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INCREASED BINDING OF CONCANAVALIN A TO α_2-MACROGLOBULIN, IgM AND IgG FROM CYSTIC FIBROSIS PLASMA

Emmanuel Shapira and Roger Menendez

The Hayward Genetics Center
Departments of Pediatrics and Pathology
Tulane University School of Medicine
New Orleans, Louisiana 70112

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SUMMARY

Alpha$_2$-macroglobulin from patients with cystic fibrosis was shown to have significantly increased binding of fluorescein conjugated concanavalin A as compared to that from healthy controls or from patients with chronic lung diseases other than cystic fibrosis ($t=5.2$; $p<0.001$). Obligate heterozygotes for cystic fibrosis revealed values intermediate between the controls and the cystic fibrosis group. This phenomenon was not unique to α_2-macroglobulin but was shown with two other plasma glycoproteins. The binding of concanavalin A to IgG or IgM from cystic fibrosis patients was significantly higher ($p<0.001$) than in the binding in the control groups ($t=7.3$ and $t=7.9$ respectively).

INTRODUCTION

The basic defect in cystic fibrosis (CF), the most common significant autosomal recessive disorder affecting Caucasians, remains as yet unknown (1). Abnormal glycoproteins or abnormalities in some aspects of glycoprotein metabolism were suggested as the possible underlying defect in cystic fibrosis and were recently summarized (1,2). Most studies have been done using crude glycoprotein preparations from tissues or secretions of CF patients (2).

Alpha$_2$-macroglobulin (α_2M) is one of the major plasma glycoproteins, containing 8.5% carbohydrates (3). Altered functional and kinetic properties of α_2M from plasma of CF patients and obligate CF heterozygotes have been described (4-9). In the present study, we measured the binding ratio of the lectin concanavalin A (Con A) to the oligosaccharide moiety of α_2M from healthy controls, patients with chronic lung disease other than CF, patients with CF

Abbreviations used: CF, cystic fibrosis disease; α_2M, α_2-macroglobulin; Con A, concanavalin A; PBS, phosphate buffered saline.

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and obligate heterozygotes for CF. To determine if the difference revealed between CF patients and controls was unique for α_2M or might also be present with other glycoproteins, we compared the binding ratio of Con A with IgG and IgM.

MATERIALS AND METHODS

Concanavalin A (lot 58C-7440), fluorescein isothiocyanate (isomer 1, lot 108C-5059), α-methyl-D-mannoside (grade III), soybean trypsin inhibitor (1-S chromatographically prepared) and Trasylol (Aprotinin) were all from Sigma Chemical Company (St. Louis, MO). Immunobead^R reagent was from Bio-Rad Laboratories (Richmond, CA). The beads specific for human α_2M (lot 16192) had antigen binding capacity of 0.9 µg/mg beads, the beads specific for human IgM (lot 15660) had antigen binding capacity of 1.12 µg/mg beads and the beads specific for human IgG (lot 17965) had antigen binding capacity of 0.38 µg/mg beads. Sephadex G-25 coarse was from Pharmacia Fine Chemicals (Piscataway, NJ). All other reagents were reagent grade.

Blood specimens (2 ml) were obtained, following signed informed consent, from healthy adult controls, obligate CF heterozygotes, CF patients, and an age matched group of patients with chronic lung disease other than CF (patient control). Blood was collected using plastic equipment and contact with glass surface was avoided. The blood specimen was mixed with 0.2 ml of anticoagulant (EDTA 10^{-2} M, soybean trypsin inhibitor 5 mg/ml and trasylol 100 units/ml). The plasma was separated and stored at -20°C until tested.

Conjugation of fluorescein isothiocyanate to concanavalin A: Fluorescein isothiocyanate (20 mg) was dissolved in 5 ml of borate buffer (0.05 M, pH 9.0) and 200 mg of Con A were added and stirred at room temperature. After 15 minutes, 1 ml of phosphate buffer (0.5 M, pH 8.0) was added and the stirring continued for another two hours. The fluorescein conjugated Con A was separated from the free fluorescein by column chromatography (Sephadex G-25C, 2 x 110 cm) using 0.01 M phosphate buffer in 0.14 M sodium chloride, pH 6.5 (PBS, pH 6.5) as an eluent. The first eluted peak was concentrated by negative pressure ultrafiltration and dialyzed, against 4 liters of the same buffer, at 4°C for 48 hours. The denatured protein fraction was separated by centrifugation (40,000 rpm for 60 minutes)

The molar binding ratio of fluorescein to Con A was very similar (1.4 to 1.6) in four preparations used in the study. This binding ratio was determined both by optical absorbency (280 nm to 489 nm ratios) and from the protein concentration (Lowry method) to the fluorescent measurement (excitation at 485 nm and emission at 525 nm).

Pre-treatment of the immunobeads with concanavalin A: Each of the various lyophilized immunobead preparations (50 mg) was mixed with 20 ml of 0.01 M phosphate buffer, pH 6.5, containing 100 mg of Con A and stirred gently for one hour at room temperature. The beads were separated by centrifugation and washed three times with 30 ml of phosphate buffer 0.01 M, pH 7.5, containing 0.14 M sodium chloride (PBS, pH 7.5). Following this washing, the beads were dispersed in 24 ml of PBS, pH 6.5 (2 mg/ml) and had very low binding to fluorescein conjugated Con A.

Quantitative determination of Con A to α_2M binding ratio: The plasma specimens were thawed once and diluted 1:40 with PBS, pH 6.5. To 100 µl of the diluted plasma, 150 µl of the fluorescein conjugated Con A (10 mg/ml) were added and incubated for 30 minutes at 37°C. In such Con A excess, no precipitable complexes were formed. Following the incubation, 200 µl of the immunobead suspension (Con A pretreated) were added, mixed and incubated for another 30 minutes at 37°C. To this incubation mixture, 4 ml of PBS, pH 7.5 were added, mixed, and the beads separated by centrifugation. The supernatant was removed from the pellet by aspiration and the pellet washed once with 5 ml of PBS, pH 7.5.

The washed pellet was dispersed in 1 ml of the buffer and the fluorescence measured at excitation wave length of 485 nm and emission wave length of 525 nm. The non-specific binding of fluorescein conjugated Con A to the immunobeads was determined in one set of tubes in which the fluorescein conjugated Con A was incubated with the buffer prior to the addition of the beads and in another set in which it was incubated with 0.2% of human serum albumin in the same buffer. The non-specific background binding was found to be identical for the buffer and for the albumin solution and was less than 10% of the fluorescence measured with the plasma specimens. This non-specific background was subtracted and the specific binding of fluorescein conjugated Con A to the α_2M bound to the beads was calculated using 1 ml of fluorescein conjugated Con A solution (10 µg/ml) as standard. The α_2M concentrations in plasma of controls and CF patients were previously shown (6) to be between 0.9-2.5 mg/ml. Thus the plasma used in the test was calculated so that the final α_2M concentration in the aliquots would be in a 5-20 fold excess of the beads' binding capacity. In some control experiments, identical fluorescence was measured when 50 µl or 200 µl of the diluted plasma were used for the test. This indicates that under these experimental conditions, a constant aliquot of α_2M is bound from the plasma and that the excess of fluorescein conjugated Con A was such that saturation of the α_2M binding was reached.

In order to document the binding specificity of the Con A to the α_2M on the immunobeads, the following experiments were done: After completing the measurement of the Con A binding as described above, the immunobead pellets were separated by centrifugation. Duplicate pellets were dispersed in increasing concentrations of α-methyl-D-mannoside solution in PBS, pH 6.5. Following incubation for 30 minutes at 38°C, the beads were separated by centrifugation and the fluorescence released to the supernatant was measured. Nearly 90% of the fluorescein conjugated Con A was released from the pellet by 0.25 M α-methyl-D-mannoside solution and 97% at 0.5 M concentration. These experiments document the sugar specificity of the Con A binding to the α_2M as indicated by the α-methyl-D-mannoside competition.

The binding of fluorescein conjugated Con A to IgM was determined in a similar set of experiments but using 200 µl aliquots of the plasma dilution. Thus the final IgM concentration range and the binding capacity of the IgM immunobeads were very similar to those of the α_2M experiment. In the experiments where the binding of fluorescein conjugated Con A to IgG was measured, only 50 µl of the diluted plasma were incubated with 75 µl of fluorescein conjugated Con A and 0.4 ml of the anti-IgG immunobead suspension were added. By these modifications, the concentration ratios for IgG were also similar to those of the α_2M and the IgM experiments.

RESULTS

The binding of Con A to α_2M from plasma specimens from normal controls, CF patients, CF heterozygotes, and patients' control are shown in the figure. Although there was different scattering of the values in the control group and in the patients' control group, the differences between these two groups were non-significant ($t=0.435$; $p>0.5$). The Con A bound to α_2M from the CF samples (22.5± 5.7 µg/µg α_2M) was significantly higher than that bound in the control group (12.4±3.4 µg/µg α_2M; $t=5.2$; $p<0.001$) and in the patient control group (13.1± 4.3 µg/µg α_2M; $t=4.5$; $p<0.001$). The binding values in the obligate hetero-

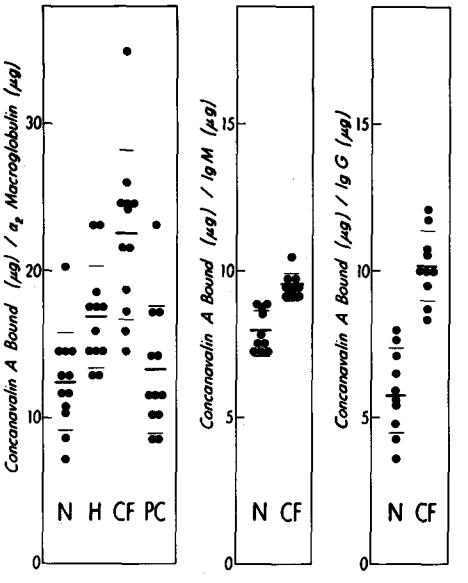

Figure
The binding ratio of fluorescein conjugated concanavalin A (Con A) to three plasma glycoproteins: α_2-macroglobulin (α_2M), IgM and IgG. Healthy control group (N), cystic fibrosis patients (CF), obligate heterozygotes for cystic fibrosis (H), and patients with chronic lung disease other than cystic fibrosis (PC). The bold line in each group indicates the mean value and the fine lines indicate $\pm$ one standard deviation.

zygotes group (16.9±3.5 μg/μg α_2M) were significantly higher than in the control group ($t=3.2$; $p<0.01$) and significantly lower than those of the CF group ($t=2.9$; $p<0.02$).

A similar phenomenon, increased binding of Con A to CF glycoproteins was shown also with the binding to IgM and IgG (Figure 1). The range of the values obtained for the binding of Con A to IgM was much narrower than the one observed for α_2M in both the control group and in the CF patients. The binding of Con A to IgM from CF patients (9.5±0.4 μg/μg IgM) was significantly higher than that of the control group (7.9±0.7 μg/μg IgM; $t=5.95$; $p<0.001$). The binding of Con A to the plasma IgG revealed a broad range of binding ratios, similar to the range shown with the α_2M preparations. Still, the value for the CF binding (10.2± 1.2 μg/μg IgG) was significantly higher than that for the control group (5.9± 1.4 μg/μg IgG; $t=7.28$; $p<0.001$).

DISCUSSION

Most of the previous studies indicating abnormal glycoproteins in cystic fibrosis were done using crude glycoprotein preparations from tissues or secre-

tions of CF patients (2). Recently, altered composition of "heparin-precipitated" serum glycoproteins was documented in cystic fibrosis (9). With a purified lysosomal glycoprotein enzyme, liver α-L-fucosidase, a differential Con A binding was observed between CF and controls (10). In the present study, a highly significant ($p<0.001$) increase in the binding of fluorescein conjugated Con A to three plasma glycoproteins, α_2M, IgM and IgG, was shown for cystic fibrosis patients. In these experiments, the oligosaccharide specificity of the Con A binding was documented by the competition with α-methyl-D-mannoside. The binding of Con A to α_2M from patients with chronic lung disease other than CF was similar to that of the healthy control group and significantly lower than that of the CF and the CF heterozygote groups. These findings indicate that the observed differences in CF are secondary to the inherited disorder and not to the disease process.

Recently, Ben-Yoseph et al (11) compared the carbohydrate composition of highly purified α_2M preparations from controls, CF patients and CF heterozygotes. They showed normal amounts of total hexose but as much as a 40% decrease in the sialic acid content. The binding of a constant amount of α_2M preparation to increasing amounts of agarose bound Con A was decreased for α_2M from CF patients as compared to the controls. Their findings (11) might be explained by either a decreased affinity of α_2M from CF patients to Con A or the relative saturation of the constant Con A binding site with a smaller number of α_2M molecules from CF patients. The findings in the present study suggest the latter.

Our observations and those of others (11) of altered carbohydrate moiety in α_2M from CF patients suggest that the previous findings (4-9) of varied α_2M functional properties might be secondary to a structural change in its oligosaccharide component. Another α_2M property which is more directly related to each carbohydrate composition, the uptake of α_2M complexes by fibroblasts, was recently shown to be altered in cystic fibrosis (12). The observation in the present study that the increased binding of Con A was not unique to α_2M, but a similar change was also shown with two other glycoproteins, IgG and IgM, as well

as α-fucosidase (10) suggests that this might be a general phenomenon in CF glycoproteins. As the synthesis of the carbohydrate side chains of glycoproteins is determined by parallel enzyme reactions, rather than by a genetically determined template, a large degree of variation is possible in the product. The primary product of the genes are the enzymes participating in the glycoprotein synthesis and degradation. A mutation in one of these enzymes might lead to a different extent of branching of the oligosaccharide moiety of the many different glycoproteins. That could lead to a different accessibility of specific sugars for the binding to Con A and other lectins. In the present study, as in many other observed variations in CF, a statistically significant difference among the various groups was observed although an overlap of the range for individual values exists. As the heterogeneity of the carbohydrate moiety of a glycoprotein is a direct consequence of the mechanism of its synthesis, some degree of overlap among the groups would be expected.

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