

Measurement of IgG Levels Can Serve as a Biomarker in Newly Diagnosed Diabetic Children

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Summary This study was undertaken to determine humoral immune response to the presence of anti-immunoglobulin antibodies in children with newly diagnosed type 1 diabetes mellitus, using as a target cow immunoglobulins, in an attempt to elucidate further complex immuno-pathogenetic interactions of the disease. Serum immunoglobulin G (IgG) concentrations were measured by ELISA in 30 children with type 1 diabetes mellitus and 30 healthy matched normal children. It was found that normal children had a mean IgG level of 7.41 mg/ml while diabetic individuals had a mean IgG level of 8.52 mg/ml ($p < 0.00004$). On the contrary, the mean level of IgG in diabetic sera after purification from anti-cow immunoglobulins was determined to be 7.52 mg/ml. Therefore, there was no significant difference in IgG level in patients with type 1 diabetes mellitus after removal of anti-cow immunoglobulin antibodies compared to normal children ($p < 0.58$). Visualization of IgG and immuno-precipitation confirm that anti-cow 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 in children with type 1 diabetes and may well play a part in the complex immuno-pathogenetic interactions.

Key Words: type 1 diabetes mellitus, IgG, antibodies

Introduction

Type 1 diabetes mellitus is an autoimmune disease associated with the presence of different types of autoantibodies. The presence of these antibodies and the corresponding antigens in the circulation leads to the formation of circulating immune complexes (CIC). The sequence of this process is still mostly unknown, and it may take years before β -cell destruction has proceeded so far that the disease becomes overt [1–4]. Damage to the target organs in organ-specific

autoimmunity can occur as the result of direct cellular damage by humoral or cell-mediated mechanisms or by stimulating autoantibodies or blocking autoantibodies [5]. Young age and definite signs of islet cell-directed autoimmunity are known to be factors that affect the speed of the β -cell destructive process [6].

It has been reported that the incidence of childhood type 1 diabetes mellitus has increased in recent years [7, 8]. Various exogenous triggers, such as certain dietary factors and viruses, are thought to induce the immune-mediated process leading to extensive cell destruction and ultimately to the clinical manifestation of type 1 diabetes [9]. The measurement of antibodies has been essential for understanding the epidemiology of childhood diabetes. In this study, we investigated whether serum level quantification in children with newly diagnosed

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type 1 diabetes mellitus specific antibodies and immunoglobulin G (IgG) together furnished novel insight into infection and immunity.

A number of humoral immune factors are present in the circulation in the early stages of type 1 diabetic patients and are thought to play an important part in the complex immuno-pathological interactions occurring during β -cell destruction [10–13]. Immunoglobulins, which bind other immunoglobulins or antibodies, add another facet to the abnormal immune response of type 1 diabetes mellitus. The human antibodies, which reacted in this way, were termed anti-ruminant antibodies [14–16].

The present study was therefore designed to investigate the presence of these anti-immunoglobulin antibodies in diabetic children. Anti-immunoglobulin antibodies were defined using cow immunoglobulins as a target to determine the effect of anti-immunoglobulin antibodies on the measurement of the humoral immune response in children with newly diagnosed type 1 diabetes mellitus in an attempt to clarify some immuno-pathogenetic aspects of B cell activation during diabetic disease.

Subjects and Methods

Anti-human IgG antiserum (raised in rabbit), human IgG, rabbit anti-human IgG conjugated to horseradish peroxidase (HRP), donkey anti-rabbit IgG conjugated to HRP, diaminobenzidine, 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).

Subjects

The study population consisted of 30 normal healthy children as a control group and 30 children with newly diagnosed type 1 diabetes mellitus. Sera from normal or diabetic children were allocated to two groups (A and B). Each serum sample in group A (from normal children) was divided into two and assigned to groups 1 and 2 and each sample in group B (from diabetic children) was assigned to groups 3 and 4. Groups 1 and 3 were untreated while groups 2 and 4 were treated with cow immunoglobulins as outlined in Materials and Methods (Purification of diabetic sera from the effect of antibodies that interact with cow immunoglobulins). Normal and diabetic sera were pre-treated with cow immunoglobulins to investigate whether this would affect the IgG level as measured by ELISA. Both group A and B were matched for sex and age with mean age 10.8 years (mean \pm SD = 10.8 \pm 0.5 years). Medical history, physical examination and routine laboratory investigations were completely normal in all subjects of group A. Patients, had no clinical or laboratory findings indicating other diseases such as rheumatoid arthritis and viral infection.

Only 30 patients with type 1 diabetes mellitus (group B) fulfilled these diagnostic criteria and they did not use any insulin medication prior to this study. 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. All sera were collected within four months and stored in small aliquots at -80°C until tested under code.

Electrophoresis of immuno-precipitates on polyacrylamide gel

Human serum samples were immuno-precipitated with anti-human IgG developed in rabbit in the presence of cow immunoglobulins. Human serum samples (25 μl) were diluted with 1 \times PBS (475 μl) and, in addition, 75 μl of anti-IgG antiserum with 1 \times PBS (425 μl). After dilution, the antiserum and serum were mixed to give a final volume of 1 ml and incubated for 1 h at room temperature. The precipitate was removed by centrifugation at 13,000 rpm for 5 min in micro centrifuge and then washed with 100 μl 1 \times PBS. The antigen-antibody precipitate was dissolved in 50 μl of 2 \times Laemmli sample buffer (0.125 M Tris-HCl pH (6.8), 4% (w/v) SDS, 20% (w/v) glycerol, 10% (v/v) β -mercaptoethanol, 0.005% (w/v) bromophenol blue) and then incubated at 95°C for 3 min [17]. A fraction of this mixture (25 μl) was electrophoresed overnight on a 10% polyacrylamide gel at a constant voltage of 45 V at room temperature. Following electrophoresis, proteins in gel were visualized by staining with Coomassie blue staining [18].

Sample preparation for Immunoblotting

Diabetic serum samples (4 μl) were mixed with 20 μl of 2 \times 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.

Immunoblotting of gel

After electrophoresis, the protein was electroblotted onto a sheet of nitrocellulose (Millipore HAHY 00010) at 500 mA for 1 h [19, 20]. 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 IgG 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) CoCl₂, 0.03% (w/v) ammonium phosphate to which 5 µl of 100 Vol. H₂O₂ was added immediately prior to staining [21].

Human immunoglobulin G measurement by turbidimetric assay

Human serum samples were titrated previously against antisera to obtain the optimum optical densities (optimal precipitation) [22]. Briefly, 8 µl of human serum samples was diluted with 492 µl of 1× PBS and, in addition, 25 µl of anti-IgG 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 IgG 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 IgG (mg/ml) was calculated from the standard dilution series.

Human immunoglobulin G measurement by ELISA

Coating antibody (anti-human IgG 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 [23, 24]. After overnight incubation at 4°C the plate was washed 4 times with PBST20 (0.1% (w/v) Tween 20 in 1× PBS). Sites unoccupied by antibody were blocked by addition of 5% (w/v) Marvel 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 IgG 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) H₂SO₄ to each well after that the absorbance was measured at 450 nm. The concentration of IgG (mg/ml) was calculated from the standard dilution series. A standard curve was constructed by plotting absorbance against concentration for the standard solutions and the concentration of IgG in the samples was determined.

Purification of diabetic sera from the effect of antibodies that interact with cow immunoglobulins

Cow immunoglobulins were isolated previously from cow serum by affinity chromatography using the appropriate sepharose-bound antibody (protein A sepharose CL-4B, Amersham Pharmacia Biotech UK Ltd). The final purified antibody preparation contains only antigen-specific active antibody plus a small amount of denatured antibody resulting from elution procedure. Cow immunoglobulins, 200 µl, at a concentration of 10 mg/l in PBS, pH 7.2 were mixed with 200 µl of human serum samples from each of 30 diabetic individuals (diluted 1 in 10) to minimize further cross-reactivity to human sera. The absorption was carried out for 1h at 37°C, followed overnight at 4°C. The diabetic sera were clarified by centrifugation at 10000 x g for 15 min at 4°C before testing [25, 26]. The absorption of diabetic sera with cow immunoglobulins completely removed the positive reaction of these sera, and then the amount of IgG 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.

Results

The probability that immunoglobulins from diabetic sera bind and co-precipitates with cow immunoglobulins more than do immunoglobulins from unaffected individuals was investigated using immuno-precipitation. Five sera from diabetic children and five serum samples from an unaffected participant were immuno-precipitated in the presence of cow immunoglobulins and the immuno-precipitates were electrophoresed on a polyacrylamide gel (Fig. 1). Visual examination of Fig. 1 shows that diabetic sera with cow immunoglobulins were co-precipitates with the antigen-antibody complex and co-migrates with the IgG heavy chain.

It is not possible to differentiate between IgG and other immunoglobulin heavy chains using polyacrylamide gel electrophoresis therefore, immunoblotting technique with anti-human IgG was carried out to determine if this increased interaction included IgG. Visual examination of Fig. 2 shows that the band intensities in diabetic sera purified from anti-cow immunoglobulin antibodies was lower than that seen in diabetic sera without pre-treatment. These results were validated by measuring the concentration of serum IgG in 5 selected diabetic and normal sera using a turbidimetric assay (Table 1).

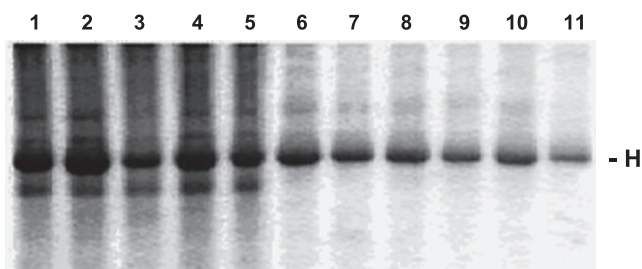


Fig. 1. Immuno-precipitation and polyacrylamide gel electrophoresis of human serum samples with anti-human IgG. Five diabetic serum samples (lanes 1–5) and five normal sera (lanes 6–10) were immuno-precipitated with anti-human IgG developed in rabbit in the presence of cow immunoglobulins. Lane 11 represents standard human IgG. H indicates positions of immunoglobulin heavy chains. The precipitates were washed, dissolved in Laemmli sample buffer and analyzed by polyacrylamide gel electrophoresis.



Fig. 2. Visualization of IgG by immunoblotting after denaturing polyacrylamide gel electrophoresis. IgG heavy chain was detected by immunoblotting. Lanes 1 & 2 contained purified diabetic serum samples and lanes 3, 4, 5, 6 & 7 contained non-purified diabetic serum samples.

Therefore, the absorption of diabetic sera with cow immunoglobulins was carried out to eliminate the positive reaction of these sera and/or investigate whether this would affect the IgG level as measured by ELISA. Results (Fig. 3) demonstrated that pretreatment of diabetic sera with cow immunoglobulins prior to ELISA affected IgG levels where this dramatically reduced the level of IgG in the sera from diabetic patients (group 4) while sera from normal children

(group 2) were barely affected by this treatment. The quantitative analysis of serum IgG level (mean \pm SD) found that group 1 had a mean level of IgG (7.41 ± 0.72 mg/ml) lower than group 3 (8.52 ± 0.73 mg/ml). These represented significant increases in IgG level in the sera of group 3 compared to group 1 ($p < 0.00004$). On the contrary, the mean level of IgG in the sera of group 4 was (7.52 ± 0.85 mg/ml) within the normal level and there was no significant difference between group 4 and group 1 ($p < 0.58$). The effect of cow immunoglobulins treatment on the IgG levels in the sera of the control group (group 2) was previously investigated and concluded that the IgG level was within the normal level after treatment (7.34 ± 0.71 mg/ml; $p < 0.68$). Our results in this study did not address any statistical significant differences in sex variation (data not shown).

Discussion

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

Type 1 diabetes mellitus is an organ-specific autoimmune disease in which the insulin-producing beta cells in the pancreatic islets are selectively eliminated. The autoimmune attack destroys the beta cells resulting in decreased production of insulin and consequently increased levels of

Table 1. Effect of anti-cow IgG antibodies on turbidimetric assay.

Normal serum sample ID	Group 1		% Decrease in IgG concentration	Diabetic serum sample ID	Group 3		% Decrease in IgG concentration
	Serum IgG level (mg/ml)	Serum IgG level (mg/ml)			Serum IgG level (mg/ml)	Serum IgG level (mg/ml)	
6	7.00	6.90	1.43	1	8.20	7.20	12.19
7	7.81	7.80	0.13	2	8.70	7.00	19.54
8	7.50	7.48	0.27	3	9.10	8.50	6.59
9	6.90	6.90	0.00	4	8.70	6.70	22.99
10	7.70	7.66	0.52	5	8.40	7.10	15.48

Serum samples from diabetic and normal children were investigated using polyacrylamide gel electrophoresis (Fig. 1). Groups 1 and 3 were untreated while groups 2 and 4 were treated with cow immunoglobulins.

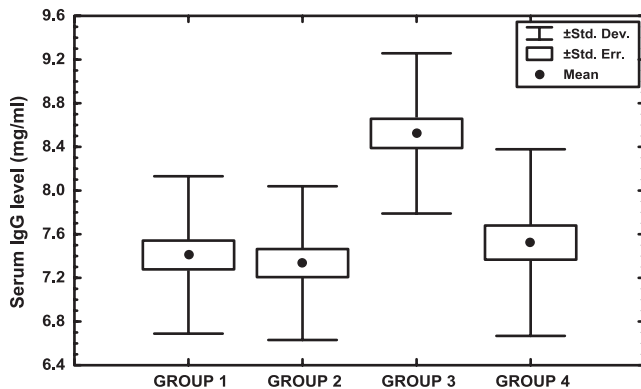


Fig. 3. Serum IgG levels in groups of diabetic children and unaffected control. Comparison of average serum IgG (mean \pm SD).

blood glucose. B-lymphocytes play a major pathogenetic role by the generation of autoantibodies. Autoantibodies to beta cells may also contribute to cell destruction by facilitating either complement-mediated lysis or antibody-dependent cell-mediated cytotoxicity. However, antibodies to beta cell proteins are also generated and may be used for predicting disease in at-risk populations [27]. High levels of IgM and IgG autoantibodies are associated with many autoimmune diseases. Evidence suggested that IgG autoantibodies are usually pathogenic, whereas secreted IgM, including IgM autoantibodies produced naturally or as part of an autoimmune response, may lessen the severity of autoimmune pathology associated with IgG autoantibodies [28]. The high prevalence of elevated anti-IgG/antibody interactions in diabetic patients enhances the clinical utility of this immune marker due to polyclonal B cell activation or autoantibodies generation. However, the time course for the development of antibodies before onset of clinical type 1 diabetes is unknown, which might be most sensitive or specific for predicting future development of the disease severity.

The measurement of IgG levels can serve as surrogate marker to discriminate between antibody positive subjects at high or low risk for rapid development of diabetes. Anti-ruminant antibodies effect noticed by a target cow immunoglobulins showed that the major dramatic effect was accompanied diabetic children using ELISA measurement. Therefore, it was necessary to examine with other non-immunochemical assay, such as immuno-precipitation 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 diabetic 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-cow

immunoglobulin antibodies found in diabetic sera is able to form a cross-linked structure when mixed with antiserum. Hence, an antibody in antiserum binds to antibodies in diabetic sera is not indicative that the bound antibody is the antigen.

Rabbit antisera were reliable for quantitation of serum IgG in diabetic patients based on ELISA absorbance. Anti-cow immunoglobulin antibodies found in diabetic sera greatly overestimate serum IgG concentration measurements in diabetic children, regardless of the source of antibody used. The possibility of interference with the antigen-detection immunoassay for diabetic patients by anti-sera developed in sheep or goat was previously investigated and concluded that antigen detection ELISA for captures and detection might misdiagnose diabetic 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 [16, 29, 30]. The increased IgG levels in the sera of diabetics suggest a possible role for IgG in the pathogenesis of the vascular complications of diabetes mellitus [27, 31, 32].

In conclusion, the presence of these anti-cow immunoglobulins in type 1 diabetes mellitus 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 immunoprecipitation as well as a circulating immunoglobulin which is capable of binding other autologous immunoglobulins that may well interact with other immune factors [33]. Moreover, this study indicates the risk factor of antibodies reacting with human immunoglobulins in sera from diabetic children. Furthermore, the autoimmune process begins years before the beta-cell destruction becomes complete, thereby providing an opportunity for early intervention that may be used for predicting disease in at-risk populations. 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 the beta cell from autoimmune attack and the immunogenicity of different insulin's produced in several animal species.

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