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New Alterations of Serum Glycoproteins in Alcoholic and Cirrhotic Patients Revealed by High Resolution Two-Dimensional Gel Electrophoresis

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Plasma protein are synthesized and secreted by the liver. Several reports have shown that excessive consumption of ethanol interferes with the hepatic protein synthesis and/or secretion. This study was undertaken to identify the plasma/serum proteins altered in two groups of patients with different alcohol-related diseases: actively drinking alcoholic patients group without liver disease and alcohol cirrhotic patients group. Two-dimensional gel electrophoresis was used to separate proteins with high resolution. Proteins were detected by silver staining and glycoproteins were specifically visualized and analyzed after lectin blotting followed by chemiluminescence detection. Different protein alterations were identified in each group of patients. In the alcoholic group, two new glycosylation modifications of serum proteins were identified. An abnormal microheterogeneity of haptoglobin and α1-antitrypsin was detected in the serum of all alcoholic patients. We also characterized by two-dimensional gel electrophoresis the carbohydrate deficient transferrin. The modifications of haptoglobin, α1-antitrypsin and transferrin present a similar change of charge and molecular weight in the two-dimensional gel electrophoresis pattern. These qualitative estimations support the hypothesis of a general mechanism of liver glycosylation alteration of serum proteins induced by excessive alcohol consumption. The immunoglobulin alterations were easily visualized and identified for the cirrhotic and the alcoholic patients. And finally, the decrease of haptoglobin and albumin spots for cirrhotic patients was confirmed.

Circulating plasma proteins, with the exception of immunoglobulins (Ig) and some apolipoproteins, are synthesized and secreted by the liver parenchymal cells (hepatocytes). The most abundant plasma protein is albumin, which accounts for 55 to 60 per cent of the total plasma proteins. In human, these proteins, with only a few exceptions such as albumin and C-reactive protein, are glycosylated (1, 2). The liver is the primary organ responsible for the metabolism and detoxification of ethanol. There have been numerous in vitro studies demonstrating that addition of ethanol to liver cellular preparations inhibits the synthesis and secretion of plasma proteins (3, 4, 5). Studies carried out in animals have reported variable effects of ethanol consumption on hepatic protein synthesis and an inhibitory effect on plasma protein secretion (6, 7, 8). The ethanol-induced inhibition of hepatic secretion of plasma proteins appear to mediated by acetaldehyde, the first metabolite of ethanol oxidation. To our knowledge, no systematic attempt has been done to determine which plasma proteins are affected by this liver function impairment. Since plasma proteins carry out numerous important functions, their impaired secretion may alter many homeostatic and physiologic processes in the patient with liver disease.

In this work, plasma/serum proteins from 2 groups of patients were analyzed in order to identify

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Abbreviations used: Ig, immunoglobulin; 2-D PAGE, two-dimensional polyacrylamide gel electrophoresis; IEF, isoelectric focusing; CDT, carbohydrate-deficient transferrin; RT, room temperature; IPG, immobilized pH gradient; WGA, wheat germ agglutinin; RCA, ricinus communis agglutinin; ALD, alcoholic liver disease.

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and compare the protein alterations associated to different alcohol related disease. The first group was composed of 10 actively drinking alcoholic patients without any liver disease and the other group of 6 patients with alcoholic cirrhosis. High resolution two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) was used to separate the proteins (9). The interest of this highly reproducible method is to allow the analysis, in a single experiment, of a large number of proteins from complex biological samples. The general plasma/serum protein patterns of patients and controls were analyzed after silver staining of the gels. Each modified protein was rapidly and efficiently identified using a reference 2-D PAGE plasma protein map (10, 11). It is known that excessive ethanol intake has widespread effects on the metabolism and turnover of glycoproteins (12, 13). We therefore combined the high separation capacity of 2-D PAGE with the lectin blotting technique (14) to detect specifically the plasma/serum glycoproteins and analyze their carbohydrate structures.

MATERIALS AND METHODS

Apparatus. The isoelectric focusing (IEF) separation was performed with a Multiphor II (LKB, Bromma, Sweden) connected to a Model 3000 Xi power supply (Bio-Rad, Hercules, CA). SDS-PAGE was performed on a vertical slab gel (160×200×1.5 mm) using a Protean II Multi-Cell (Bio-Rad, Hercules, CA). The gels were cast in a Protean Multi-Cell Casting Chamber (Bio-Rad, Hercules, CA) using a Model 395 Gradient Former (Bio-Rad, Hercules, CA) and a Masterflex microprocessor pump drive (Cole-Parmer, Chicago, II). Electrophoretic transfer was done using a Trans-Blot Cell (Bio-Rad, Hercules, CA). The developed X-ray films and the silver stained 2-D PAGE were scanned using a 300A laser scanner (Molecular Dynamics, Sunnyvale, CA) and quantitative analyzes were performed with the Melanie II software (Bio-Rad, Hercules, CA) (15). After silver staining of the gel, the optical density of altered protein spots from pathological samples were compared to controls. This allows a semi-quantitative estimation of the modifications.

Reagents. Acrylamide, DTE, Tween-20 and glycine were obtained from Fluka (Buchs, Switzerland). PDA, ammonium persulfate, TEMED and dye reagent for protein assay (Bradford) were obtained from Bio-Rad (Hercules, CA). Enhanced luminol chemiluminescent reagent (ECL Kit) and horseradish peroxidase-linked donkey anti-rabbit were supplied by Amersham International (Buckinghamshire, UK). Sigma (St-Louis, MO) supplied CHAPS, iodoacetamide, rabbit anti-human α-1 antitrypsin polyclonal antibody and peroxidase-labeled Extravidin. Rabbit anti-human transferrin and haptoglobin polyclonal antibodies were purchased from Dako (Glostrup, Denmark). Biotinylated lectins were obtained from Boehringer-Mannheim (Indianapolis, IN). All other chemicals were analytical grade.

Plasma/serum samples. Pathological specimens were obtained from patients with well characterized diseases. All patients (10 males) in the alcoholic without liver disease group fulfilled the DSM IV criteria for alcohol dependence (16). We measured for these samples the carbohydrate-deficient transferrin (CDT) using the CDT ect Pharmacia (kabi-Pharmacia, Uppsala, Sweden). The CDT serves as a sensitive and specific marker of abusive consumption of alcohol, appearing after regular intake of 60 g ethanol/day for at least 1 week and normalizing within 10 days of alcohol abstinence (12). We selected for this study 10 patients with a CDT at least 3 times higher than the normal value (CDT higher than 60 U/L).

For the second group, 6 serum samples from subjects with alcoholic cirrhosis (5 males, 1 female) were obtained from patients admitted to the University Hospital of Geneva for this alcohol related disease. Six normal control plasma samples were obtained from blood donors. Ethanol consumption by these controls was estimated to be 0-10 g/day based on personal interviews.

Blood samples were collected by venipuncture. Plasma or serum were collected after a centrifugation of blood at 2300 × g for 10 min. Plasma/serum samples were stored at −80°C until use. The protein content of all samples was determined by the method of Bradford. We used plasma for control samples and serum for pathological specimens. The only difference between the plasma/serum spot patterns is the absence of the fibrinogen chains in serum (Fig. 1).

2-D PAGE. Plasma/serum proteins were denatured as follows: 8 μl of plasma was added to 10 μl of a denaturing solution containing 10% (w/v) SDS and 2.32% (w/v) DTE and heated at 100°C for 5 min. After a cooling time of 2 min at room temperature (RT), 482 μl of a second denaturing solution containing 65 mM DTE, 65 mM CHAPS, 9 M urea and 35 mM Tris was added. For the IEF, a constant amount (75 μg) of denatured proteins was loaded onto a rehydrated immobilized sigmoidal pH gradient (IPG) strip gel (Pharmacia-LKB, Uppsala, Sweden). The procedures for the first and the second dimension gel electrophoresis were described elsewhere (9, 17). Briefly, after equilibration (14), the IPG strip gel was transferred to a 6–13% (v/v) or a 9–16% (v/v) gradient polyacrylamide gel. The 6–13% gradient allows a better separation of most plasma/serum proteins located in the middle part of the gel. SDS-PAGE was performed at 40 mA per gel at 10°C.

One factor which determines the electrophoretic behavior of transferrin is its iron content. In order to study the 2-D PAGE pattern of transferrin, the iron saturation of serum samples from alcoholic patients was performed according to De Jong et al. (18) before the denaturation process.

Silver staining of 2-D PAGE. The fixation and ammoniacal silver staining were done according to the method described by Oakley et al. (19) and modified by Hochstrasser et al. (20) and Rabilloud (21).

Protein blotting. Before the western blotting procedure, the gels were equilibrated in transfer buffer i.e. Towbin buffer diluted 1:2 with distilled water (12.5 mM Tris, 96 mM glycine and 10% (v/v) methanol) for 10 min. The electrotransfer was carried out using a tank buffer transfer system with 90 V for 1 h and then 40 V was applied overnight at 10°C. At the end of the transfer, nitrocellulose membranes (0.45 μm, Schleicher & Schuell, Dassel, Germany) were washed (3 × 5 min) with distilled water.

Lectin blotting. This protocol was previously described in details (14) and is summarized here. Following the protein transfer, the membrane was first treated for 30 min with 100 ml blocking solution containing 0.5% (w/v) Tween 20 in PBS at RT. All further incubations and washing steps were carried out in the same blocking solution and at RT. The blot was then incubated for 2 h in biotinylated lectin (1 μg/ml) and washed for 1 h with 6 changes of PBS-Tween 20. Horseradish peroxidase-labeled Extravidin diluted 1:2000 was added for 1 h. The membrane was again washed 6 times with the blocking solution. Chemiluminescence detection of peroxidase activity was performed according to the manufacturer's instructions (Amersham). After incubation with luminol reagent, the blot was wrapped in plastic sheeting and exposed to X-ray film (X-OMAT S, Kodak, Rochester, NY) for periods of 2 to 20 s. The exposure time depends on the lectin used and on the amount of proteins loaded onto 2-D PAGE.

Immunoblotting. For immunodetection, the membrane was blocked as described previously using the PBS solution containing 0.5% (w/v) Tween 20. The blot was then incubated for 2 h at RT or overnight at 4°C in blocking solution containing the primary antibody (dilution of 1:7000 for the rabbit anti-human transferrin, haptoglobin and α-1 antitrypsin antibodies). After the washing step, the membrane was incubated at RT for 1 h with the secondary antibody which was horseradish peroxidase-linked donkey anti-rabbit IgG (dilution of 1:5000 in blocking solution). The chemiluminescence detection of peroxydase activity was also performed on these blots.

RESULTS AND DISCUSSION

Serum protein pattern of alcoholic patients without hepatic disease. A typical silver stained gel of serum from an alcoholic patient is shown in Fig. 1a. When compared to normal control plasma (Fig. 1b), four important protein alterations are present in the pathological samples. The spot patterns of transferrin, haptoglobin and α-1 antitrypsin show some qualitative abnormalities and the IgA response is elevated. The 9 other serum samples of alcoholics analyzed in this study showed the same altered spot pattern.

The abnormalities in transferrin, haptoglobin and α-1 antitrypsin appear from the presence of additional spots at the basic (cathodic) end of the immobilized pH gradient (Fig. 1). These spots are easily visualized after the immunodetection of proteins on the nitrocellulose membrane (Fig. 2). The additional spots of transferrin in alcoholic patients (identified by arrow in Fig. 2A) represent the carbohydrate-deficient transferrin. These molecules of transferrin have a lower global chemical

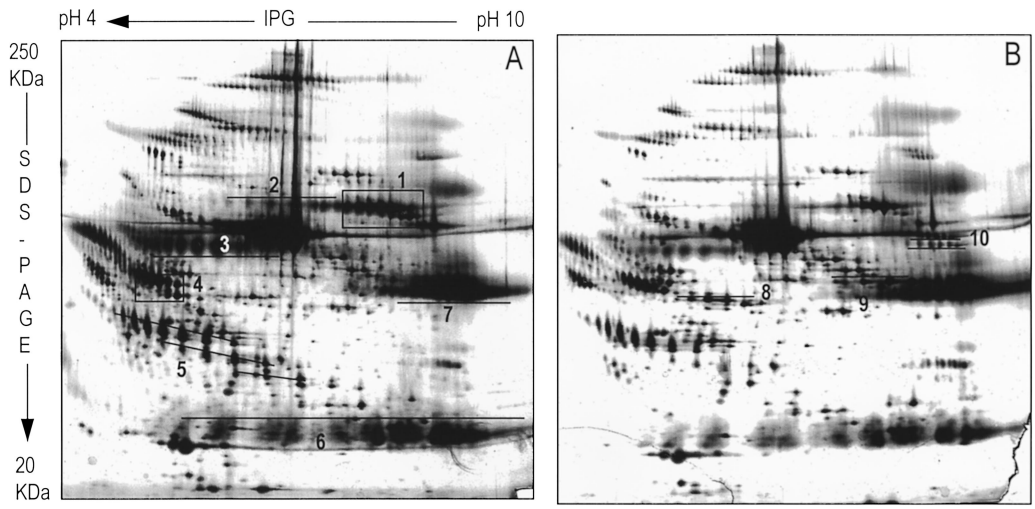

FIG. 1. Silver-stained serum/plasma proteins after 2-D PAGE from alcoholic patient (A) and control subject (B). (1) transferrin, (2) IgM μ chain, (3) IgA α chain, (4) α1-antitrypsin, (5) haptoglobin β chain and haptoglobin cleaved β chain, (6) Ig light chains, (7) IgG γ chain, (8) fibrinogen γ chain, (9) fibrinogen β chain, (10) fibrinogen α chain.

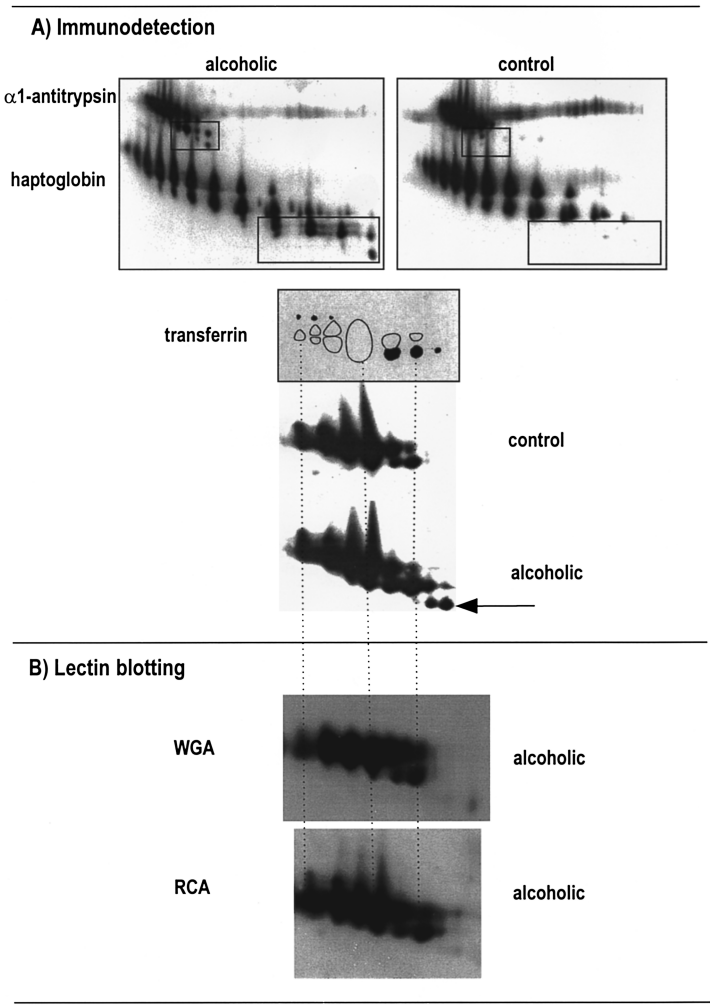

FIG. 2. (A) Blot patterns of transferrin, α1-antitrypsin and haptoglobin detected by immunoblotting and revealed by chemiluminescence after 2-D PAGE of serum proteins from alcoholic and control subjects. In alcoholic patients, the additional spots of transferrin are identified by an arrow and of haptoglobin and α1-antitrypsin by the framed areas. (B) Blot patterns of transferrin from alcoholic patients probed with WGA and RCA and revealed with chemiluminescecnce.

charge due to the loss of some negatively charged sialic acid residues (12). Transferrin with reduced sialic acid content represents a pool of up to 21% of the total transferrin in alcoholics (22). This percentage is sufficient to allow their visualization on 2-D gel.

Several glycoproteins have been studied for changes similar to those of transferrin. It is the first time that an abnormal microheterogeneity for haptoglobin and α-1 antitrypsin is observed for alcoholic patients without liver disease (Fig. 2A, framed areas). Takase et al (13) had previously reported some abnormal isoelectric focusing bands of α-1 antitrypsin but for alcoholic patients with liver disease.

The abnormal transferrin, haptoglobin and α-1 antitrypsin present also a lower molecular weight. This could be explained by the loss of more than one sugar residues on the glycan chain of these proteins. To support this hypothesis, we studied the carbohydrate structure of blotted proteins by lectin incubations. Because of their specificity for carbohydrates, lectins were used here to detect glycoproteins separated by 2-D PAGE and transferred to nitrocellulose membrane as described

previously (14). Two different lectins were used: wheat germ agglutinin (WGA) which detects terminal sialic acid and N-acetylglucosamine residues and ricinus communis agglutinin (RCA 120) which is specific for galactose residues, located at the penultimate position in the complex type glycans (Fig. 3). Fig. 2B shows the transferrin signal of an alcoholic subject detected with RCA and with WGA. The signal was generated on a X-ray film after chemiluminescence detection. For all serum samples of alcoholic patients, the lectin blotting incubations with WGA or RCA revealed a normal transferrin pattern. The additional basic spots of this glycoprotein associated to the lack of sugar residues were not detected by either lectin. The same results were obtained with the haptoglobin and α1-antitrypsin signals obtained after the lectin blotting (not shown). This suggests that transferrin, haptoglobin and α-1 antitrypsin lack also, on top of terminal sialic acid, the galactose residue (RCA detection negative) and the N-acetylglucosamine residue (WGA detection negative) which are located, respectively, at the penultimate and antepenultimate position of the glycan chains (Fig. 3). These results confirm the previous findings of Stibler et al. (23) for the CDT. The 2 new glycosylation alterations of haptoglobin and α-1 antitrypsin, which are similar to those of transferrin, suggest the involvement of a common mechanism.

During the last 2 decades, an increasing number of studies have shown that the CDT represents a new, sensitive and specific diagnostic marker of alcohol abuse. The mechanism behind the transferrin abnormality is still unknown. It was suggested that ethanol metabolism may interfere with several steps in glycosylation turnover. In fact, two effects of the alcohol abuse on glycoprotein in men have been demonstrated: the impaired tubulin polymerization, which is necessary for the secretory process (24) and the reduced activity of 3 glycosyl transferases in serum. The acetaldehyde-mediated inhibition of glycosyl transfer has been proposed as the responsible mechanism of the abnormal transferrin (25). Our findings further support this hypothesis and suggest a broader effect of alcohol abuse on different serum glycoprotein.

Furthermore, transferrin, haptoglobin and α-1 antitrypsin represent the 3 most abundant serum glycoproteins secreted by the liver (26). It is therefore possible that the alcohol abuse induce an alteration of the hepatic glycosylation affecting all serum proteins but due to analytical method limits, the modifications are only visible for the more concentrated proteins. Also, since the alteration of the glycosylation affects a relatively small portion of the total amount of protein (22), it is probable that the 2-D electrophoresis, in the conditions used in this work, lacks to reveal some of the small modifications. Nevertheless, this method proved useful to visualize, in a single experiment, different changes of charge and molecular weight associated to the glycosylation modification of proteins.

The amount of serum IgA α chain from alcoholic patients was increased 2-3 fold compared to controls (Fig. 1). Only 2 out of 10 alcoholic patients had an elevated IgM μ chain. The other visible immunoglobulins on 2-D PAGE (IgG γ chain and Ig light chains) appeared to be in the normal range. As discussed below, the IgA alteration is also found in the serum of patients having alcoholic cirrhosis. Indeed, early IgA elevation has been considered a characteristic of alcoholic liver diseases. However, there is evidence that prolonged consumption of ethanol, even in the absence of a liver disease, can produce an increase of serum IgA concentration (27). IgA-producing cells are particularly located in the gastrointestinal tract and it is possible that elevation of this Ig may reflect a concomitant alteration of the gastrointestinal system, which is frequently affected in chronic alcoholics (28).

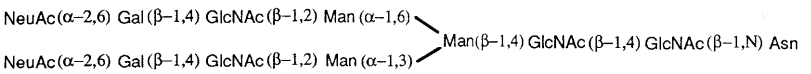

FIG. 3. Oligosaccharide units of the main forms of normal transferrin, haptoglobin and α1-antitrypsin. Two, 8 and 3 N-linked glycans of complex type are present in each molecule of transferrin, haptoglobin and α-1 antitrypsin, respectively. NeuAc, neuraminic acid; Gal, galactose; GlcNAc, N-acetyl glucosamine; Man, mannose; Asn, asparagin.

Since the amount of IgA in alcoholic patients is elevated, it is not surprising that the same protein has elevated RCA and WGA signal (not shown), reflecting the proportional increase in sugar moieties.

Serum protein patterns of cirrhotic patients. In alcoholic liver disease, the liver function is seriously impaired, largely because of injury to hepatic parenchymal cells with accompanying fibrosis. It would seem that a consequence of alcoholic liver disease would be significant changes in the level and composition of the plasma protein pool caused by impairment of protein secretion.

As shown in Fig. 4, cirrhotic patients present a silver stained serum protein pattern differently altered from alcoholic patients without liver disease. The gel presented in Fig. 4 is representative of the 6 samples of cirrhotic patients studied in this work. The major modified proteins in cirrhotic patients are the immunoglobulins. A massive polyclonal stimulation of light chains leads to the appearance of numerous distinct clones between the cloud-like spot area of light chains (29). All heavy chains present in the gels are increased: the IgA α chain, the IgM μ chain and the IgG γ chain. The main increase (15 to 20 fold) was in IgA α chain. The amount of IgA is so important that they formed a broad band extending almost completely over the pH gradient of the strip. Hypergammaglobulinaemia characterized by increased in serum IgA, IgM and IgG concentrations is generally found in patients with alcoholic liver disease (ALD). However, it can also be observed in patients with liver disease not caused by alcohol. The mechanisms of this hypergammaglobulinaemia are no better understood in ALD than they are in other forms of chronic liver disease. Two different mechanisms may be operative: 1) a normal increased stimulation of Ig-producing cells (due to increased absorption of antigens from the intestinal mucosa, to increased release into the circulation of hepato-biliary antigens due to liver necrosis and/or to decreased clearance of antigens by the reticuloendothelial system), or 2) a hyperactive immune response to normal antigen stimulation which could result from a defect in suppressor T-cell function (28).

Since the liver secretes IgA into the bile, the important increase of IgA in serum may also be related to an altered hepatic transport of IgA (27).

Another evident protein modification in cirrhotic patients, which is probably directly associated to the liver secretion or production impairment is the decrease of the haptoglobin spots. Agostoni et al. (30) and Tissot et al. (31) also observed this protein alteration in some cirrhotic patients. In 2 patients, the level was so low that there was virtually no visible haptoglobin spot (as represented in Fig. 4). A tendency towards low level of haptoglobin in cirrhosis has been attributed to the chronic hemolytic state so often associated with liver disease, although some authors could find no

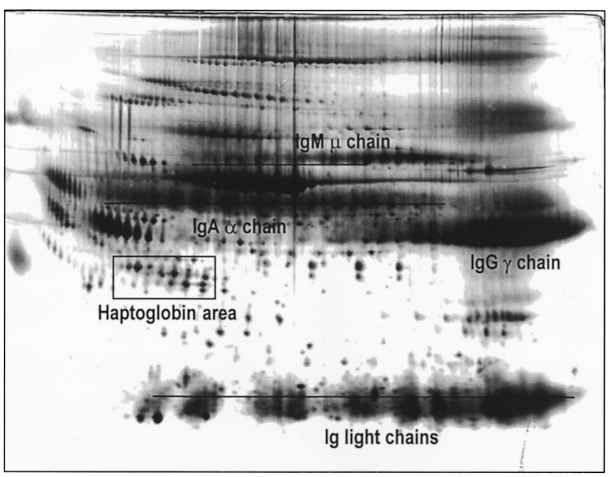

FIG. 4. Silver-stained serum proteins from cirrhotic patient separated by 2-D PAGE.

direct correlation with measurements of red cell survival (32). Finally, the amount of albumin seems to be decreased in all cirrhotic patients. Habitually, the albumin spots form vertical streaking due to the abundance of this protein into the gel (Fig. 1). In Figure 4, no streaking is visible and the albumin spot area is smaller. As previously reported (33), the decrease in serum albumin is one of the protein changes occurring in liver disease associated with parenchymal damage.

Lectin blotting incubations with WGA and RCA were also performed with these samples. No additional alteration in the glycosylation of serum proteins was revealed (not shown). As expected, the amount of Ig and haptoglobin/albumin, which were respectively increased and decreased in silver stained gel, had a proportional modification of their sugar composition.

It appears from these 2-D protein patterns of patients having alcoholic cirrhosis that, despite an important impairment of hepatic function in these patients, the protein and glycoprotein synthetic and secretory capacity seem to be, in general, weakly affected. Except for the protein alterations mentioned above, all other serum proteins in the 2D silver stained gel present normal spot size and form. However, the apparently normal concentrations of serum proteins from cirrhotic patients may reflect compensatory mechanisms such an increase of the rate of protein synthesis in the liver or a decrease of the rate of their catabolism by both hepatic and extrahepatic tissues, or both. Also, the very sensitive silver staining detection method used in this work may lack to detect some very small changes of shape or abundance of certain 2D spots.

In conclusion, with the present 2-D PAGE method, combining the high-resolution and reproducibility for the separation of proteins, it was possible to screen plasma proteins from alcoholics and cirrhotic patients for abnormalities in protein patterns and in glycosylation when using the lectin blotting technique. We therefore identified and confirmed different protein alterations associated with both diseases. Further studies are needed to determine the specificity and the sensitivity of the 2 new glycosylation alterations found for the haptoglobin and α-1 antitrypsin.

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REFERENCES

1. Schreiber, G. (1987) *in* The Plasma Proteins (Putnam F. W., Ed.), Vol. V, pp. 294–347, Academic Press, New York.
2. Zakim, D., and Boyer, T. D. (1990) Hepatology. A Textbook of Liver Disease p. 124, Saunders, Philadelphia.
3. Tuma, D. J., Jennett, R. B., and Sorrell, M. F. (1981) *Hepatology* **1,** 590–596.
4. Morland, J., and Bessessen, A. (1977) *Biochim. Biophys. Acta* **474,** 312–316.
5. Sorrell, M. F., Tuma, D. J., and Schafer, E. C. (1977) *Gastroenterology* **73,** 137–141.
6. Sorrell, M. F., Naus, J. M., and Donohue, T. M. (1983) *Gastroenterology* **84,** 580–587.
7. Volentine, G. D., Tuma, D. J., and Sorrell, M. F. (1984) *Gastroenterology* **86,** 225–230.
8. Volentine, G. D., Tuma, D. J., and Sorrell, M. F. (1986) *Gastroenterology* **90,** 158–164.
9. Hochstrasser, D. F., Harrington, M. G., Hochstrasser, A. C., Miller, M. J., and Merril, C. R. (1988) *Anal. Biochem.* **173,** 424–435.
10. Golaz, O., Hughes, G. J., Frutiger, S., Paquet, N., Bairoch, A., Pasquali, C., Sanchez, J. C., Tissot, J. D., Appel, R. D., Walzer, C., Balant, L., and Hochstrasser, D. F. (1993) *Electrophoresis* **14,** 1223–1231.
11. Sanchez, J. 0C., Appel, R. D., Golaz, O., Pasquali, C., Ravier, F., Bairoch, A., and Hochstrasser, D. F. (1995) *Electrophoresis* **12,** 722–735.
12. Stibler, H. (1991) *Clin. Chem.* **37,** 2029–2037.
13. Takase, S., Takada, A., Tsutsumi, M., and Matsuda, Y. (1985) *Alcohol* **2,** 405–410.
14. Gravel, P., Golaz, O., Walzer, C., Hochstrasser, D. F., Turler, H., and Balant, L. P. (1994) *Anal. Biochem.* **221,** 66–71.
15. Appel, R. D., Hochstrasser, D. F., Funk, M., Vargas, J. R., Pellegrini, C., Muller, A. F., and Scherrer, J. R. (1991) *Electrophoresis* **12,** 722–735.
16. American Psychiatric Association. (1994) Diagnostic and Statistical Manual of Mental Disorders. 4th ed. APA, Washington, DC.
17. Gravel, P., Sanchez, J. C., Walzer, C., Golaz, O., Hochstrasser, D. F., Balant, L. P., Hughes, G. J., Garcia-Sevilla, J., and Guimon, J. (1995) *Electrophoresis* **16,** 1152–1159.

18. DeJong, G., and Van Eijk, H. G. (1988) *Electrophoresis* **9,** 589–598.
19. Oakley, B. R., Kirsch, D. R., and Morris, N. R. (1980) *Anal. Biochem.* **105,** 361–363.
20. Hochstrasser, D. F., and Merril, C. R. (1988) *Appl. Theor. Electrophor.* **1,** 35–40.
21. Rabilloud, T. (1992) *Electrophoresis* **13,** 429–439.
22. Stibler, H., Sydow, O., and Borg, S. (1980) *Pharmacol. Biochem. Behav.* **13,** 47–51.
23. Stibler, H. and Borg, S. (1986) *Alcohol. Clin. Exp. Res.* **10,** 61–64.
24. Matsuda, Y., Takada, A., Kanayama, R., and Takase, S. (1983) *Pharmacol. Biochem. Behav.* **18,** 479–482.
25. Stibler, H., and Borg, S. (1991) *Scand. J. Clin. Lab. Invest.* **51,** 43–51.
26. Baenziger, J. U. (1984) *in* The Plasma Proteins (F. W. Putnam, Ed.), Vol. IV, pp. 271–315, Academic Press, Orlando.
27. Paronetto, F. (1981) *Seminars liver disease* **1,** 232–243.
28. MacSween, R. N. M., and Anthony, R. S. (1982) *J. Clin. Lab. Immunol.* **9,** 1–5.
29. Hochstrasser, D. F., and Tissot, J. D. (1993) *in* Advances in Electrophoresis (A. Chrambach, Ed.), Vol. 6, pp. 267–375, VCH, Weinheim.
30. Agostoni, A., Vergani, C., Stabilini, R., and Marasini, B. (1969) *Clin. Chim. Acta* **26,** 351–355.
31. Tissot, J. D., Schneider, P., James, R. W., Daigneault, R., and Hochstrasser, D. F. (1991) *Appl. Theor. Electrophor.* **2,** 7–12.
32. Murray-Lyon, I. M., Clarke, H. G. M., McPherson, K., and Williams, R. (1972) *Clin. Chim. Acta* **39,** 215–220.
33. Teloh, H. A. (1978) *Ann. Clin. Lab. Sci.* **8,** 127–129.