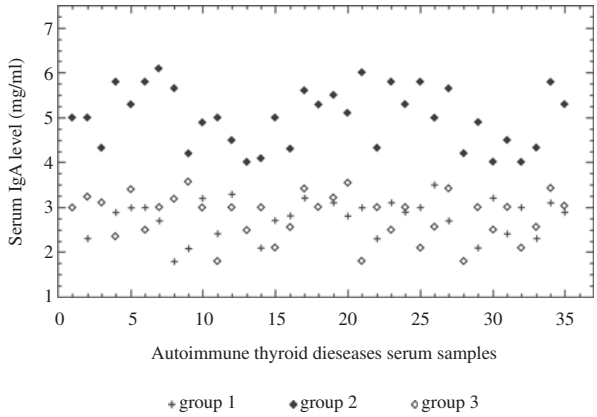

Fig. 2. Scatter diagram showing the effect of anti-goat immunoglobulin antibodies on ELISA IgA determinations

levels where this dramatically reduced the level of IgA in the sera from ATD patients. The quantitative analysis of serum IgA level (mean ± SD) found that normal individuals (group 1) had a mean level of IgA (2.75 ± 0.44 mg/ml) lower than untreated ATD subjects (group 2) (5.01 ± 0.66 mg/ml). These represented significant increases in IgA level in the sera of group 2 compared to group 1 ($p<0.0004$). On the contrary, the mean level of IgA in the sera of treated ATD subjects (group 3) was (2.81 ± 0.51 mg/ml) within the normal level and there was no significant difference between group 3 and group 1 ($p<0.63$). The effect of goat immunoglobulins treatment on the IgA levels in the sera of the control group was previously investigated and concluded that the IgA level was within the normal level after treatment (2.74 ± 0.45 mg/ml; $p<0.68$). However, non-significant differences were found (Table 1) in both IgG and IgM level of ATD patients of group 2 compared to normal individuals of group 1 ($p<0.20$ and $p<0.34$). Our results in this study did not address any statistical significant differences in sex variation (data not shown).

Discussion

Since autoantibodies are markers of disease activity, it follows that, at least under some circumstances; autoantibodies should be able to predict disease. This approach is especially promising for diseases with a long preclinical period, a feature of many organ-specific autoimmune diseases. The aim of disease prediction is disease prevention. Autoimmune diseases, which affect as least 5% of the population, might be prevented by avoiding those environmental factors that trigger the disease (primary prevention) or by use of therapy that modulates the destructive process before the onset of clinical symptoms (secondary prevention). Accurate disease prediction is vital for secondary prevention, so that therapy is given only to those individuals who are likely to become clinically ill [16, 17].

Table 1. Statistical analysis of IgG and IgM levels measured by ELISA

Study group	Case	Serum IgG level (mg/ml)	Serum IgM level (mg/ml)
1 (n=35)	normal	9.67±1.09	1.81±0.27
2 (n=35)	ATD patients	10.17±1.18$^{\#}$	1.92±0.28^{+}

Values are MEAN±SD: $^{\#}$ $p<0.20$; $^{+}$ $p<0.34$ compared with normal individuals.

In an attempt to elucidate further complex immunopathogenetic interactions of autoimmune thyroid diseases; our finding of anti-immunoglobulin antibody in ATD sera, leading to inaccuracies in immunoglobulin A estimation by ELISA, represents a move in diagnostic research towards other possible immunological factors likely to be present in ATD. In order to overcome this problem of interfering due to the effect of anti-immunoglobulin antibodies on ELISA detection, the human ATD sera were pre-treated with goat immunoglobulins to eliminate cross-reaction of other irrelevant antibodies found in ATD sera. Interestingly, the levels of these antibodies decline after purification; hence this proves that it is not reliable to use the human ATD sera directly without previous purification. Thus, this sort of interaction led to over-estimation of immunoglobulin levels in ATD sera, which in turn produces unreliable results.

Autoantigens for autoreactive T cells and autoantibodies have been extensively explored in various autoimmune diseases. These explorations are important not only for understanding the pathogeneses of autoimmune diseases, but also for establishing antigen-specific immunotherapies. Predictive antibodies may not be directly responsible for specific manifestations of the disease, but are markers of future disease in presently healthy individuals [18]. Almost all autoimmune diseases are associated with circulating autoantibodies, which bind self-protein. Furthermore, for many diseases these autoantibodies are found in serum samples many years before disease onset [19].

The measurement of IgA levels can serve as surrogate marker to discriminate between antibody positive subjects at high or low risk for rapid development of ATD. Anti-ruminant antibodies effect noticed by a target goat immunoglobulins showed that the major dramatic effect was accompanied ATD patients using ELISA measurement. Therefore, it was necessary to examine with other non-immunochemical assay, such as immunoprecipitation using polyacrylamide gel electrophoresis and/or immunoblotting technique, to find out what sort of complex can be seen due to this interaction. Results suggested that other antibodies in ATD sera 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 anti-goat immunoglobulin antibodies found in ATD sera is able to form a cross-linked structure when mixed with antiserum. Hence, an antibody in antiserum binds to antibodies in ATD sera is not indicative that the bound antibody is the antigen. However, as no antibody assay offers 100% specificity, and the results are strongly dependent on the assay method used to measure the autoantibodies, it is essential to use assay methods with high diagnostic specificity, to minimize false positives and obtain a high positive predictive value [20].

Rabbit antisera were reliable for quantitation of serum IgA in ATD patients based on ELISA absorbance. Anti-goat immunoglobulin antibodies found in ATD sera greatly overestimate serum IgA concentration measurements in ATD subjects, regardless of the source of antibody used. The possibility of interference with the antigen-detection immunoassay for ATD patients by antisera developed in sheep or goat was previously investigated and concluded that antigen detection ELISA for captures and detection might misdiagnose ATD serum immunoglobulin concentration (results not shown). Results indicate that this bias will be avoided if reagents for capture and detection are derived from different species such as rabbit and this is in agreement with previous reports [21-24].

In conclusion, the presence of these anti-goat immunoglobulins in ATD may reflect the increase 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 which is capable of binding other autologous immunoglobulins that may well interact with other immune factors. Moreover, this study indicates the risk factor of antibodies reacting with human immunoglobulins in sera from ATD patients. This study provides an opportunity for early intervention that may be used for predicting disease in at-risk populations where the presence of certain autoantibodies may have some predictive utility in ATD. The results of the present paper point out that the production of specific antibodies is essentially a question of species specificity. In addition, address an important question on the protection of autoantibodies from autoimmune attack and the immunogenicity of the diseases. This observation holds out the prospect of screening the general population to identify individuals at high risk for some autoimmune diseases. Such cases might be willing for therapy either to prevent progression to clinical disease or to limit the impact of disease.

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