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Altered expression and glycosylation of plasma proteins in rheumatoid arthritis

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Abstract Altered glycosylation of plasma proteins has been directly implicated in the pathogenesis of rheumatoid arthritis (RA). The present study investigated the changes in the *Concanavalin*-A (Con-A)-bound plasma proteins in the RA patients in comparison to that of the healthy controls. Two proteins (MW ∼32 kDa and ∼62 kDa) showed an alteration in expression while an altered monosaccharide profile (high mannose) was observed in the ∼62 kDa protein in the samples collected from RA patients. The 2-dimensional polyacrylamide gel electrophoresis analysis of the Con-A-bound plasma samples showed a large number of protein spots, a few of which were differentially expressed in the RA patients. Some unidentified proteins were detected in the RA patients which were absent in the control samples. The present study, therefore, enunciates the role of carbohydrates as well as that of the acute phase response in the disease pathogenesis.

Keywords Rheumatoid arthritis · *Concanavalin A* · Altered glycosylation · Plasma proteins · 2-dimensional gel electrophoresis

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease with immune dysfunction affecting 2% of the world population. Although the precise etiology of RA is unknown, genetic and environmental factors seem to be involved in its pathogenesis [1,2,3]. It is characterized by chronic inflammatory symptoms leading to damage of synovial tissue and formation of rheumatoid nodules and rheumatoid pannus, capable of eroding adjacent cartilage and bone causing subsequent joint destruction [2].

Human plasma is rich in glycoproteins, many of which exist in different glycoforms. Aberrant glycosylation of cell surface glycoconjugates and serum glycoproteins are known to be involved in a variety of biological phenomena such as inflammation, cell differentiation, infection, tumor progression, and metastasis. Changes in protein glycosylation are early indicators of cellular changes in many such diseases, providing useful diagnostic markers and insights into disease progression and pathogenesis [4].

Alteration in the glycosylation of IgG (agalactosylated IgG/IgG_0) has been directly implicated in the pathogenesis of RA [5]. The IgG_0 levels correlate with the disease severity in patients with rheumatoid arthritis. Rheumatoid factors, the characteristic autoantibodies which bind to the Fc region of agalactosylated IgG molecule, are increased in the sera of patients with RA [6]. Another plasma protein alpha-1-acid glycoprotein (AGP produced by the liver) is known to be present in different glycoforms, and alteration in its glycosylation was reported in several pathological conditions including rheumatoid arthritis [7].

In rheumatoid arthritis, cytokines produced at inflammatory lesions are believed to travel via the circulation to the liver where they induce the production of acute phase proteins by hepatocytes [8]. Some proteins are over-expressed whereas the levels of some others are decreased, termed as positive and negative acute phase proteins respectively [9]. Acute phase proteins are not merely associated with inflammation; rather, they are contributing to the disease

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pathogenesis as well; for example, *C*-reactive protein has been implicated in the atherosclerotic disease [10]. Particularly in the area of joint inflammation acute phase proteins related to complement cascade have been found in excess. One such protein, namely, mannose-binding lectin, due to its strong affinity for the exposed GlcNAc of IgG_0 (agalactosyl IgG), has been found to stimulate the complement pathway following its binding with IgG_0. Such complexes have been recovered from the synovial fluid of RA patients [11]. Hence, it appears that differential expression pattern of proteins, as acute phase response, may be significant in the pathogenesis of RA.

With this perspective, here we have investigated differential expression and/or altered glycosylation of proteins in the Con-A-bound plasma of RA patients in comparison with that of normal individuals.

Materials and methods

Blood samples from 25 patients with rheumatoid arthritis were collected at the Department of Rheumatology, Army Hospital, Research and Referral, New Delhi, India. All patients fulfilled the American College of Rheumatology classification criteria for the disease. Blood samples from 30 healthy individuals served as controls. Age in the control group was 40 ± 5 years and for the RA patients was 45 ± 6 years. The cohort was sex, age, and ethnicity matched. Informed consent was obtained from each patient and normal individual. The Institute and Hospital ethics committees approved the study.

Plasma was stored at -70°C.

Albumin removal using Con-A sepharose affinity column

The total amount of protein in plasma samples of both the RA patients and the healthy controls was calculated by the Lowry method [12]. Affinity columns were packed with 1 ml of *Concanavalin*-A (Con-A) sepharose 4B (Sigma) and washed repeatedly with Tris-buffer saline (TBS, pH 7.2, containing 10 mM Tris, 0.9% NaCl and 1 mM each of $MnCl_2$, $MgCl_2$ and $CaCl_2$). Con-A lectin is used as it non-specifically binds to glucose and mannose [13]. A small fraction of glycoproteins would be eliminated with the unbound fraction. Plasma samples containing equal amounts of protein from both the RA patient and the healthy control were loaded onto the Con-A columns separately and left for 30 min for proper binding of the glycoproteins with the lectin. The unbound fraction including the albumin was washed off with TBS until the OD of the elute at 280 nm became zero. The Con-A-bound glycoproteins were eluted with 200 mM α-methyl mannopyranoside (Sigma). This elute was dialyzed against TBS (10 times diluted) and then against triple distilled water to remove traces of salts. The dialyzed samples were passed through Centricon-3 (Amicon INC. USA) to remove all traces of salts. The samples were concentrated in vacuum evaporator, and total protein concentration was estimated by Lowry method. Each sample was processed separately.

SDS-polyacrylamide gel electrophoresis

Equal amounts of protein from the desalted and dealbuminated samples of both the controls and RA patients together with protein molecular weight markers were loaded into a homogenous 12% SDS-PAGE gel. Electrophoresis was carried out at a constant 10 mA in the buffer containing 250 mM glycine, 25 mMTris and 0.1% SDS (Sigma). The gels were stained by Coomassie Brilliant Blue *R*-250 (Sigma) and scanned for differential expression of proteins in controls and RA patients as 16 bit grey scale Tif-images with a AlphaDigidoc 1201 scanner (Alpha Innotech Corp.). All the samples were analyzed, but results of some samples are presented in the figures, as the results are very similar for all the samples.

Lectin blotting

A 12% acrylamide gel was run using equal amounts of bound proteins both from the controls and the RA patients as described above. The proteins from the gel were transferred on to a nitrocellulose membrane using protein transfer apparatus (BioRad). The membrane was stained with Ponceau-S (Sigma) stain to confirm the complete transfer of proteins. The stain was washed off with distilled water. The unoccupied sites of the membrane were blocked by incubating the membrane with 1% BSA (Bovine serum albumin, Sigma chemicals, USA) in phosphate-buffered saline (PBS, pH 7) containing 0.05% Tween-20 (PBST) for 2 h at room temperature. The membrane was washed twice with PBST and incubated with biotinylated *Pisum sativum* (5 μg/ml) lectin (PSL, isolated by affinity chromatography and biotinylated using biotin labeling kit from Sigma chemicals, USA) in PBST containing 1% BSA (PBST-1% BSA) for 2 h at room temperature. The membrane was washed with PBST and left for 1 h in streptavidin peroxidase (Sigma) diluted 1:1000 in PBST-1% BSA. After washing the membrane with PBST, substrate (25 ml PBS containing 15 mg of 4-chloronapthol dissolved in 5 ml methanol with 0.1% hydrogen peroxide) was added to develop the blot.

The lectin blot was also carried out as described above wherein instead of PSL, 2.5 μg/ml biotinylated *Bandeiraea simplicifolia* lectin (BSL II, Vector Lab, USA) was used.

2D gel electrophoresis of Con-A-bound plasma

Sample preparation and isoelectric focusing

The lyophilized proteins (150 μg) purified by the Con-A affinity columns were dissolved in 120 μl of rehydration buffer containing 8 M Urea, 2% Triton X-100, 65 mM dithiothreitol (DTT, Sigma), and 0.2% ampholytes separately (Bio-Rad). The samples were then loaded into the rehydration tray, and the 7 cm IPG strips (pH 4-7) (BioRad) were rehydrated in these samples overnight. The strips were transferred to the focusing tray, and the IEF (Bio-Rad 3000 apparatus) was carried out using a multistep protocol (25 min at 250 V, 2 h 50 min at 4000 V and finally to reach at 10000 Vh). The strips were then either stored at -70°C or equilibrated for 25 min in 8 M Urea, 1.5 mM Tris-HCl (pH8.8), 2%(w/v) sodium dodecyl sulphate (SDS), 2% (w/v) DTT, and 20% (w/v) glycerol followed by 25 min in the same buffer containing 2% (w/v) iodoacetamide instead of DTT.

Second dimension electrophoresis

The strips were transferred to 1 mm thick 12% polyacrylamide gel and held in position with molten 0.4% agarose containing bromophenol blue dye. The electrophoresis was carried out at a constant 10 mA in the buffer consisting of 250 mM glycine, 25 mMTris, and 0.1% SDS.

Protein visualization and image analysis

Gels were stained using ammoniacal silver nitrate as described by Smith *et al*. [14]. The stained gels were scanned as 16 bit grey scale Tif-images with an Alpha Digidoc 1201 scanner.

Database search to identify proteins

2D gels of the Con-A-bound samples were compared using Swiss 2D-PAGE database (http://cn.expasy.org/cgi-bin/map2/noid_big?PLASMA_HUMAN) to identify reference proteins.

High performance anion exchange chromatography

Sample preparation

SDS-PAGE was carried out using equal amounts of the Con-A-bound protein samples from the controls and the RA patients as described above. The gels were stained with Coomassie Brilliant Blue R-250 and scanned as 16 bit grey scale Tif-images with a AlphaDigidoc 1201 scanner. The proteins which were differentially expressed in the controls and the RA patients were eluted from the gel using a multistep protocol without reduction and alkylation steps [15]. The eluted protein was digested with peptide N-glycosidase (PNGase F) as described earlier [16]. The released oligosaccharides were passed through AG50 W resin and treated with 4 M HCl at 80°C for 6 h [17], and acid was removed by evaporation. Hydrolysate was dissolved in 200 μl of deionized water, and analysis of 20 μl sample was performed in HPAE-PAD (Dionex DX500 BioLC).

HPAE-PAD analysis

The analysis of monosaccharides was carried out using an isocratic 18 mM NaOH eluant followed by a gradient of sodium acetate (1 M) and NaOH (100 mM). A 15 min column wash with 200 mM NaOH followed by a 15 min equilibration with the starting eluant was required for reproducible retention times. The flow rate was maintained at 1 ml/min at ambient temperature. The detection of the separated monosaccharides was by PAD using a gold electrode. Triple pulsed amperometry was used and the following pulse potential duration was given: E1 $= +0.05$ V for 0.4 s, E2 $= +0.065$ V for 0.2 s, and E3 $= -0.15$ V for 0.4 sec. Integration was done from 200 to 400 m s. The response time of the PAD was set to 5 s. The standard sugar solutions were prepared in deionized water injected at a 50 μM. The standard mixture of sugars was run before and after analysis of each sample. The identification of sugar peaks was based on comparison of standards using a detector sensitivity of 50 nC full scale.

Results

In the present study, SDS-PAGE of Con-A-bound plasma proteins from 30 controls and 25 RA patients showed a number of protein bands, out of those, two protein bands appear to stain differentially in the patients and controls. These are evident from the results shown with 4 representative samples from each group (Fig. 1). The extrapolated molecular weight of the two proteins against the protein molecular weight markers gives an approximate molecular weight of 62 kDa and 32 kDa. The lower molecular weight protein (32 kDa) appears to be over-expressed in the RA patients while the 62 kDa protein showed a decreased expression in the RA patients (Fig. 1) as revealed by commassie blue staining. The PAS (Schiff's reagent) staining of the SDS-PAGE gel confirms the glycoprotein nature of the proteins (data not shown) with similar differential staining pattern.

Lectin blot using biotinylated PSL also shows a similar decrease in the lectin binding to the 62 kDa protein in RA patients (Fig. 2), while the 32 kDa band was not detectable by the same lectin blot.

The BSL blot shows an overexpression of a protein with the molecular weight of 50 kDa in the RA patients as

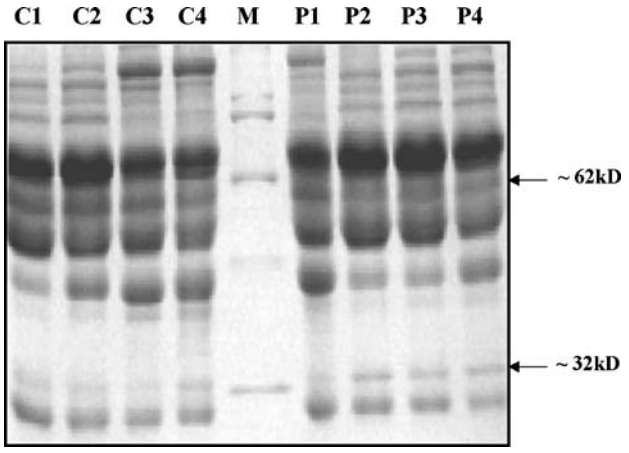

Fig. 1 SDS-PAGE of the Con-A bound plasma samples of controls and RA patients. Lane C1-C4: control samples. Lane M: molecular weight markers. Lane P1-P4: RA patient samples

compared to the controls (Fig. 3). This protein appeared to be heavy chain of immunoglobulin G (IgG_0) which is frequently reported to be increased in rheumatoid arthritis [5,6]. This was further confirmed by SDS-PAGE wherein IgG was loaded in one of the wells along with the Con-A-bound samples and a BSL blot was performed (data not shown).

The 2D gel electrophoresis of Con-A-bound plasma and the synovial fluid samples were visualized by silver staining. A number of protein spots were obtained but the albumin fraction was absent from the Con-A-bound samples (Fig. 4). The database search was carried out to identify proteins. Apolipoprotein A1 was considered as the reference protein for the comparison of different spots in the controls and the RA patients. Some proteins were observed in the RA

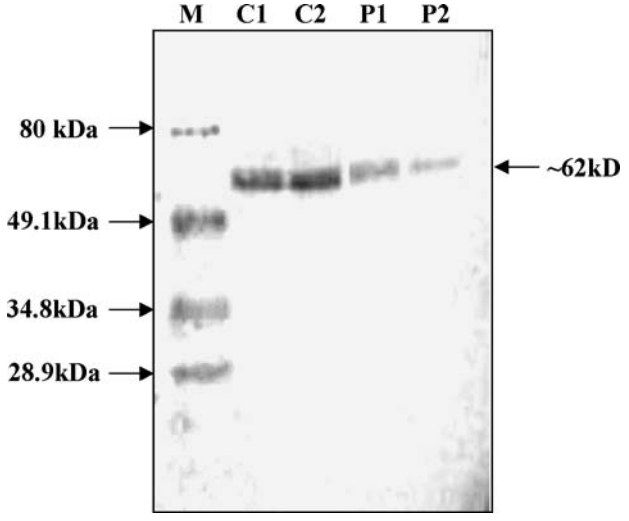

Fig. 2 PSL (*Pisum sativum* lectin) lectin blot of Con-A bound control and RA patient plasma samples. Lane M: prestained molecular weight markers. Lane C1, C2: control samples. Lane P1, P2: RA patient samples. C1, C2 and P1, P2 are respectively representative of 20 and 16 samples

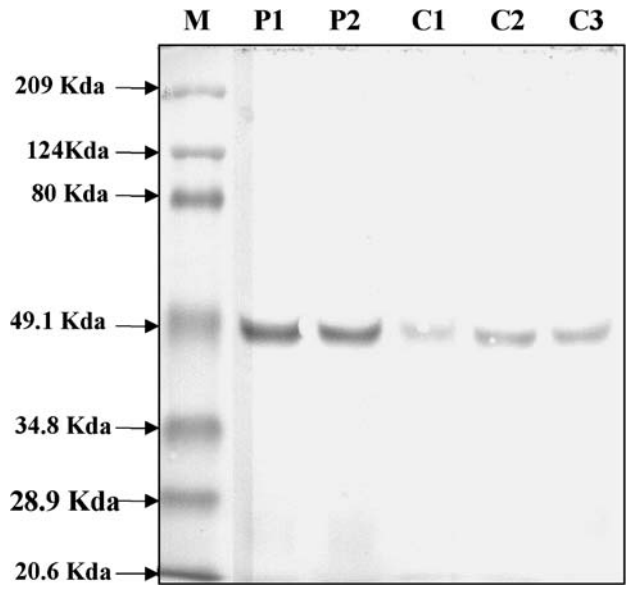

Fig. 3 BSL-II (*Bandeiraea simplicifolia* lectin-II) blot of Con-A bound control and RA patient samples. Lane M: prestained molecular weight markers. Lane P1-P2: RA patient samples. Lane C1-C3: control samples. P1, P2 and C1-C3 are respectively representative of 16 and 20 samples

patients but were absent in the controls. These low molecular weight proteins could not be identified using the database search. The level of haptoglobin $\alpha 2$ chain was increased in the RA patients.

The HPAE-PAD analysis of the 32 kDa and the 62 kDa proteins was carried out. The monosaccharide analysis of the 62 kDa protein showed a significant difference between the controls and the RA patients (Fig. 5). The amount of all the monosaccharides was observed to be more in the control samples as compared to the RA patients and therefore qualitative changes were observed. Most of the monosaccharides including *N*-acetyl galactosamine, galactose and other unidentified ones were observed to be proportionately similar in both the controls and the RA patients, but the ratio of glucose/mannose was reversed in the controls and the RA patients. Glucose was observed to be greater in the controls while mannose was increased significantly in the RA patients.

The qualitative analysis of monosaccharides of the 32 kDa protein from the Con-A-bound samples of controls and the RA patients did not show any difference in the proportionate sugar profile.

Discussion

From the pioneering studies of Sanchez *et al.* on human plasma maps [18], several studies have identified changes in serum glycoproteins in patients with hepatocarcinoma [19]

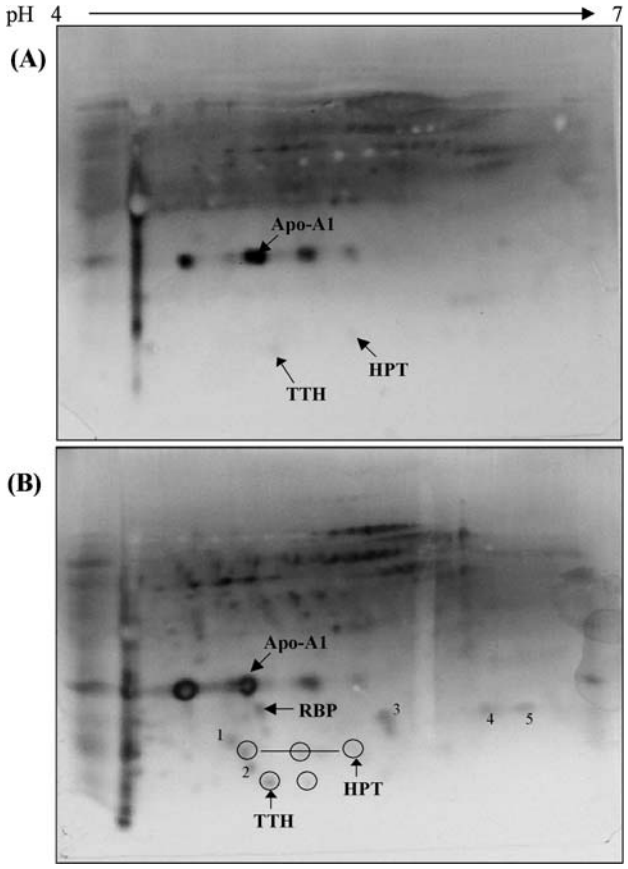

Fig. 4 2-Dimensional gel electrophoresis of Con-A bound plasma samples of control and RA patient. (A) Control sample. (B) RA patient sample. Spot 1,2,3,4,5: Unidentified spots. Apo-A1: Apolipoprotein A-1. RBP: Retinol binding protein. TTH: Transthyretin. HPT: Haptoglobin (similar results were obtained for control and patient samples, 6 of each)

and other liver diseases [20]. In the present study, as the first step in elucidating disease specific protein patterns of body fluids from patients, we started to examine carefully the Con-A-bound plasma from patients suffering from RA, and a comparison with the control group was made. By this approach we intended to search for disease-specific protein differences in the body fluids without introducing a bias potentially resulting from inter-individual differences. Various techniques have been used to identify appearance or disappearance and differential expression of some proteins in the RA patients in comparison to the controls.

The SDS-PAGE showed an overexpression of a ~32 kDa protein in the patients but difference was not visible in the PAS-stained gel. Presence of a negligible amount of monosaccharides in ~32 kDa protein as analysed by HPAE-PAD indicated the possiblity, that glycosylation may not be N-linked in this protein. The higher molecular weight (~62 kDa) protein was decreased in the Con-A-bound plasma samples of the RA patients as confirmed by the SDS-PAGE analysis.

PSL is the legume lectin with sugar specificity for glucose/mannose, but with specific carbohydrate structure. The lectin blot using PSL showed a decreased amount of ~62 kDa protein in the RA patients (Fig. 3). This might be either due to the lesser amount of the protein or its alteration in glycan structure in the RA patients. The altered glycosylation of the ~62 kDa protein was observed by the HPAE-PAD analysis. The overall monosaccharide profile of the protein was similar in both the controls and the RA patients. The qualitative analysis was performed taking the control monosaccharide profile as the reference. Of note was the ratio of mannose/glucose in the samples. The amount of mannose was higher in the RA patients while the controls were observed to have a higher amount of glucose (Fig. 5). The high mannose/glucose ratio in RA patients may suggest the presence of unprocessed oligomannose glycan structures as in cases of congenital disorders of glycosylation. An accumulation of aberrant unprocessed glycan structures, in serum glycoproteins of patients with congenital disorders of glycosylation was reported to be due to defective glycan processing step. In certain case such aberrant glycan accumulation is reported to be pentamannosyl hybrid type glycans due to defective α-mannosidase II/III [21]. The defect appears to be the inability to cleave two terminal mannose residues with α1- and α1-6 linkages to the α1-6-linked antenna of the core structure leading to the formation of pentamannosyl hybrid type glycans with varying length of α1-3-linked antenna.

BSL II, the lectin specific for terminal *N*-acetylglucosamine, showed a distinct increase of IgG molecules in the RA patients. IgG is known to be increasingly agalactosylated (IgG_0) in rheumatoid arthritis, exposing the *N*-acetylglucosamine residue as the terminal sugar [5,6]. The rheumatoid factor, an autoantibody formed against the IgG_0 molecule, increases in rheumatoid arthritis and is therefore used for the diagnosis of the disease [6]. The increase of IgG_0 molecule in the RA patients is therefore confirmed by the BSL-II lectin blot (Fig. 4).

Very few proteins from body fluids were observed in 2-D gels to migrate below pH 4. Above pH 7, gels were dominated by light chain and heavy chain immunoglobulin proteins (data not shown). Hence, for proteome analysis of the Con-A-bound plasma, proteins were separated in to immobiline gels ranging from pH 4 to 7. At first glance, the protein patterns visualized by silver-stained gels from Con-A-bound plasma of the exemplary RA patients resemble the controls closely (Fig. 4). The database search helped to identify some proteins of the 2D gels considering apolipoprotein A1 as the reference protein. The Con-A-bound plasma samples of RA patients showed an increase in the expression of haptoglobin α2 chain. The plasma concentration of acute phase proteins like haptoglobin α2 chain is known to increase after an inflammatory stimulus [9]. Thus, a causal increase of haptoglobin α2 chain is observed in the plasma

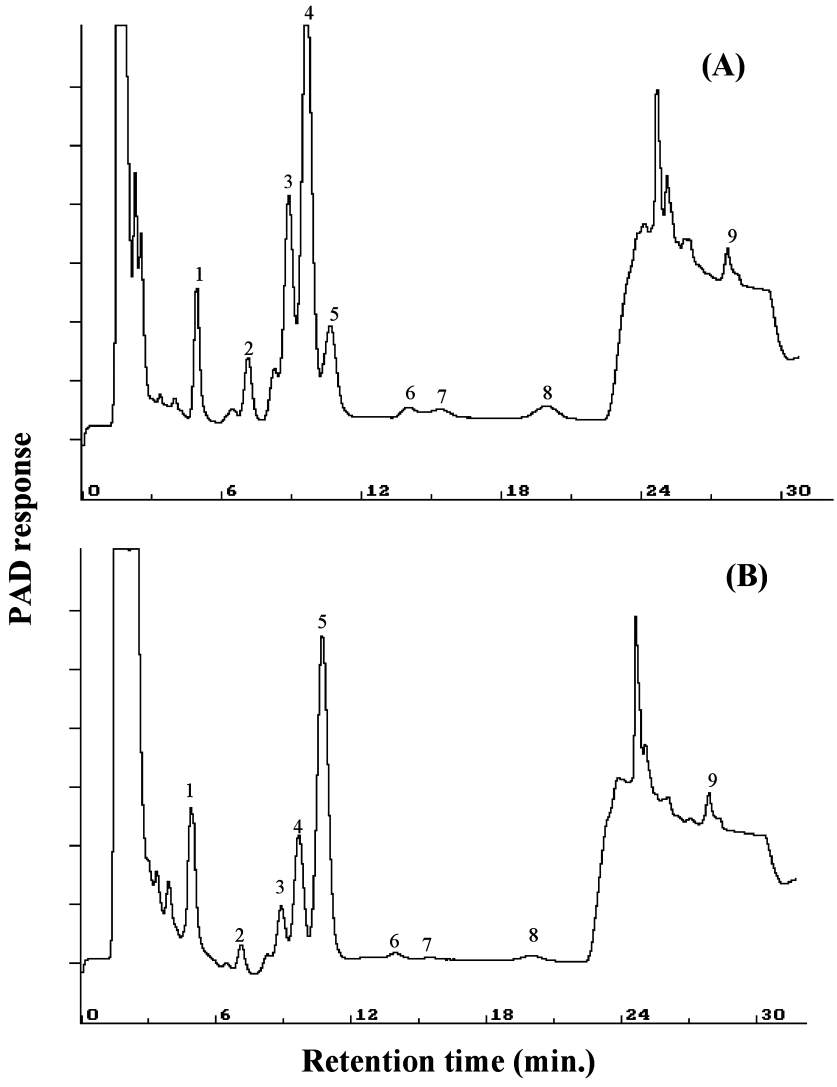

Fig. 5 HPAE-PAD analysis of N-linked monosaccaride from ~62 kDa protein from Con-A bound plasma samples of control and RA patient (the results were similar for control and RA patient samples, 6 each). (A). Control sample. (B) RA patient sample. Peak 1: 6-O-methyl-D-galactopyranoside (Internal standard); 2: Galactosamine. 3: Galactose; 4: Glucose; 5: Mannose; 6,7,8,9: Unidentified

samples of the RA patients in comparison to the healthy controls. Some unidentified low molecular weight proteins were increased in the RA patients but were either absent or were present in undetectable amounts. An altered glycosylation of these proteins leading to affinity with the Con-A matrix might be the cause of their appearance in the RA patients.

Further work to identify the proteins is in progress

Rheumatoid arthritis is causally associated as the disease of carbohydrates. The appearance of glycosaminoglycans in the synovial joints of RA patients [22] and increase in the agalctosylated IgG molecules have been reported to be associated with the pathogenesis of RA. The present study further enunciates the role of carbohydrates in the disease pathogenesis. From the perspective of the rheumatologist, it will be important to identify reliable markers of bone and cartilage destruction as well as chronic inflammation, and this could be a realistic prospect in the near future.

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