Identification of a novel 43-kDa protein in serum-circulating immune complex of type 2 diabetic patients

Shivkumar D. Chauhan*^, Nirmalendu M. Nath*, Vinay K. Tule^, Anjali V. Tule**

^Eugeniks, Genetic Research Institute, Lokmat Bhavan, Ramdaspeth, *Department of Biochemistry, RTM Nagpur University, LIT Premises, **Department of Anatomy, Government Medical College, Nagpur, Maharashatra, India

BACKGROUND: High levels of circulating immune complexes (CICs) in the serum have been reported in both, type 1 and type 2 diabetes. **METHODOLOGY:** Fasting blood samples of type 1 and type 2 diabetes mellitus (DM) subjects and normal controls of both sexes were collected (age 30-45 years) for the study. CICs were isolated by employing polyethylene glycol precipitation technique and subsequent separation of its proteins by SDS-PAGE. A neutrophil model was employed for evaluating the *in-vitro* effect of CIC protein (CICP). Cell proliferative index, oxidant/antioxidant status and ion transporting ability were chosen for the study. **RESULTS:** A significant elevated level of CIC was shown in both types of diabetes. Postexamination of CICs on SDS-PAGE demonstrated that a new protein moiety was expressed only in type 2 DM with a molecular weight of 43 kDa. *In-vitro* action of 43-kDa protein on polymorphonuclear neutrophils (PMN) showed significantly decreased cell proliferative activity, increased free radical levels and decreased antioxidant enzyme activity and decreased ionic transporters. The 43-kDa protein also exhibited protease activity when compared with trypsin. **CONCLUSIONS:** This study concluded that a 43-kDa protein present in CIC in type 2 DM demonstrated a protease activity as well as antimitogenecity. Furthermore, this protein may act as a diabetogenic factor, since it elicited a gross elevation in the level of oxidant status and depression in ionic transport in PMN when preincubated with it.

Correspondence to **Dr. Shivkumar Chauhan,** Eugeniks, Genetic Research Institute, 106/A-wing, First Floor, Lokmat Bhavan, Ramdaspeth, Nagpur-440 012, Maharashatra, India.
E-mail: shivkumar_chauhan@rediffmail.com

KEY WORDS: Circulating immune complex, diabetogenic factor, ionic transporters, oxidant/antioxidant status, polymorphonuclear neutrophils, type 2 diabetes mellitus

Introduction

Evidence of various autoimmune diseases with immune complex connection has been well documented.[1-5] In diabetes mellitus (DM), IgG antibody is known to bind with soluble antigens to form an immune complex.[6-10] Advanced glycation end-products (AGEs),[11] insulin-like growth factor binding proteins (IGF-BPs),[12] and albumin group protein bound to IgG Fc receptor[13] are depicted to form immune complexes in type 2 DM. However, the inability to obtain suitable immunological marker for ascertaining the disease state has crippled the autoimmune complex hypothesis of this disease. The failure may be due to the nonspecific interaction of autoantibodies with the autoantigens.[14] It may be recalled that positive islet cell antibodies (ICAs),[15-16] glutamic acid decarboxylase antibodies (GADs),[17-18] and insulin autoantibodies (IAAs)[19-20] do not contribute toward the diagnosis of type 2 DM.

Against the above background, it is warranted that a detailed probe of the immune complexes be made in diabetes. In the present study, an attempt has been made to examine the circulating immune complex (CIC) and its possible association with an unknown protein moiety by employing polyethylene glycol precipitation technique and subsequent separation by SDS-PAGE. We present a low molecular weight immune-complex associated protein along with its antigenic credibility. A neutrophil model was employed for evaluating the

in-vitro effect of CIC protein (CICP). Cell proliferative index, oxidant / antioxidant status and ion transporting ability were chosen for the study.

Methodology

Collection of sample

Fasting diabetic human sera from both sexes were collected (age 30-45 years) for the study. Sera of healthy, age-matched individuals from both sexes were collected as control.

Detection and estimation of CIC

Circulating immune complex was isolated by Polyethylene Glycol (PEG) precipitation method as suggested by Creighton *et al*,[21] with slight modification. To eliminate the extreme deposition of proteins from precipitation, the concentration of PEG was reduced and standardized. Normal and diabetic (types 1 and 2) human sera were diluted with 0.1 M borate buffer (pH 8.4) in the ratio 1:3. Diluted serum (0.22 ml) was mixed with 2 ml of PEG-6000 in 0.1 M borate buffer. The diluted serum (0.22 ml) was mixed with 2 ml of 0.1 M borate buffer as control. Both were incubated at 4°C overnight. The absorbance was measured in a spectrophotometer at 450 nm to assess the turbidity due to precipitation of CICs in PEG. PEG index was calculated as absorbance in PEG upon absorbance in borate buffer multiplied by 1000.

Isolation of CICP

After overnight incubation, CICs were centrifuged at 3500 rpm for 20 min and the complexes were collected as pellet. Pellet was reconstituted in 0.1 M borate buffer and dialyzed against 0.1 M borate buffer at 4°C for 12 h. The dialyzate (100 µg) was then run in a 10% SDS-PAGE and the gel was stained with 0.02% silver nitrate. A new protein from CIC of type 2 DM was isolated with the help of electro-elution technique.

Establishment of primary cell culture of neutrophils

Normal human polymorphonuclear neutrophils (PMN) were isolated by the method of Boyum.[22] Single PMN cell was picked up with the help of micro-needle and cultured in 96-well plate containing RPMI-1640 medium supplemented with 13% fetal calf serum, penicillin (10,000 IU / ml), streptomycin (100 µg / ml), gentamycin (2 µl / ml) and nystatin (40 µg / ml). For the development of single cell colony, cell was incubated at 37°C supplemented with 5% CO_2 for 4 weeks.

Cell culture study

Cultured cells were separated with the help of 0.01% collagenase P and approximately 4.2-4.3 × 10^2 numbers of cells were taken up for study. For study, cells were pretreated with 30 µM of CICP obtained from CIC of type 2 DM and incubated for 60 min at 37°C. After incubation, cells were washed twice with 0.1 M PBS and different biochemical parameters were assayed in order to ascertain antigenecity of 43-kDa CICP of type 2 DM. Known type 2 DM human PMN were isolated and served as a positive control.

Evaluation of protease activity of 43-kDa protein

Casein (0.5%) was used as a substrate for determining the activity of protease. Thirty microgram of 43-kDa CICP obtained from CIC of type 2 DM was incubated with 2 ml of 0.5% casein solution at 37°C for 60 min. The activity was stopped by adding 2 ml 30% TCA solution and centrifuged at 10,000 rpm for 20 min. The concentration of small peptides from supernatant was estimated by the method of Lowry *et al*.[23] Trypsin is used as positive control (30 µg). Activity is expressed in units and specific activity is expressed as units / mg of protein.

The study of the effect of 43-kDa CICP on the PMN cell growth

In the cell culture, 30 µg of 43-kDa CICP was incubated with 4.3 × 10^2 number of PMN cells at 37°C for 60 min. After incubation, cells were washed with 0.1 M PBS and incubated further up to 72 h for cell growth study and the cell numbers counted after every 6-h intervals.

In-vitro effect of 43-kDa CICP on biochemical parameters

The activities of Nitric oxide radical, superoxide radical, Cu-Zn superoxide dismutase (Cu-Zn SOD), Na^+/K^+-ATPase and Ca^{++}/Mg^{++}-ATPase were estimated by the method of Green *et al.*,[24] Johnston *et al.*,[25] Misra and Fridovich,[26] Post and Sen[27] and Lynch and Cheung,[28] respectively and inorganic phosphate was estimated by the method of Fiske and Subbarow.[29]

Statistical analysis

The data were analyzed using GraphPad Prism software (version 4). The results are expressed as mean ± SD and Student's '*t*' test was used to assess the statistical significance. *P*-values < 0.05 were considered as significant.

Results

An elevated level (~threefold) of CIC in the sera obtained from both types of diabetics when compared to normal control ($P < 0.001$) is depicted in [Figure 1]. Though the CIC level was significantly higher in type 1 DM

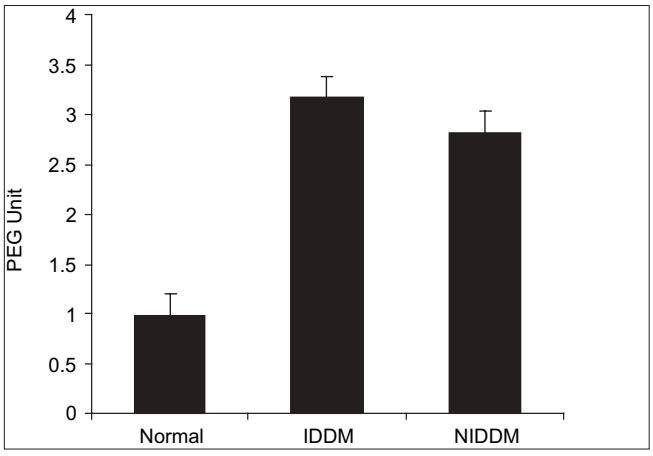

Figure 1: Level of circulating immune complexes in normal and both types of diabetes ($P < 0.001$ when compared with normal)

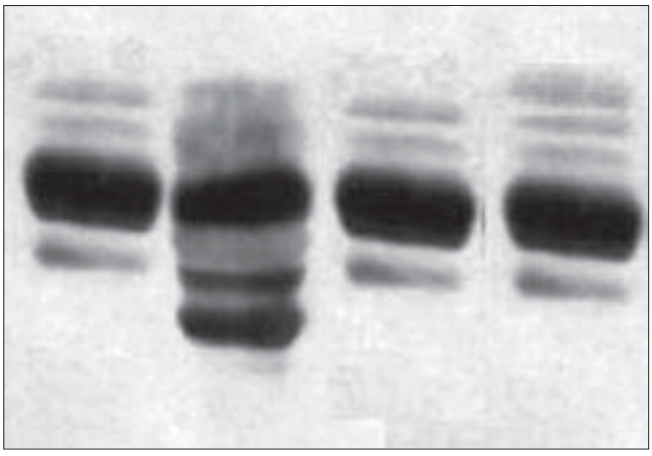

Figure 2: Electrophoretic pattern of CICs on 10% SDS-PAGE. Lane 1: Normal subject, Lane 2: Type 2 DM, Lane 3: Type 1 DM and Lane 4: Bronchial asthma

as compared to type 2 DM, the level of significance ($P < 0.02$) was low. Post-examination of CICs on SDS-PAGE along with 2-mercaptoethanol demonstrated that a new protein moiety was expressed only in type 2 DM with a molecular weight of 43 kDa, but neither in normal or type 1 DM nor in positive autoimmune diseases such as bronchial asthma and rheumatoid arthritis. However, in case of positive controls, separate expression of protein band was demonstrated in bronchial asthma with molecular weight of 60 kDa [Figure 2]. All these newly expressed entities are CICPs. A confirmation of 43-kDa CICP was obtained by isolating the protein through electro-elution technique and running with type 2 DM control [Figure 3]. It is interesting to note that in the absence of 2-mercaptoethanol during SDS-PAGE, the 43-kDa protein moiety of type 2 DM was not separately expressed [Figure 4].

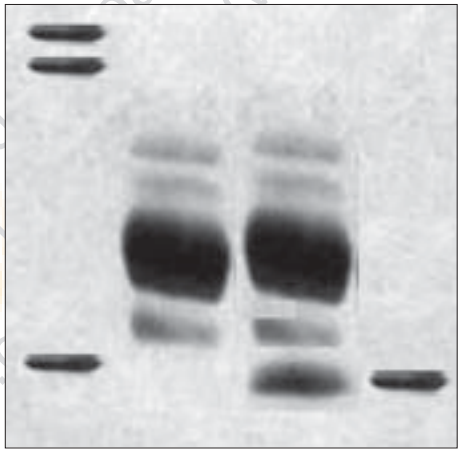

Figure 3: Isolation of 43 kD CICP with electro-elution technique and running on 10% SDS-PAGE. Lane 1: Molecular weight marker, Lane 2: Normal CIC, Lane 3: Type 2 DM CIC and Lane 4: Isolated 43-kD CICP from type 2 DM

Figure 5 represents the partial N-terminal sequential analysis of the 43-kDa protein. It was found that aspartic acid was the N-terminal amino acid along with five neutral and polar amino acids at position 3 to 7. Additionally, it was found to be rich in hydrophobic amino acids, which are present at position 10 to 15 and at position 17. Further, four basic amino acids in positions 8, 9, 16 and 18 were also present. This protein possesses a protease activity quite comparable to that of trypsin [Figure 6].

In-vitro action of 43-kDa protein on cell growth rate on PMN has been demonstrated in Figure 7. A significant decrease in cell proliferative activity was noticed ($P < 0.001$). The oxidant/antioxidant activity of PMN post-treatment with CICP showed an elevated level of free radicals along with a concurrent decrease in the activity of Cu-Zn superoxide dismutase (Cu-Zn SOD)

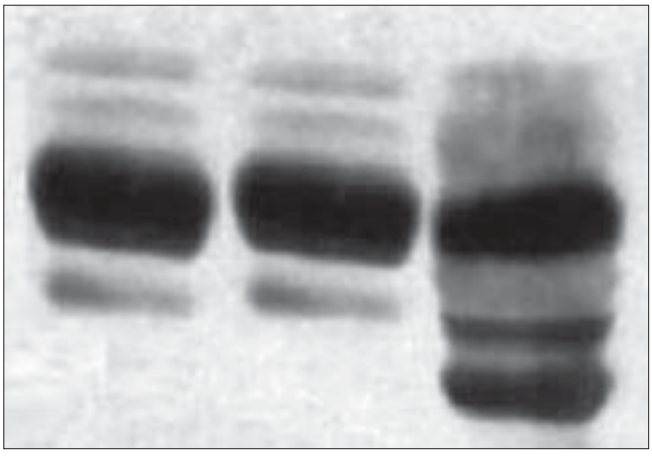

Figure 4: Action of β-mercaptoethanol (β-ME) on CICs. Lane 1: Normal CIC, Lane 2: Without β-ME treated CIC of type 2 DM and Lane 3: β-ME treated CIC of type 2 DM

^{N} **D-D-Q-S-T-G-Y-R-R-M-M-V-P-W-F-K-I-L-**

Figure 5: Partial N-terminal sequential analysis of 43-kDa CICP obtained from CIC of type 2 DM

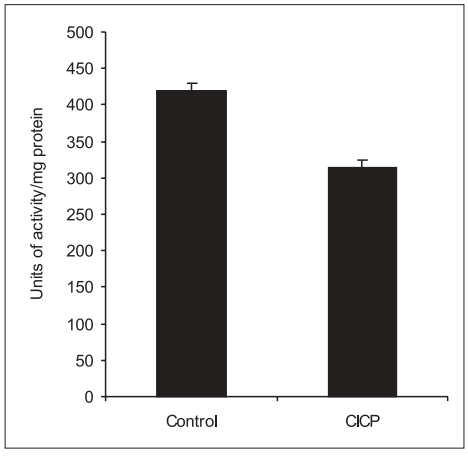

Figure 6: Protease activity of 43-kDa CICP of type 2 DM. Trypsin is used as control. Result expressed as mean ± S.D. of seven experiments. $P < 0.001$ when compared with control

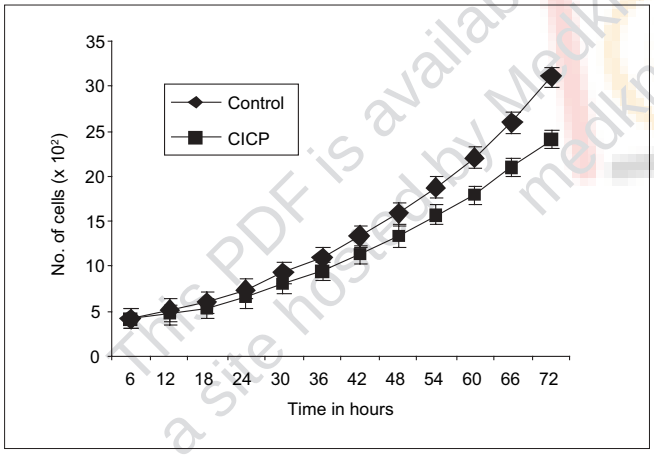

Figure 7: Action of 43-kDa CICP obtained from CIC of type 2 DM on normal PMN growth rate. Result expressed as mean ± S.D. of four experiments

[Table 1]. Significant decrease in the Na^+, K^+ and Ca^{++} pump activity has been demonstrated in Table 1. It may be noted that the degree of aberrations obtained in the above parameters after treatment with CICP was found to be quite parallel to that of diabetic PMN.

Discussion

The incapability of the adaptive immune system in responding to autoantigens generated in various autoimmune diseases results in the formation of CICs

Table 1: *In-vitro* effect of 43-kDa CICP on the level of free radicals, superoxide dismutase and ionic transporter of normal PMN

Parameters	Normal control	Diabetes control	Normal treated with CICP
Nitric oxide radicals (nM/mg protein)	3.57 ± 0.31	6.30 ± 0.40*	6.09 ± 0.37*
Superoxide radicals (nM cytochrome C reduced/mg protein)	1.8 ± 0.13	3.4 ± 0.25*	3.1 ± 0.22*
Superoxide dismutase (Units of activity/mg protein)	6.07 ± 0.3	3.8 ± 0.21*	3.71 ± 0.31*
Na^+/K^+-ATPase (μM Pi librated/ min/mg protein)	9.20 ± 0.37	4.50 ± 0.29*	4.72 ± 0.25*
Ca^{++}/Mg^{++}-ATPase (μM Pi librated/ min/mg protein)	21.10 ± 0.77	10.60 ± 0.57*	11.46 ± 0.63*

Result expressed as mean ± SD of seven experiments, *$P < 0.001$ when compared with normal control

with the said autoantigens.[30-31] Hence, it becomes difficult to obtain an immunological marker for the detection of autoantigens. The failure in the attempt of obtaining a suitable immunological marker in diabetes can thus be explained. Although various workers observed the possible presence of such autoantigens as AGEs,[11] IGF-BPs,[12] albumin group protein bound to IgG Fc receptor,[13] circulating proinsulin[32] and islet amyloid[33] in type 2 DM, none of them attempted to evaluate the CIC for the presence of the unidentified autoantigens. In the present study, we have not only shown the elevated levels of CICs in both types of diabetes, but also demonstrated the presence of a CICP in type 2 DM with a molecular weight of 43 kDa by employing polyethylene glycol gradient isolation of the complex and subsequent SDS-PAGE separation in the presence of 2-mercaptoethanol. The elevated levels of CICs in either types of diabetes are in consonance with the findings of earlier workers.[8-10] One of the interesting outcomes from our study is the unique presence of a 43-kDa CICP in type 2 DM, which was neither witnessed in type 1 DM nor in the CICs of positive control, namely bronchial asthma. No possible explanation for the absence of CICP in type 1 DM can be offered at the present juncture. However, CICs from positive control[34-35] did contain 2-mercaptoethanol sensitive unidentical CICP with varied molecular weight. It may also be seen that the CICP obtained from diabetic CIC remains unassociated if 2-mercaptoethanol treatment was withdrawn. This shows that the associated protein is linked with CIC by a sulfur-sulfur conjugation. This

finding needs further exploration.

The N-terminal sequence analysis of the novel protein obtained from CIC of type 2 DM demonstrated the presence of aspartic acid at the N-terminal end followed by serine, threonine and glycine. A possible bend in the molecule is envisaged.[36-37] The presence of hydrophobic amino acid in a cluster and the subsequent basic amino acids in the chain indicates a chemical flexibility of the molecule for interaction.[38-40] It may be noted that this new protein had a protease activity, a result indicative of its antigenecity. It may be recalled that several workers have reported protease activity in various autoantigens.[41-42] It could be presumptious to term it as a serine-threonine protease at the moment till its complete amino acid sequence is performed.

Post-treatment of CICP with normal PMN *in-vitro* depicted the retardation in cell growth rate indicating its antiproliferative activity. This finding is interesting especially against the backdrop of unavailable information regarding the antimitogenic activity of other known autoantigens. The diabetic PMN were found to show enhanced oxidant status with a significant elevation in free radicals and depressed activity of the antioxidant enzyme. Interestingly, when CICP was pre-incubated with normal PMN for 60 min, the oxidant/antioxidant status was found to be aberrated in the similar fashion as was witnessed in the diabetic control. Furthermore, similar decrease in the activities of Na^+/K^+-ATPase and Ca^{++}/Mg^{++}-ATPase were seen in both diabetic PMN as well as CICP-treated normal PMN. These aberrations indicate that CICP probably behaves as a diabetogenic factor.

Conclusions

It can be concluded from our findings that the sera obtained from both types of diabetes showed enhanced levels of CICs. A novel 43-kDa protein from the CIC of type 2 DM was detected with its N-terminal sequence. This unique protein had possible disulphide ligation with its CIC. This protein demonstrated a protease activity as well as antimitogenecity. Furthermore, the protein acted as a diabetogenic factor, since it elicited a gross elevation in the level of oxidant status and depression in ionic transport in PMN when preincubated with it.

Contributors

All authors assisted with statistical analysis, interpretation of data and writing of the manuscript.

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Source of Support: Nil, **Conflict of Interest:** None declared.

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