Nonenzymatic Glycation of Immunoglobulins Does Not Impair Antigen–Antibody Binding

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We glycated immunoglobulins from commercial kits designed to measure human ferritin, thyrotropin, and transferrin, and compared the calibration curves for assays utilizing glycated antibodies with those of assays utilizing non-glycated antibodies. Glycation was verified by borate affinity chromatography and assay with thiobarbituric acid reagent. We found no evidence that antigen–antibody binding is impaired by nonenzymatic glycation of antibodies. Our results provide no evidence in support of the supposition that glycation may be a contributory factor in the decreased resistance of diabetics to infection.

Diabetics have impaired resistance to infection. Some causes for this are known, such as impaired granulocyte and lymphocyte function (1), but the underlying mechanism is not understood. Nonenzymatic glycation of immunoglobulins is well established, so it has been suggested that glycation may impair their function and thereby contribute to increased susceptibility to infection (2). Dolhofer et al. (3) found a marked decrease in complement-fixing capacity of antisera that had been incubated with high concentrations of glucose in vitro. Except for this single, still unconfirmed report, we know of no other study examining the effect of glycation on immunoglobulin function. We elected to examine the effects of immunoglobulin glycation on the specific binding of antibodies to their antigens, using the antibodies supplied in commercial immunoassay kits as models.

We glycated both monoclonal and polyclonal antibodies, labeled and unlabeled, in both solid and liquid phases. In our experiments we used kits designed for measurement of human ferritin, thyrotropin (thyroid stimulating hormone, TSH), and transferrin. Nonenzymatic glycation was verified by borate affinity chromatography and assay with thiobarbituric acid. Impairment of antibody function was assessed by comparing calibration curves for assays in which glycated or non-glycated antibodies were used.

Materials and Methods

The following immunoglobulins were incubated for as long as 10 days in the presence of 0, 120, 240, or 480 mmol of glucose per liter of 66 mmol/L sodium–potassium phosphate pH 8.2 buffer (sodium azide, 1 g/L, as preservative) at ambient temperature: (a) monoclonal mouse antiferritin on plastic beads (Hybritech, San Diego, CA 92121); (b) monoclonal mouse antiferritin conjugated to bovine alkaline phosphatase (Hybritech); (c) monoclonal mouse antiTSH on plastic beads (Abbott Laboratories, North Chicago, IL 60064); and (d) polyclonal goat antitransferrin, which was neither labeled nor bound to matrix (Atlantic Antibodies, Scarborough, ME 04074).

Immunoglobulins in solution were fractionated into glycated and non-glycated protein on columns of "GlycoGel" borate affinity support (Pierce Chemical Co., Rockford, IL 61105). Quantification was by turbidimetry on the Cobas-Bio centrifugal analyzer (Roche Diagnostics, Nutley, NJ 07110), with use of antibodies and protocol from Atlantic Antibodies. Glycation of immunoglobulins on beads was estimated by thiobarbiturate assay (4).

We assessed the effect of glycation on immunoglobulin function by comparing calibration curves for assays of each analyte with glycated and non-glycated antibodies, the assays being done according to the respective manufacturer’s calibrators and protocols.

Results and Discussion

It is evident from Figure 1 that immunoglobulins are readily glycated when incubated with glucose in vitro. Within four days of incubation in the presence of 480 mmol of glucose per liter, over 80% of immunoglobulins are sufficiently glycated to be retained on borate agarose. Thereafter, glycation progresses more gradually to the tenth day, when over 90% are retained. Even at 120 mmol of glucose per liter, the extent of glycation is remarkable. Our estimation of glycation of immunoglobulins immobilized on plastic beads (Figure 2) indicates that saturation concentrations...
are equivalent to 1.4–1.6 mmol of glucose per liter. This is consistent with a sevenfold to eightfold molar ratio of glucose to immunoglobulin, based on a concentration of approximately 0.20 nmol (30 µg) of immunoglobulin per bead (personal communication, Dale Sevier, Hybritech). This facile glycation of immunoglobulins was fully anticipated, because about 7% of the internal amino acid residues are lysine residues (5).

In view of the ready glycation of immunoglobulins, the presence of several presumably glycatable lysines within 15 amino acid residues of the N terminal of each chain (antigen binding site), the decreased resistance of diabetics to infection, and the report of Dolhefer et al. (3), we anticipated finding pronounced impairment of either antibody specificity or avidity, or both. Figure 3 illustrates the hypothetically anticipated responses should specificity, avidity, or both be impaired.

Instead, we found no significant deviations in the calibration curves of glycated and non-glycated assays. Figure 4a shows our results with the ferritin assay on using unglycated beads and beads glycated over a period of six days. There is virtually no significant difference in the results with beads glycated for three days (not shown) were even less different. It could even be argued from the data that glycation, particularly less than maximal glycation, enhances immunoglobulin binding of antigen. These essentially negative results led us to conjecture that immobilized immunoglobulins are possibly already impaired in their ability to bind antigen and that, if anything, glycation may alter the tertiary structure sufficiently to enhance their function slightly.

To eliminate the possibility that the results with the antiferritin beads were unique to this particular assay, we examined another bead-based assay, Abbott’s TSH. As evident from Figure 4b, the data revealed no altered binding properties. Our attention then turned to non-immobilized immunoglobulins. Figure 4c shows the results with nonglycated and glycated antiferritin immunoglobulins conjugated to alkaline phosphatase. Again, the results suggested no alteration of binding properties, with the possible exception of a slight, but equivocal, negative effect at maximum glycation.

Finally, we turned to immunoglobulins that were not immobilized, not conjugated, and not monoclonal. We examined a turbidimetric assay for transferrin (see Figure 4d). Again, results suggested no clear impairment of antigen–antibody binding. Again, there was instead an equivocal suggestion of possible binding enhancement with moderate glycation and very slight impairment with maximum glycation.

The sevenfold to eightfold ratio of glucose to immunoglobulins in our bead experiment and the approximately 7% of the residues that are lysyl (5) indicates that less than 10% of these lysine residues are glycated. The N-terminal acid of the heavy chains and lambda light chains is pyrrolid-2-1-5 carboxylic acid (sic) and is unreactive. Kappa light chains generally have aspartic acid as an N-terminal amino acid, and they could be glycated. At our experimental pH of 8.2, and more so at physiological pH, the ionization constant of the epsilon ammonium group of lysine (9.5–10.5) suggests only limited Schiff base formation. The irreversible Amadori rearrangement, however, would shift the equilibrium in favor of the overall glycation. In continuing experiments we will attempt to “titrate” immunoglobulins, using glycation as a tool. We will also extend our studies to include glycation of epsilon ammonium groups at higher pH. It should be useful to determine the minimum molar ratio of glucose to immunoglobulin required for immunoglobulins to be retained on borate affinity support. Some questions remain about possible differences in response of human versus non-human antibodies. Commercial assays in which human antibodies are used are not available, so comparable studies with human antibodies would require that we first develop appropriate assays using human antibodies. It may be possible to use advantageously some serological tests for human antibodies.

While we have been able to confirm unambiguously that immunoglobulins are readily non-enzymatically glycated, we were not able to find any clear evidence that antigen–antibody binding is impaired with glycated immunoglobulins. There is equivocal evidence that glycation possibly may alter immunoglobulin structure sufficiently to cause slight enhancement or slight impairment of antigen–antibody binding. When we consider that the conditions we used were near optimum for glycation (2), that exceptionally extensive glycation was attained, and that this extent of glycation exceeds what we have found in vivo (6), it seems to us very unlikely that impairment of immunoglobulin bind-

![Fig. 3. Hypothetical responses for specificity, avidity, and mixed antibody impairment](image-url)
ing functions contributes to decreased resistance to infection in diabetics. Although our conclusion is tempered by our use of non-human antibodies, it is not unreasonable to expect that human antibodies would respond in like manner. Furthermore, our conclusion is consistent with the clinical observation that diabetic response to infection improves promptly on rectification of hyperglycemia (1), which ought not to be the case were these glycated immunoglobulins impaired.

References

Analysis for Carboxyhemoglobin by Gas Chromatography and Multicomponent Spectrophotometry Compared

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Measurements of carboxyhemoglobin (HbCO) by gas chromatography (GC) were compared with those obtained by multicomponent spectrophotometric analysis (MCA). Correlation was good for HbCO ranges (proportion of total hemoglobin) of 1.5 to 5% (GC = 0.94 MCA + 0.37%, n = 25, r2 = 0.98); 5.0 to 10.0% (GC = 0.89 MCA + 0.63%, n = 18, r2 = 0.93); >10% (GC = 1.01 MCA - 0.02%, n = 22, r2 = 0.99); and >0% (GC = 1.01 MCA - 0.10%, n = 95, r2 = 1.00). The correlation is lower for the clinically normal range of 0 to 1.5% (GC = 0.65 MCA + 0.24%, n = 29, r2 = 0.87). The gas-chromatographic method is linear for HbCO proportions of 30% to as little as 0.15%. Re-analysis of blood samples after two-month storage at 4°C showed that samples remained stable under these conditions.

Additional Keyphrases: neonates • pediatric chemistry • control materials • assessing exposure to CO

The carboxyhemoglobin (HbCO) content of blood is measured to assess the effects of exposure to carbon monoxide from environmental sources (1, 2). In addition, this determination is used to assess hemolysis in newborns, a process that yields endogenous CO (3, 4). For routine HbCO measurements, several methods are available, based mainly on spectrophotometry (5, 6). However, gas chromatography (GC) is, perhaps, the method of choice for determining very low proportions of HbCO (<1.5% of total hemoglobin). Furthermore, samples to be assayed by GC are more stable than those used for spectrophotometry, and the required sample volume is smaller (7). Thus GC is particularly suitable for use with blood samples from infants and in cases where immediate on-site analysis is not possible.

Recently, a sensitive GC method (7) and a multicomponent analysis spectrophotometric (MCA) method (8) have been reported for determination of HbCO. Although the GC method was not validated against other, non-GC methods, the MCA HbCO determination has been validated with a chemical titrimetric method (9). We undertook the present study to compare the HbCO values measured in whole blood by GC with those measured by the MCA method. Furthermore, we investigated the linearity of the GC method for HbCO proportions down to approximately 0.15%. Finally, because no assayed quality-control samples exist at present for measurement of HbCO by GC, we describe a method for preparing whole-blood control samples for internal quality-control purposes.

Materials and Methods

Subjects and Samples

Blood samples were obtained from specimens sent to the routine clinical laboratory for analysis for hemoglobin derivatives. Samples were from both smokers and nonsmokers. Most of the samples with HbCO proportions >5% were obtained by tonometry of patients’ blood with pure CO (1, 5).

Preparation of Control Samples

HbCO control samples were prepared from outdated whole blood in a blood bag obtained from the Stanford University Medical Center Blood Bank. Per 500 mL of blood, we added 500 mg of saponin (Sigma Chemical Co., St. Louis, MO) dissolved in 5 mL of distilled water. This was mixed well, then centrifuged for 10 min at 3000 × g. The “low control” hemolyzed blood in the supernate was transferred to pediatric HbCO sample tubes (see below) with a syringe. For the “high control” sample, we mixed 0.6 mL of CO gas (purity 999 mL/L) with 100 mL of the hemolytically supernate (total hemoglobin concentration approx. 126 g/L) in a glass syringe. After 2 h of incubation, with periodic