Complement mediated bactericidal activity and humoral immune response in type 2 diabetes mellitus

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Abstract
Since diabetic patients suffer from protracted infections, the aim of this study was to determine the functions of serum complement and its relationship with immunoglobulin profiles in patients with type 2 diabetes mellitus (DM). A total of 55 DM patients and 54 healthy volunteers were included in this study. Serum IgG was measured by nephelometry, complement components C3 and C4, and IgA were measured by immunoturbidometric methods. The bactericidal activities of complement from fresh serum and complement-inactivated serum were assessed against Escherichia coli DH5α cells using the standard plate count method. In the DM patients, IgG (p<0.001), IgA (p<0.02) and complement component C4 (p<0.001) were found to be significantly elevated whereas the levels of C3 were slightly lowered (p<0.4). The complement mediated bactericidal activity in diabetic patients was significantly lower than the controls (p<0.01). In contrast, while the serum complements were inactivated by heat treatment, the DM patients had significantly higher (p<0.01) bactericidal activity associated with heat-stable immune effector molecules, possibly elevated levels of serum IgG and IgA. The results of this study indicate that serum complement mediated bactericidal activity was impaired in type 2 diabetic patients, which might be a cause for delayed wound healing and repeated infections. (Int J Diabetes Metab 14: 92-97, 2006)

Keywords: Bactericidal activity · C3 · C4 · IgA · IgG ·

Introduction
Pathogenesis of insulin-resistance is the key feature of type 2 diabetes that includes low-grade inflammation. Indeed, elevated concentrations of various inflammatory markers in the circulation have been reported in humans with insulin-resistance. These include IL-6, TNFα, soluble TNF receptors, low albumin and C-reactive protein. Obesity has been found to be associated with elevated circulating levels of IL-6, TNFα as well as CRP. Therefore, elevated plasma levels of different inflammatory markers may sometimes reflect obesity rather than insulin-resistance per se, although obesity plays an important role in insulin-resistance as well as type 2 diabetes.

Several studies have shown that elevation of soluble markers of inflammation including CRP, sialic acid, and proinflammatory cytokines are associated with the future development of myocardial infarction, stroke, and peripheral vascular disease and with cardiovascular mortality. Serum sialic acid has been shown to be cross-sectionally related to coronary heart disease in type 2 diabetes and to predict future cardiovascular mortality in the patients independently of baseline atherosclerosis. Taken together with the evidence that inflammation also predicts type 2 diabetes independently of atherosclerosis, these studies suggest that activation of the innate immune system is likely to be at least one of the long-postulated common antecedents of both atherosclerosis and type 2 diabetes.

The functions of the complement system include control of inflammatory reactions and chemotaxis, clearance of immune complexes, cellular activation and antimicrobial defense. In children, the deficiency of complement protein C3 has been found to result in overwhelming bacterial infection. Because of repeated infection episodes, the functions of serum complement in killing intracellular bacteria could be impaired in the diabetic patients. In addition, the role of humoral immune response has not been extensively studied in diabetic patients. However, a study conducted in Nigeria has shown that humoral immunity provided by complement C3c, C4 and IgM is more deranged in Type 2 diabetic patients compared with Type 1. Elevated levels of serum total γ-globulin, a nonspecific measure of the adaptive immune system, has been shown to predict the development of Type 2 diabetes in the Pima Indians. In view of the scarcity of data, this study was undertaken to evaluate humoral immune response with respect to complement mediated bactericidal activity and immunoglobulin profiles in patients with Type 2 diabetes mellitus.

Materials and methods

Study subjects and sample collection
A total of 55 DM patients (28 males and 27 females), selected randomly, were enrolled in this study. The patients routinely visited the Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorder (BIRDEM) Hospital for their medical check-up. Fifty-four controls, healthy, non-diabetic subjects (30 males, 24 females), not matched for age with the patients, were enrolled in this study. About
three mL of peripheral blood were drawn from the DM patients and control subjects with their full consent to take part in this study. The general information of the patients was recorded on preformed questionnaires. Serum was collected from each blood sample and preserved at -80ºC until analyzed.

Assay of complement mediated bactericidal activity

*Escherichia coli* DH5α were grown in nutrient broth for 14 h at 37°C in an orbital shaker. The bacterial cells were harvested, washed twice using excess of PBS and the suspension was adjusted to 0.600 O.D. at 620 nm. Immediately, aliquots of 200 µL of the bacterial cell suspensions (BCS) were taken into separate tubes, 20 µL of serum was added to each tube and the mixture was incubated for 30 min at 37°C. At the end of incubation, the remaining viable cells were serially diluted with PBS to 1:10,000. An aliquot of 20 µL of this dilution was spread on each of 3 agar plates and incubated for 16 hours at 37°C. The number of colonies formed was counted and the mean value for each serum was taken from the readings of three plates. For the negative control experiments, 20 µL of PBS was added to the BCS, incubated and then serially diluted with PBS to 1:50,000. An aliquot of 20 µL of this dilution was spread on each of three agar plates.

Assay of complement inactivated bactericidal activity

To evaluate the function of serum complement in killing bacteria, complement proteins were inactivated by heat treatment at 56°C for 30 min in a water bath and a 20 µL aliquot was used to test for bactericidal activity. Both the negative control preparation and bacteria treated with inactivated serum were serially diluted to 1:50,000. The rest of the procedure was as described before.

Calculation of bactericidal activity

Complement mediated bactericidal activity was calculated using the following formula. For the negative control, if the mean colony-forming unit (cfu) on the plate was Nc, then one mL of the original bacterial cell suspension contained Nc X 50 X 50,000 cfu. For the test serum, if the mean cfu on the plate was Ns, then 1 mL of the bacterial cell suspension treated with serum complement contained Ns X 50 X 10,000 cfu. Therefore, % bactericidal activity =

\[
\frac{(Nc \times 50 \times 50,000) - (Ns \times 50 \times 10,000)}{Nc \times 50 \times 50,000} \times 100
\]

The bactericidal activity (%) of the inactivated serum for the mean cfu, Ni, was

\[
\frac{(Nc \times 50 \times 50,000) - (Ni \times 50 \times 50,000)}{Nc \times 50 \times 50,000} \times 100
\]

Determination of serum C3, C4, IgG and IgA

Quantitative estimates of complement components C3, C4, and IgA were performed using a TURBOX Plus Analyzer, Orion Diagnostica, Finland. The method was based on the principle of an immunoprecipitation reaction of a specific antibody with its antigen, according to the manufacturer’s recommended protocol. The light scattering caused by antigen-antibody complexes was measured after incubation. The intensity of the scattered light was directly proportional to the concentration of the tested component present in the serum sample. IgG was measured by immunonephelometry using DADE Behring reagents (USA) and an autoanalyzer. The results were expressed in g/L.

Determination of total serum protein

A serum sample (20 µL) was added to 1000 µL of the working biuret reagent (Randox Laboratories Ltd., Diamond Road, Crumlin, UK), mixed and incubated at 37°C for 30 min. Absorbance was measured at 546 nm against the reagent blank. The total protein concentrations of the serum samples were determined and the results expressed in g/L.

Statistical analysis

Data analyses were carried out using the Statistical Package for Social Sciences (version 11.5 for Windows, SPSS Inc., Chicago, USA) and SigmaStat (version 2.0, SPSS Inc., USA). Independent t-test was done for the comparison of two groups (control and patients) and the differences were considered significant when p was <0.05. Pearson’s correlation analysis of the data was made using the statistical program.

Results

Baseline characteristics of the study subjects

The baseline characteristics of the study subjects were determined from the preformed questionnaires. The mean age of the DM patients was 49.7 ± 11.1 years (range: 30-75 years) and duration of diabetes was 11.7 ± 5.8 years. Their mean BMI was 24.9 ± 4.1 (male: 24.2 ± 3.6; female: 25.6 ± 4.6) and random plasma glucose (PG) level was 9.9 ± 3.3 mM (glucose oxidase method). The mean age of the control subjects was 35.3 ± 10.4 years (range: 28-65 years) and BMI was 23.2 ± 2.1. The random PG levels of the control subjects were normal and the mean was 4.75 ± 0.98 mM.

Complement mediated bactericidal activity

It was found that in the negative control experiments (n = 52), *Escherichia coli* DH5α colonies grown on agar plates varied from 310–1030X10⁶ cfu/mL. After treating the BCS with serum from control healthy subjects (n = 54) the numbers of colonies varied from 0.3-7.5x10⁶ cfu/mL (Figure 1). The numbers of colonies grown after treating the BCS with serum from DM patients (n = 55) varied from 0.13 –145x10⁶ cfu/mL. Compared to the negative control experiments, both types of sera exhibited highly significant bactericidal activities (p<0.001).

The bactericidal activity of serum complements from the DM patients varied from 93.5-99.8% compared to about
Figure 1: Photograph of *Escherichia coli* DH5α colonies formed on agar plates after treatment of the bacterial cells with (a) PBS and (b) serum complements. The dilutions used were 1 : 50,000 for plate (a) and 1 : 10,000 for plate (b).

**Figure 2:** Percentage bactericidal activity on *Escherichia coli* DH5α cells by serum complements from (a) control subjects and (b) diabetic patients; and by complement inactivated serum from (c) control subjects and (d) diabetic patients. Each point represents the bactericidal activity of one individual expressed as the mean of three separate experiments.

Table 1: Comparison of complement mediated and complement inactivated bactericidal activities between healthy controls and diabetic patients

<table>
<thead>
<tr>
<th>Study subjects</th>
<th>Complement mediated bactericidal activity (%) (Mean ± SD)</th>
<th>Complement inactivated bactericidal activity (%) (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetic patients (n=55)</td>
<td>98.5 ± 1.8 (range: 93.5-99.8)</td>
<td>22.3 ± 7.7 (range: 7.5-37.5)</td>
</tr>
<tr>
<td>Control subjects (n=54)</td>
<td>99.3 ± 0.6 (range: 97.1-99.9)</td>
<td>18.2 ± 6.9 (range: 6.2-35.3)</td>
</tr>
<tr>
<td>p values</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
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97.0-100% in the control healthy subjects (Figures 2a and 2b). The DM patients had significantly lower bactericidal activity than that of the control subjects ($p<0.01$, Table 1).

**Effect of serum inactivation on bactericidal activity**

The bactericidal activity of the complement inactivated serum (BACIS) from the DM patients varied from 7.5–37.5% whereas that from the control subjects varied from about 6.0–35.0%. The results are shown in Figures 2c and 2d. The BACIS from the DM patients was found to be significantly higher ($p<0.01$, Table 1).

**Levels of complement component C3**

The levels of complement component C3 in 46 out of 55 DM patients (83.7%) were found within the normal range (0.9–2.1 g/L) whereas 7 (12.7%) had elevated, and only 2 (3.6%) had lower levels. In the 50 control subjects, 39 (78%) had normal and 11 (22%) had elevated levels of C3. The mean level of serum complement component C3 in the DM patients was 1.72 ± 0.38 g/L and in the healthy control subjects was 1.78 ± 0.35 g/L.

**Levels of complement component C4**

The levels of complement component C4 in 49% of the DM patients were found within the normal range (0.1–0.4 g/L), 49% had elevated, and 2% had lower values. In the control subjects, 89% had normal, 6% had elevated, and 5% had lower levels of C4. It was found that the mean value of C4 in the DM patients was 0.44 ± 0.18 g/L while that in the healthy control subjects was 0.24 ± 0.10 g/L. Statistical analysis showed the level of complement component C4 in the DM patients was significantly higher than that in the control subjects ($p<0.001$).

**Levels of serum IgG**

The levels of serum IgG in the DM patients were found to vary from 11.0 – 19.9 g/L and those in the control subjects varied from 7.9–16.4 g/L (normal range: 7 - 16 g/L). The patients had a mean value of serum IgG level of 15.5 ± 1.95 g/L compared to 11.0 ± 1.93 g/L in the healthy control subjects. Statistical analysis showed that the IgG level in the DM patients was significantly higher than that in the controls ($p<0.001$).

**Levels of serum IgA**

It was found that serum IgA levels in the DM patients varied widely from 1.18 – 7.38 g/L (normal range: 0.7 – 4.0 g/L). In contrast, the control subjects had normal levels of serum IgA that varied from 0.82 – 3.4 g/L. The mean value of serum IgA in the DM patients was 2.64 ± 1.18 g/L and that in the healthy control subjects was 2.14 ± 0.64 g/L. Statistical analysis showed serum IgA level in the DM patients was significantly higher than that in the control subjects ($p<0.02$).
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Levels of total protein
The total protein level in 98% of the DM patients was within the normal range (66–87 g/L), the remaining 2% had elevated values. Similarly, 96% of the control subjects had a normal level of total protein in serum; the remaining 4% had elevated values. The mean level of total protein in the DM patients was 75.5 ± 5.5 g/L and that in the control subjects was 78.0 ± 5.3 g/L. The patients had significantly lower levels of total protein in serum than the control subjects (p<0.02) although the values were within the normal range.

Correlation analysis of the data
In the patients, the fasting level of plasma glucose showed highly significant direct correlation with the duration of diabetes (r=0.395, p<0.01) as well as with the glucose level at 2 hours after breakfast, ABF (r=0.729, p<0.001). The level of complement protein C3 also showed a significant linear relationship with ABF (r=0.372, p<0.01), level of C4 (r=0.437, p<0.01) and with total protein in serum (r=0.306, p<0.02, Figure 3a). Like the DM patients, the level of complement protein C3 showed significant positive correlation with that of C4 (r=0.409, p<0.02), but unlike the patients, BACIS showed significant direct correlation with the level of total protein in the control subjects (r=0.300, p<0.03, Figure 3b).

Discussion
Patients with diabetes have an increased risk of infections, but information on their immune response is incomplete and contradictory. It has been suggested that chronic low-grade inflammation may be involved in the pathogenesis of insulin resistance and type 2 diabetes.22 The complications of diabetes contribute to compromised delivery of nutrients to surrounding tissues, poor elimination of metabolic waste products, impaired wound healing, immunosuppression, and susceptibility to bacterial infection.23 In diabetes, glycosylation of immunoglobulin G with glucose leads to covalent incorporation of the sugar into the protein that causes marked decrease in biological activity including complement fixation.24 There are reports of impaired neutrophil function and significant diminution in intracellular bactericidal activity in subjects with poorly controlled diabetes compared with healthy controls.25,26

Insulin resistance and cardiovascular disease share common pathophysiological mechanisms, such as the chronic activation of the innate immune system.27 Complement proteins play an important role in the innate immune system. In this study, the test bacteria *Escherichia coli* DH5α is a nonpathogen and so no appreciable levels of antibody against this organism should be present in the sera of normal healthy subjects. Therefore, killing of bacteria would be solely due to serum complement. It was found that the serum complement of the DM patients exhibited significantly lower bactericidal activity than those of the healthy controls.

The reasons for lower bactericidal activity might be the decreased level of complement proteins. Measurement of the major complement proteins C3 and C4 showed the concentration of C3 was slightly lowered while that of C4 was significantly higher in the DM patients. Previous workers have found lowered28,29 as well as elevated levels of complement C3 in diabetic patients.30-32 Lower levels of C3 might be associated with its high catabolic rate. Furthermore, there have been reports of both elevated30,31 and decreased28,29 levels of C4 in DM patients. Elevated levels of C4 indicate that activation of the classical pathway, which is linked to inflammation, might be showing a low turnover rate.

The findings on complement inactivated bactericidal activity indicated the presence of heat-stable molecules in the sera. Determination of the immunoglobulin profiles showed significantly elevated levels of IgG and IgA in the patients. Other researchers made similar observations for IgG33-35 and IgA36-38 Elevated levels of serum IgG and IgA suggest that, since the diabetic patients suffered from different types of bacterial infections, their sera containing these nonspecific antibodies might have killed the bacterial cells while the complement had been destroyed by heat inactivation.
Under physiological conditions, complement promotes the clearance of immune complexes, an important way of eliminating antibody-coated bacteria. If, however, immune complexes cannot be eliminated, complement becomes chronically activated leading to increased consumption of the components. This might be the reason for lower levels of complement C3 that could affect the formation of membrane-attack complex and lower bactericidal activity. The depressed bactericidal activity might be due to lower activation of the classical pathway complement as evidenced by significantly elevated levels of C4 arising from either low consumption or because of defects in other complement components. Hyperglycemia associated with DM might have affected complement functions by non-enzymatic glycosylation.

In the present study, a significantly decreased level of serum total protein was found in the DM patients. Previous workers reported decreases in albumin and gamma globulin values and an increase in α2-globulin that were roughly proportional to the extent of complications in the DM patients. We found a significant linear correlation between the levels of total protein and BACIS in the control subjects but such relationship was not found in the DM patients, although the levels of immunoglobulins and BACIS were high. In conclusion, the altered levels of serum complement and immunoglobulins might be responsible for depressed immune response in patients with type 2 diabetes.

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