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PURIFICATION OF GLYCATED HEMOGLOBIN FREE OF HEMOGLOBIN A_{1c} AND ITS USE TO PRODUCE MONOCLONAL ANTIBODIES SPECIFIC FOR DEOXYFRUCTOSYLLSINE RESIDUES IN GLYCOHEMOGLOBIN

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Hemoglobin nonenzymatically glycated at *E*-amino groups of lysine residues was purified from human erythrocyte lysates and used for immunization of BALB/c mice. Hybridomas secreting monoclonal antibodies for glycated hemoglobin were produced by fusion of mouse spleen cells with SP 2/0 myeloma cells. Immunoblotting with purified monoclonal antibody demonstrated specificity for glycated hemoglobin, with no reaction with HbA_0. Glycated hemoglobin was effectively separated from other hemoglobins upon application of erythrocyte lysates to an affinity column of monoclonal antibody immobilized onto Sepharose 4B. A small fraction of purified HbA_{1c} adsorbed to the monoclonal antibody affinity column, indicating that glycation can occur at both *E*-amino lysine and *N*-terminal valine positions in the same molecule. HbA_{1c} did not react with the antibody after removal by immunoadsorption of molecules containing glycated lysine, confirming specificity of the antibody for deoxyfructosyl-lysine residues. The findings indicate that these monoclonal antibodies are site specific for glycated lysine amino groups in hemoglobin, and can provide rapid and efficient separation and identification of glycated hemoglobin in human erythrocyte lysates.

The major products of the nonenzymatic condensation reaction between glucose and hemoglobin are hemoglobin A_{1c}, which is identical to hemoglobin A_0 except that glucose is linked to the amino-terminal valine residue of the beta chain, and hemoglobin glycated at other positions along the alpha and beta subunits (non-A_{1c} glycated hemoglobin), which is identical to hemoglobin A_0 except that glucose is linked to epsilon amino groups of lysine residues of the alpha or beta chains. The lysine residues that undergo glycation *in vivo* are, in order of prevalence, beta-lys-66, alpha-lys-61, and beta-lys-17 (1, 2). Formation of these glycated hemoglobin adducts, like that of HbA_{1c}, is increased in diabetic patients, and glycated hemoglobin can represent 10% or more of the total hemoglobin (3, 4). Unlike HbA_{1c}, however, modification by glucose at these other sites does not usually result in changes in electrophoretic or ion exchange chromatographic properties, and separation of glycated hemoglobin from HbA_0 or HbA_{1c} and its measurement as a distinct entity is not

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easily accomplished with traditional analytic methods. In this report, we describe the purification of glycated hemoglobin free of HbA_{1c} and HbA_0, its use to produce monoclonal antibodies that specifically recognize glycated lysine epitopes residing in hemoglobin, and the utilization of these antibodies to separate and identify glycated hemoglobin in human erythrocyte lysates.

Materials and Methods

Authentic glycated hemoglobin was purified from lysates of human erythrocytes by ion exchange chromatography to separate hemoglobin A_{1c} from hemoglobin A_0 and hemoglobin glycated in positions other than the A_{1c} configuration, followed by affinity chromatography on phenylboronate to separate unglycated hemoglobin from the glycated species. In brief, ion exchange chromatography was performed on a column of Bio-Rex 70 equilibrated with 0.05M potassium phosphate, pH 6.5, containing 0.01M KCN and 0.015M NaCl. Fast-moving hemoglobins modified at the *N*-terminal position (HbA_{1a}, A_{1b} and A_{1c}) elute under these conditions. The major hemoglobin A_0 peak (which also contains non-A_{1c} glycated hemoglobin) was then eluted by making the buffer 1.0M in NaCl. The fast moving peak was reapplied to Bio-Rex and eluted with a linear salt gradient (0.01 - 0.15M NaCl) for further purification of HbA_{1c}. The hemoglobin A_0 peak containing glycated hemoglobin was dialyzed against 0.01M acetate buffer, pH 8.5 containing 0.75 gm/L asparagine, 1.25 gm/L taurine, 0.746 gm/L methionine, 0.15M NaCl and 10 mM $MgCl_2$, and applied to a column of phenylboronate agarose equilibrated with the same buffer. The non-adsorbed fraction (HbA_0) elutes with this buffer, and the adsorbed (glycated hemoglobin) fraction is then eluted by making the buffer 100 mM in sorbitol and lacking the $MgCl_2$ and NaCl. If necessary, the glycated hemoglobin isolate can be further purified by repassage on phenylboronate. The amount of glycation in these preparations was determined by assay of hemoglobin (Sigma Chem Co.) and hydroxymethylfurfuraldehyde HMF (5). Glycated hemoglobin and HbA_{1c} purified from normal red cells respectively contained 1.0 mol HMF/mol of monomer and 1-2 mol HMF/mol tetramer. HbA_0 (non-adsorbed to PBA) contained less than 0.05 mol HMF/mol of monomer.

Female BALB/c mice were immunized with glycated hemoglobin over a four week period, followed by fusion of the mouse spleen cells with SP 2/0 myeloma cells and establishment of hybridoma colonies according to standard techniques (6). Resulting colonies with binding activity for glycated hemoglobin were cloned at least four times by limiting dilution. Binding activity was determined by ELISA, performed by immobilizing antigen onto plastic microtiter wells, blocking, washing, incubating with hybridoma supernatant, washing, and detection with alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma), substrate and amplification system (Bethesda Research Laboratories). The colony designated E85 showed excellent discrimination between glycated hemoglobin and HbA_0 in the ELISA and was used for further studies, performed with purified monoclonal antibody prepared from mouse ascites fluid by protein-G monoclonal antibody purification system.

Duplicate individual samples of HbA_0, HbA_{1c} and glycated hemoglobin were denatured with SDS and subjected to electrophoresis on 12% SDS-polyacrylaminde gels at 25-35 mA/gel. After electrophoretic transfer to 0.4 um nitrocellulose, one of each duplicate set of transferred gels was stained for protein with 0.5% amido black, and the other was blocked by soaking in a solution of 1% milk in Tris-buffered saline, pH 8.0, for one hour, then incubated for 2 hours in a solution of E85 (2 ug/ml) in the same buffer containing 0.01% milk. After washing, the nitrocellulose strips were soaked in a solution of alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (Bio-Rad; 1:3000 dilution in the same buffer) and developed with BCIP/NBT color development substrate (Promega) for AP BCIP immunoblot. Agarose gel electrophoresis was performed in Corning citrate agarose gels for 35 minutes at 90V in 0.1M sodium citrate, pH 6.2. Immunoaffinity chromatography was performed on a column of E85 immobilized onto Sepharose; the

monoclonal antibodies were coupled to CNBr-activated Sepharose 4B according to the manufacturer's instructions (Pharmacia). The column was equilibrated with 0.1M acetate buffer, pH 8.5 containing 0.15M NaCl and non-adsorbed hemoglobin species were eluted with the same buffer. Glycated hemoglobin binding to the immobilized E85 monoclonal antibodies were eluted with the above buffer containing 0.05M diethylamine, titrated to pH 11.5.

Results and Discussion

Figure 1 depicts HPLC analysis of HbA_{1c} and HbA_0 purified by the methods described above. There is wide separation between HbA_{1c} and HbA_0 and the peaks are discrete, confirming the absence of HbA_{1a}, A_{1b} or A_0 in the HbA_{1c} preparation and the absence of HbA_{1c} in the HbA_0 preparation. However, HPLC does not detect the presence of contaminating glycated hemoglobin in either peak, presumably because the charge modification resulting from glycation of lysine amino groups is insufficient to be registered by this analytic system. Absence of glycated species in the HbA_0 preparations was documented by the barely detectable glyco groups per mole of hemoglobin with HMF analysis (see above). On the other hand, preliminary ELISA and immunoblots of the HbA_{1c} preparation with monoclonal antibody E85 showed minimal reactivity, suggesting

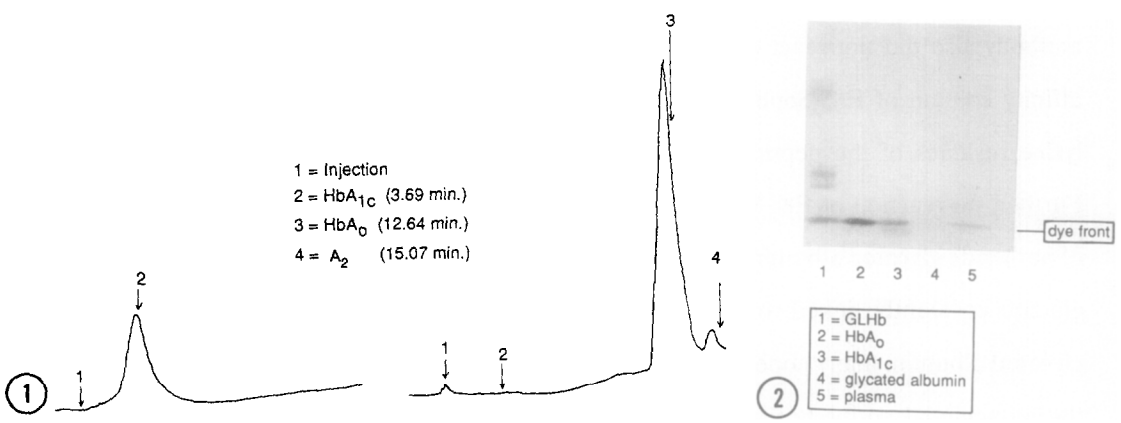

Figure 1
HPLC of purified preparations of HbA_{1c} (left) and HbA_0 (right). Performed on Waters HPLC with SP 5Pw resin. The column was pre-equilibrated with 96% buffer A (0.0124M NaH_2P0_4, 0.0032M KCN, 10% acetonitrile [v/v] added to each liter of buffer; pH titrated to 7.0) and 4% buffer B (0.12M NaH_2P0_4; 10% acetonitrile [v/v] added to each liter of buffer; pH titrated to 6.54) and run at a flow rate of 1.2 ml/min for a total of 16 minutes. The elution was programmed to change the ratios of buffer A to buffer B from 96/4 at time 0, 70/30 at 5 minutes, 30/70 at 10 minutes, and 0/100 at 11 minutes.

Figure 2
Immunoblotting on nitrocellulose with monoclonal antibody E85 of purified glycated hemoglobin, HbA_0, HbA_{1c}, glycated albumin, and human plasma. The HbA_{1c} preparation had been pre-adsorbed to an immunoaffinity column of immobilized monoclonal antibody. See text for details.

that some of this material contained glyco groups at *E*-amino lysine as well as *N*-terminal valine sites. This was confirmed by subjecting the HbA_{1c} preparation to an immunoaffinity column of E85, and subsequently analyzing the nonadsorbed and adsorbed material (see below).

On SDS-gel electrophoresis, the purified hemoglobin preparations migrated as expected showing monomer, dimer and tetramer bands at molecular weights of approximately 16,000, 32,000 and 64,000, respectively. The electrophoretic mobilities of the three preparations were similar, although some micro-heterogeneity was observed, consistent with minor modification of charge and molecular weight as a result of nonenzymatic glycation of the *N*-terminal valine in HbA_{1c} and of free amino groups of lysine residues in glycated hemoglobin. On agarose gel electrophoresis, HbA_{1c} migrated substantially faster than HbA_0, consistent with charge modification of the *N*-terminal valine; glycated hemoglobin migrated slightly more toward the anode than did HbA_0, consistent with slightly greater electronegativity as a result of glycation of lysine amino groups (not shown). After electrophoretic transfer of SDS gels and immunoblotting with monoclonal antibody E85, bands identical to those observed on amido black staining were visualized with glycated hemoglobin but not with HbA_0 (Figure 2). The monoclonal antibody also did not react on immunoblot with HbA_{1c} that had been passed through an affinity column of E85-Sepharose, indicating binding to glucose in covalent linkage with lysine residues of the peptide chain but not with *N*-terminal valine residues (Figure 2). Further, no reaction of E85 was observed after immunoblotting on nitrocellulose transfers of authentic glycated albumin or human plasma (Figure 2); since glycated albumin contains glucose covalently linked to *E*-amino lysine groups and plasma contains, in addition to glycated albumin, many nonenzymatically glycated proteins (7-9), these results indicate that the monoclonal antibody recognizes glycated epitopes residing in hemoglobin but not in other proteins.

Application of lysate from human erythrocytes to an affinity column of E85-Sepharose effectively separated glycated hemoglobin from unglycated hemoglobin (Figure 3A), confirmed with subsequent immunoblotting and agarose gel electrophoresis of the nonbound (HbA_0) and bound (glycated hemoglobin) fractions. When purified HbA_{1c} showing a single uniform peak on HPLC was applied to the E85 affinity column, a small portion bound to the immobilized monoclonal antibody (Figure 3B), indicating that some

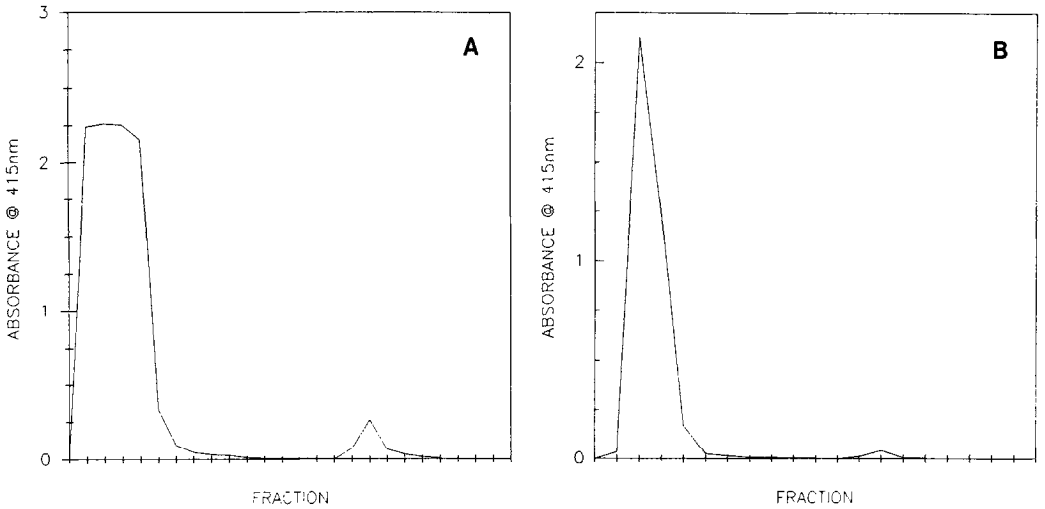

Figure 3

Immunoaffinity chromatography on E85-Sepharose of human erythrocyte lysate (A) and HbA_{1c} (B). 2 ml fractions were collected. Diethylamine elution applied at fraction 15 in A and at fraction 11 in B. See text for details.

hemoglobin A_{1c} molecules also are glycated at *E*-amino lysine sites. HbA_{1c} not binding to the affinity column showed no reaction on ELISA or immunoblot with E85, whereas the bound fraction reacted with the monoclonal antibody.

The foregoing results describe the purification of glycated hemoglobin free of HbA_0 and HbA_{1c} and demonstrate the successful utilization of this material to generate monoclonal antibodies that specifically recognize glycated lysine epitopes residing in hemoglobin. Since the eliciting antigen was not subject to modifying manipulation such as borohydride reduction and was purified by phenylboronate chromatography, it is presumed that the antibody recognizes the epitope in native (deoxyfructosyl-lysine) configuration. This contrasts with other antibodies described in the literature that might recognize glycated hemoglobin, wherein the eliciting antigen had been borohydride reduced and the resultant antibodies bind to glucitol-lysine but do not recognize the unreduced glycated adduct (10-12). According to Fluckiger (13), the site of *in vivo* glycation of most proteins can be predicted on the basis of local factors including the pK of the amino group, proximity of a catalytic proton donor/acceptor group, and primary structure, with a lys-lys sequence providing the most favorable milieu. These considerations suggest that the monoclonal antibodies described herein recognize glycated lysine in the beta-66 and/or alpha 61 positions, given that residues 63-67 in the beta chain and 58-62 in the alpha chain contain the identical sequence His-Gly-Lys-Lys-Val. The specificity of this antibody

provides opportunities for convenient, sensitive and accurate quantification of glycated hemoglobin and for its rapid and efficient separation from HbA_0 and HbA_{1c}.

Acknowledgments

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