

Monoclonal antibodies against non-enzimatically glycosylated (NEG) proteins. Their use in quantitative ELISA for NEG serum proteins measurement.

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SUMMARY

Specific monoclonal antibodies (Mabs) against nonenzymatic glycosylated (NEG) proteins were produced from immunized Balb/c mice with glycosylated homologous serum. The four Mabs assayed shown different affinity for glucosylated residues in glycosylated human serum albumin. In competition ELISA Mabs recognized glycosylated serum from different species and different glycosylated proteins. Non insulin dependent diabetics patients with peripheral vascular disease showed significant higher level of reductively NEG serum proteins than non diabetic atherosclerotic patients. A positive correlation was found between the level of NEG serum proteins and the level of fructosamine, glycosylated hemoglobin and glycemia. A sandwich ELISA using a Mab against glycosylated proteins and sheep antibodies against human apolipoprotein B allowed the detection of *in vitro* glycosylated low density lipoprotein.

RESUMEN

A partir de ratones Balb/c inmunizados con suero homólogo glicosilado fue posible obtener anticuerpos monoclonales (AcMs) que reconocen específicamente proteínas glicosiladas. Los cuatro AcMs analizados mostraron diferente afinidad por los residuos de glucosilolisisina en la albúmina sérica humana glicosilada. Utilizando un ELISA de competencia se demostró que estos AcM reconocen sueros glicosilados de distintas especies animales y proteínas glicosiladas de distinta naturaleza. Pacientes diabéticos no insulino dependientes con enfermedad arterial periférica tuvieron niveles de proteínas

séricas glicosiladas significativamente superiores a pacientes ateroscleróticos no diabéticos. Se encontró una correlación positiva entre el nivel sérico de estas proteínas y los de fructosamina, hemoglobina glicosilada y glicemia. Utilizando un ELISA tipo sandwich con el empleo de uno de los AcM obtenidos y anticuerpos de carnero anti apoproteína B humana fue posible la detección de lipoproteínas de baja densidad glicosiladas *in vitro*.

INTRODUCTION

Non-enzymatically glycation (NEG) of proteins is the result of the attachment of glucose to epsilon amino group of proteins without the participation of any enzyme. This reaction occur normally in blood to a wide spectrum of plasma proteins, but the levels of these proteins are abnormally high in diabetic patients, mostly in those with a maintained hyperglycemia. Then, the levels of these proteins have been used as an indicator of diabetes control (Bunn, 1981; Armbruster, 1987). On the other hand, NEG altered protein functions and there are increasing amount of evidences to suggest that they are involved in long-term

complications of the diabetes (Jennings and Barnett, 1988; Brownlee et al 1984; Cerami *et al.*, 1988).

A variety of methods have been developed for measurement the level of NEG serum proteins. Recently different immunochemical techniques have been described (Go *et al.*, 1987; Nakayama *et al.*, 1987; Ohe *et al.*, 1987). These methods are very sensitive and specific but they need antibodies that recognize NEG proteins and not the corresponding native proteins. Curtiss and Witztum reported a method for generating monoclonal antibodies (Mabs) that recognize specifically glyated proteins (Curtiss and Witztum, 1983).

We report here the production of hybridoma cell lines secreting Mabs against glyated proteins and they use in a solid phase immunosorbent assay (ELISA) that recognizes specifically different glyated proteins and allows the measurement of the level of NEG serum proteins.

MATERIALS AND METHODS

Serum of Balb/c mice was glyated in the presence of glucose (180 mg/mL serum) and the reducing agent sodium cyanoborohydride (NaCNBH₃, 12.5 µg/mL serum) in sterile conditions for one week at 37°C (Curtiss and Witztum, 1983). Female Balb/c mice were injected subcutaneously with 0.1 mL of this serum emulsified in complete Freund's adjuvant. On days 7, 14 and 21 the injections were repeated in incomplete Freund's adjuvant. Blood was collected from animals seven days after the last injection and the serum antibody responses were determined using an indirect ELISA as follow.

Microtiter polystyrene plate was coated with glyated (by the same method described above) and non glyated human serum albumin (HSA) at 5 µg/mL in coating buffer (bicarbonate 0.1 M, pH 9.6) overnight at 4°C. Plate was washed with PBS-Tween 20, 0.05 % and remained free sites were blocked with 1 % bovine serum albumin (BSA) in PBS-Tween. Dilutions of the serum samples in the same blocking solution were added in duplicate and incubated for 3 hours at 37°C. After washing three times a

peroxidase-conjugated (HRP-conjugate) sheep anti-mouse was added to the wells and incubated 1 hour at room temperature. The plate was washed five times and then the substrate (4 mg of ortophenylenediamine in 10 ml of citrate buffer, pH 5.0 with 4 µL of a 30% solution of H₂O₂), was added. The color reaction was stoped adding 50 µl of sulfuric acid, 2.5 M and the absorbance at 492 nm was determined in a plate reader (Multiskan, MCC 340). The animal with the high title in antibodies received a booster of 0.3 mL of the glyated serum intraperitoneally three days before fusion.

Fusion

Spleen cells of the selected animal were fused with P3 X63-Ag8.653 mouse myeloma in a ratio 10:1 lymphocyte/myeloma, in presence of polyethyleneglycol (Kholer and Milstein, 1975) with some modifications (Sorell *et al.*, 1986). Cell culture supernatants were collected and the presence of specific antibodies was assayed using an ELISA similar to above, using as samples 100 µL of the supernatants. A positive response was considered a value of absorbance four times higher than those for cell culture supernatant from non specific hibridoma cell lines. Positives cells were cloned twice by limiting dilution and store frozen in liquid nitrogen or inoculated in the peritoneal cavity of pristane primed Balb/c mice for ascitis.

Characterization and purification of the Mabs.

The type of heavy chains of the secreted Mabs were established in concentrated supernatants (20 times) by double immunodiffusion in agarose gel (Ouchterlony and Nielsen, 1967) using comercial immunotyping antisera (ICN Immunobiologicals). The purification of Mabs was performed by affinity chromatography with protein A Sepharose (Pharmacia Fine Chemicals). The specificity of the antibodies was asseyed using glyated and non glyated serum from different species (human, rabbit, dog, sheep and rat) and also with different glyated proteins (insulin, bovine gelatin, horse globulin, bovine lactoglobulin, human alfa globulin and tripsin inhibitor) in a competition ELISA.

Competition ELISA

Microtiter polystyrene plates were coated overnight at 4°C with 100 µL of glyated human serum at a protein concentration of 4 µg/mL in coating buffer. Plates were blocked as described before.

Mixtures of 30 μL of appropriate dilutions of the different serum or glycosylated proteins and 70 μL of a specific Mab to glycosylated proteins (266/116/6) conjugated to peroxidase (Nakane and Kawaoi, 1974), were incubated for three hours. Then, 100 μL of these mixtures were added to the wells in duplicated and incubated for three hours. After washing five times the specific reaction was followed as was described above. Maximal absorbance was determined in wells with conjugated Mab in absence of competitor.

Measurement of NEG serum proteins

Serum samples were obtained from 19 patients with confirmed non insulin dependent diabetes mellitus and 19 non diabetic atherosclerotic patients, both with peripheral vascular diseases diagnosed with hemodynamic and angiographic studies, in the Institute of Angiology and Vascular Surgery in Havana. Blood was collected after overnight fasting by venipuncture and let to clot two hours at room temperature. Free glucose in serum was removed passing 50 μL of serum samples through micro-columns of Sephadex G-25 (Pharmacia Fine Chemicals). The eluate was reduced with NaBH_4 (4 mg/mL serum), during one hour at 37°C. Excess of NaBH_4 was eliminated in the columns of Sephadex G-25 and protein concentration in samples was determined (Lowry *et al.*, 1951). Concentration of glycosylated hemoglobin (Fluckiger and Winterhalter, 1976), fructosamine (Johnson *et al.*, 1982) and glucose (Huggett and Nixon, 1957) were determined in the original samples.

To determine the level of NEG serum proteins a competition ELISA as described before was used. Ten μL of the processed samples and 90 μL of the HRP-conjugated Mab were incubated for three hours and then applied in duplicated onto the wells of plates coated with glycosylated human serum. As standard an *in vitro* glycosylated human serum was prepared adding 2.88 g of glucose to 20 ml of serum and let it 10 days in sterile condition at 37°C. The serum was extensively dialyzed with PBS and reduced with NaBH_4 (4 mg/mL serum). After the reduction the serum was dialyzed against and the concentration of fructosamine (Johnson *et al.*, 1982) and total proteins (Lowry *et al.*, 1951) was determined. Data were calculated as B/Bo in which B represent the amount (absorbance) of the HRP-conjugated Mab binding in the presence of competitors (samples or standard) and Bo that bound (absorbance) in the absence of competitors. The results are expressed as mol of fructosamine/mg of protein. Wilcoxon test (Wilcoxon, 1945) was used for statistical analysis of the data.

Detection of glycosylated low density lipoproteins (gly-LDL).

Low density lipoproteins (LDL) were isolated from human plasma by sequential ultracentrifugation (Havel *et al.*, 1955).

A sandwich ELISA was employed using as coating antibody, affinity purified sheep antibodies against human ApoB (López and Illnait, 1989) or a Mab against glycosylated proteins at a concentration of 10 $\mu\text{g}/\text{mL}$ in coating buffer overnight at 4°C. After blocking as was mentioned, glycosylated (as was described for others proteins) and non glycosylated LDL were incubated in different dilutions onto the plate for two hours. The amount of LDL bound to the plate was determined adding, in the first case, a HRP-conjugated Mab against glycosylated proteins or a HRP-conjugate sheep antibodies against human ApoB in the second. Specific response was evaluate as has been described.

RESULTS

Generation of monoclonal antibodies.

Immunizing Balb/c mice with glycosylated homologous sera it was possible to rise high title of specific antibodies against gly-HSA (figure 1).

Using spleen cells of the immunized animals several specific Mabs for glycosylated proteins were generated. All of them were IgG1 class. The yield of Mabs from ascitis after purification by Protein A Sepharose chromatography was around 3 mg/mL of ascitis with a purity higher than 90 % by SDS-PAGE (Laemly, 1970).

Specific dose response recognition of reductively glycosylated HSA was found using one of the Mabs (266/116/6) previously conjugated to horseradish peroxidase (Nakane and Kawaoi, 1974). Non glycosylated HSA was not recognized (figure 2).

The ability of other Mabs to compete with the HRP-conjugated Mab for reductively glycosylated HSA was assayed in a

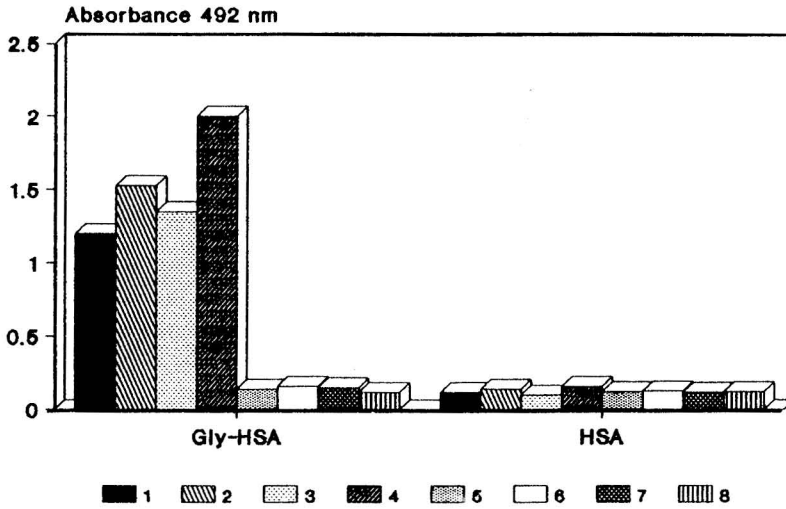


Figure 1. Levels of specific antibodies to glucocitolysine residues. Antisera at a final dilution of 1:1000 in PBS with 1 % of BSA were added to microwell plates precoated with reductively glycosylated human serum albumin (gly-HSA) and non glycosylated human serum albumin (HSA). Specific antibodies were detected with HRP-sheep antimouse antibodies: (1 to 4) represent serum antibodies title from immunized Balb/c mice with homologous glycosylated serum and (5 to 8) from non immunized mice. See details in text.

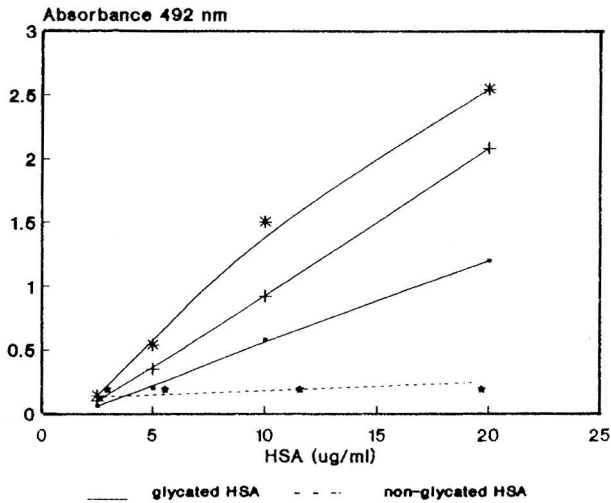


Figure 2. Specific recognition of gly-HSA with the HRP-conjugate monoclonal antibody (226/116/6). Dilution of conjugate in PBS with 1 % BSA: (*) 1:500; (+) 1:1000; (•) 1:2000.

competition ELISA (figure 3). All of the Mabs tested shown competition for reductively glycosylated HSA. The required amount of different Mabs needed for a

50 % reduction of the binding of labelled Mab to glycosylated HSA onto the plate may reflect the different affinity of these Mabs for glucocitolysine residues.

Glycated sera from different species and different glycosylated proteins were recognized for the Mabs as shown the competitions

ELISA (fig 4a, 4b). The same non glycosylated sera or proteins had not competitive effect (results not shown).

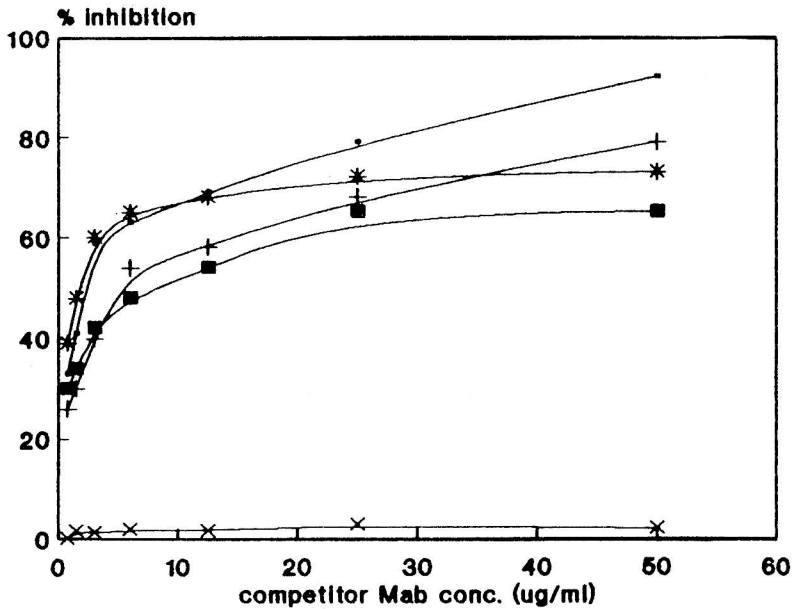


Figure 3. Competition of different monoclonal antibodies (Mabs) for glucocitollysine residues. HRP-cojugated Mab 266/116/6 was mixed with different concentrations of the competitors Mabs and then incubated in a microtiter plate coated with gly-HSA. Details in text. The Mabs assayed were: (+) 41/31/1; (*) 185/55/5 and (■) 243/77/7. Mab 266/166/16 was used as positive control (●) and a Mab against human Apo B (x) as negative control.

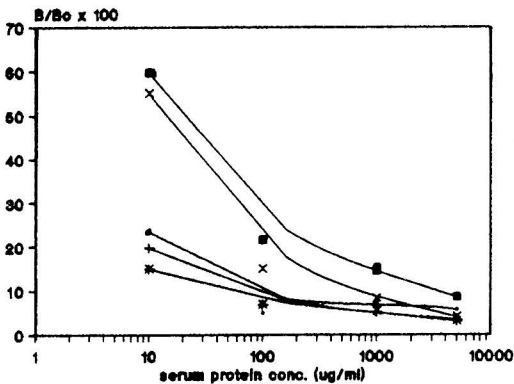


Figure 4a. Competition of different reductively glycosylated serum from different species with gly-HSA recognition by the HRP-conjugated Mab 266/116/16. Serum samples from: (■) rabbit; (x) human (●) dog; (+) sheep; (*) rat.

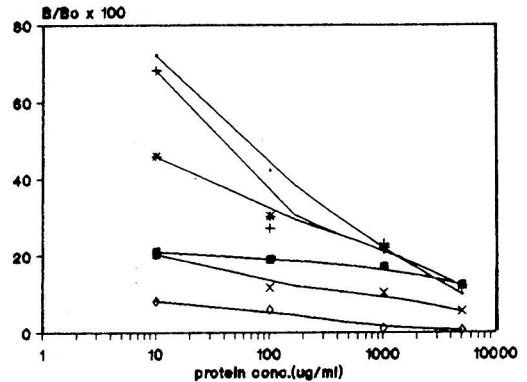


Figure 4b. Competition of different reductively glycosylated proteins with gly-HSA recognition by the HRP-conjugated Mab 266/166/16. The proteins assayed were: (●) bovine insulin; (+) horse gamma globulin; (*) gelatin; (■) bovine lactoglobulin; (x) human alfa globulin; (◇) soybean tripsin inhibitor.

Competition ELISA for NEG serum proteins measurement.

Figure 5 shows a typical curve in the competition ELISA using as competitor the standard glycated human serum in a range of 7.81 to 500 moles fructusamine/mg

protein. The intra and inter-assay coefficient of variation were 12.3% (n=14) and 14.6% (n=30) respectively.

The ELISA was used for the measurement of human NEG serum proteins. Diabetic patients showed significant higher level of NEG serum

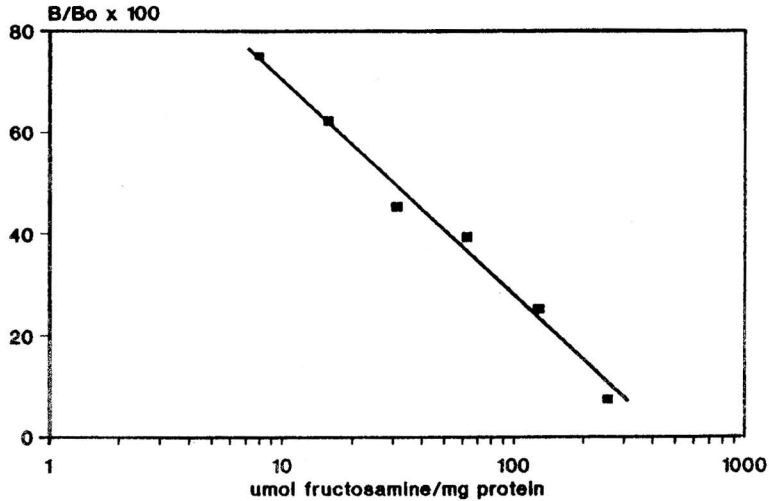


Figure 5. The standard curve in the competition ELISA for glycated serum proteins obtained by using as competitor the standard reductively glycated human serum in a range of 7.81 to 500 μ F255mol of fructosamine/mg of protein.

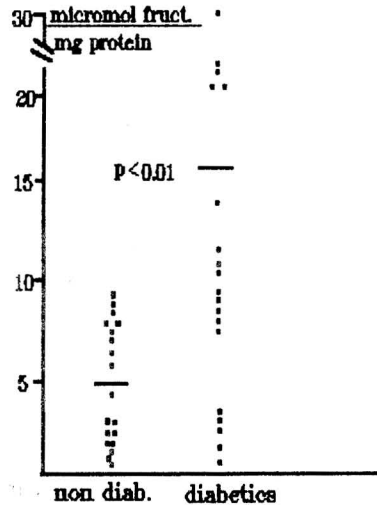


Figure 6. Level of reductively glycated serum proteins in diabetics (n=19) and non diabetic (n=19) atherosclerotic patients. Group medians are indicated by horizontal lines. Level of glycated proteins was significantly high in diabetics patients ($p < 0.01$) from non diabetics subjects.

proteins than non diabetics atherosclerotic patients ($p < 0.01$) (figure 6). It was also found a positive correlation ($p < 0,05$) between the levels of NEG serum proteins as determined by the ELISA and the values of serum fructosamine ($r = 0.30$), glycosylated hemoglobin ($r = 0.52$) and glycemia ($r = 0.43$).

Detection of glycosylated LDL

The two sandwich ELISA described here were useful to detect *in vitro* gly-LDL. The slope of the curve obtained with different dilution of the gly-LDL was higher when a Mab against glycosylated protein as capture antibody and the HRP-conjugate sheep anti-human ApoB as labelled antibody were used (A in figure 7). The slope was lower using the polyclonal antibodies in the solid phase and the HRP-conjugate Mab against

glycosylated protein in the second step (B in figure 7). These results may reflect differences in the affinity of the antibodies involved and probably some steric effects.

DISCUSSION

Curtiss and Witztum, using as immunogen homologous glycosylated LDL, were able to produce Mabs that recognize another glycosylated proteins (Curtiss and Witztum, 1983). This result shows that glucitolysine groups formed as a result of the non enzymatic reaction of glucose and epsilon amino groups in proteins is common for different proteins. According to this result we used as immunogen homologous glycosylated serum instead a purified protein to produce Mabs against glycosylated proteins. We can demonstrate that these Mabs were able to recognize serum and different

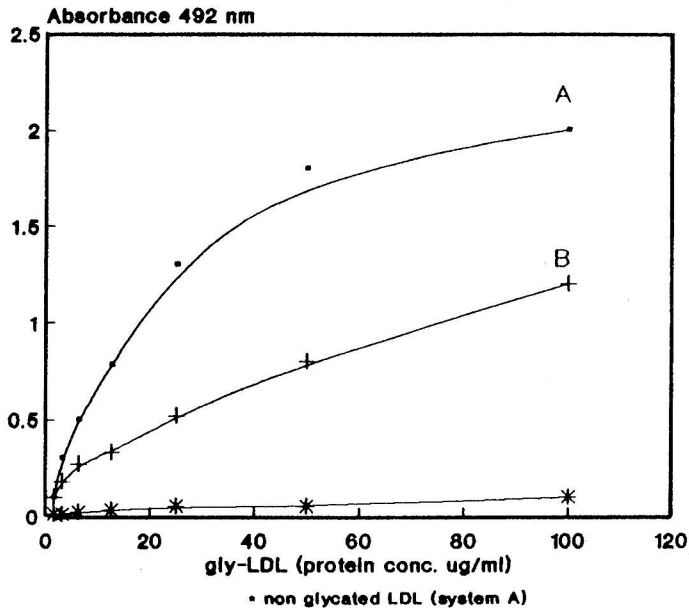


Figure 7. Sandwiches ELISA for measurement of reductively glycosylated LDL. A) Mab 266/116/16 as capture antibody and HRP-conjugated sheep antibodies against human apoprotein B as labelled antibody were used. B: Affinity purified sheep antibodies against human apoprotein B) as coating antibody and the HRP-conjugated Mab 266/166/16 as labelled antibody were used. See in text for details.

glycated proteins and not the native ones. Then it is possible to use these Mabs to the measurement of NEG proteins in serum and other biologic fluids.

Measurement of glycated proteins shows increasing promise in the assesment of hyperglycemia. Glycated hemoglobin, widely used in the last years, gives information about blood glucose level in diabetic subjects during the preceding time according to the half live of hematies (120 days). However the measurement of other NEG serum proteins with a shorter half live than hemoglobin, for example albumin (Jones *et al.*, 1983), may be useful for assesing diabetes control, specially to evaluate therapeutic squemes. Because albumin is the most predominant serum protein, the level of NEG serum proteins reflects the level of glycated albumin, then different authors used it as an index of short-term control of diabetes (Baker *et al.*, 1984; Lloyd *et al.*, 1985; Armbruster, 1987). The competition ELISA for NEG serum proteins described in this paper may be useful for these pourposes.

Our data showed that diabetic patients had a significantly higher level of NEG serum proteins than non diabetics atherosclerotic patients. It is known the presence of a premature and agressive atherosclerosis in poorly controled diabetics. Since no unique pathogenic element has been implicated in these earlier vascular complications of diabetes, it is reasonable to investigate if the level of NEG serum proteins may have a prognostic value for these complications.

On the other hand, measurement of specific NEG proteins using a sandwich ELISA as was described here for gly-LDL, using different antibodies against single serum proteins and a common Mab specific

to glycated proteins, allows the measurement of NEG proteins with different half live what may be useful to know more exactly about the dynamic of the diabetes control and the role of these single "altered" proteins in the pathogenesis of long-term complications of the diabetes.

Low density lipoproteins (LDL) are the main carrier for blood cholesterol. It is possible that gly-LDL was not appropriately recognized by cellular receptors. Different authors have claimed about the role of gly-LDL in premature atherosclerosis in diabetic patients (Witztum *et al.*, 1982; Lorenzi *et al.*, 1984). The sandwich ELISA described was usefull to detect *in vitro* reductively gly-LDL. It is reasonable to think that it would be possible to measure the level of gly-LDL in serum with this ELISA.

The principal limitation to use this methodology to assay the level of different NEG proteins in serum is the relatively higher amount of the non glycated native proteins respect to the glycated ones and the neccessity to reduce the samples previous to the test. We are working to find conditions wich allow to make this determinations more easily.

REFERENCES

- ARMBUSTER, D.A (1987). Fructosamine: structure, analysis and clinical usefulness. *Clin. Chem* 33: 2153-2163.
- BAKER, J.R; R.N. JOHNSTON and D.J. SCOTT (1984). Serum fructosamine concentration in patient with type II (non-insulin dependent) diabetes mellitus during changes in management. *Br. Med. J.* 288: 1484-1486.
- BROWNLEE, M; H. VLASSARA and A. CERAMI (1984). Nonenzymatic glycosylation and the pathogenesis of diabetic complications. *Ann. Internal Med.* 101: 527-537.

- BUNN, H.F. (1981). Evaluation of glycosylated hemoglobin in diabetic patients. *Diabetes* 30: 613-617.
- CERAMI, A; H. VLASSARA and M. BROWNLEE (1988). Role of advanced glycosylation products in complications of diabetes. *Diabetes Care* 11: (suppl 1): 73-79.
- CURTISS, L.K. and J.L. WITZTUM (1983). A novel method for generating region-specific monoclonal antibodies to modified proteins. Application to the identification of human glycosylated low density lipoproteins. *J. Clin. Invest.* 72: 1427-1438.
- FLUCKIGER, R. and K.H. WINTERHALTER (1976). *in vitro* synthesis of haemoglobin A1c. *FEBS lett* 71: 356-360.
- GO, R; T.J. QUINN and B. GONEN (1987). Enzyme linked immunosorbent assay for the measurement of nonenzymatically glycosylated proteins in serum and in tissues. *Clin. Chim. Acta* 163: 63-73.
- HAVEL, R.Y; H.A. EDER and H. BRAGDON (1955). The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* 34: 1345-1353.
- HUGGET, A and D.A. NIXON (1957). Use of glucose oxidase, peroxidase and o-dianisidine in the determination of blood and urinary glucose. *Lancet* ii: 368.
- JENNINGS, P.E and A.H. BARNETT (1988). New approaches to the pathogenesis and treatment of diabetic microangiopathy. *Diabetic Medicine* 5: 111-117.
- JOHSON, R.N.; P.A. METCALF and J.R. BAKER (1982). Fructosamine: a new approach to the estimation of serum glycosylprotein. An index of diabetic control. *Clin. Chim. Acta* 127: 87-95.
- JONES, I.R.; D.R. OWENS; S. WILLIAMS; R.E. RYDER; A.J. BIRTWELL; M.K. JONES; K. GICHERU and T.M. HAYES (1983). Glycosylated serum albumin: an intermediate index of diabetic control. *Diabetes Care* 6: 501-503.
- KOHLER, G. and C. MILNSTEIN (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256: 495-497.
- LAEMMLI, U.K. (1970). Cleavage of structural proteins during the assembly of head of bacteriophage T4. *Nature* 227: 680-685.
- LOPEZ, J. and J. ILLNAIT (1989). Desarrollo de un procedimiento sencillo para la obtención de anticuerpos contra la apolipoproteína B. *Rev. Cub. Invest. Biomed.* 8: 270-279.
- LLOYD, D.R.; M. NOTT and J. MARPLES (1985). Comparison of serum fructosamine with glycosylated serum protein for the assesment of diabetic control. *Diabetic Medicine* 2: 474-478.
- LORENZI, M; E. CAGLIERO; B. MARKEY; T. HENRIKSEN; J.L. WITZTUM and T. SAMPIETRO (1984). Interaction of human endothelial cells with glucose concentrations and native glycosylated low density lipoproteins. *Diabetologia* 26: 218-222.
- LOWRY, O.H; N.J. ROSERBROUGH; A.L. FARR and R.S. RANDALL (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265-275.
- NAKANE, P.A. and A. KAWAOI (1974). Peroxidase labelled antibody. A new method of conjugation. *J. Histochem. Cytochem.* 22: 1084-1091.
- NAKAYAMA, H; Z. MAKITA; M. KATO; S. TANEDA; H. YOSHIDA; K. YANISAWA and S. NAKAGAWA (1987). Quantitative enzyme-linked immunosorbent assay (ELISA) for non-enzymatically glycosylated serum protein. *J. Immunol Methods* 99: 95-100.
- OHE, Y; M. MATSAMURA; Y. NAKAJIMA; S. SHIN; F. HASHIMOTO; M. HIROTA and K. SHIMA (1987). Radioimmunoassay of glycosylated albumin with monoclonal antibody to glucocitol-lysine. *Clin. Chim. Acta* 169: 229-238.
- OUCHTERLONY, O. (1967). *Immunodiffusion and immunoelectroforesis. Handbook of Experimental Immunology*. Edited by D.M. Weir, Oxford and Edimburg, Blackwell Scientific Publications, p. 655.
- SORELL, L; M.E. FERNANDEZ; E. PENTON; J. LOPEZ and I.C. PEREZ (1986). Producción y características de anticuerpos monoclonales contra lipoproteínas que contienen apoproteína B. *Interferón y Biotecnología* 3: 239-241.
- WILCOXON, F. (1945). Individual comparison by ranking methods. *Biometrics Bull* 1: 80-83.
- WITZTUM, J.L; E.M. MAHONEY; M.J. BRANKS; M. FISHER; R. ELAM and D. STEINBERG (1982). *Diabetes* 31: 283-291.