the hapten to an iodinatable derivative or solid phase with a chemical bond different from that used for linking the hapten to the carrier protein immunogen (23), may prove successful for cotinine.

We have previously reported that the cotinine concentration in an untimed urine specimen shows significant correlation with self-assessed ETS exposure; our present clinical studies corroborate these findings. We have also reported that cotinine as measured by the present assay in the serum of nonsmoking mothers in the second trimester of pregnancy is inversely correlated with birthweight (13). In the present study, serum cotinine correlated significantly with self-reported exposure. Furthermore, serum and urine cotinine concentrations were highly correlated, indicating that either serum or an untimed urine specimen can reliably be used to estimate exposure to ETS. These findings agree with those of others (3, 12), who conclude that the choice of body fluid for epidemiological studies can respond to the needs of the study design rather than any inherent advantage of measuring cotinine in particular body fluids.

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References

Nonenzymic Glycation of Human Immunoglobulins Does Not Impair Their Immunoreactivity
Leo G. Morin,1,4 Garth E. Austin,1,2 Glenn E. Rodey,1 and James E. Johnson3

Diabetic patients have an increased proportion of their immunoglobulins nonenzymically glycated. To investigate the possibility that this may contribute to increased susceptibility to infection, we compared the immunoreactivity of glycated and nonglycated human immunoglobulins against rubella and hepatitis; streptococcal exoenzyme and infectious mononucleosis; human lymphocytotoxic antigens (HLA); and Vari- cella zoster in terms of antigen–antibody binding, cell agglu- tination, cytotoxicity, and complement-fixation properties, re- spectively. We found no evidence to support the supposition that glycated immunoglobulins are functionally impaired.

Diabetic patients are generally—though not universal-ly—recognized to have impaired resistance to infection (1). Two recent reports lend support to the speculation that

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impaired immunoglobulin activity, resulting from nonenzymic glycation, may be a contributing factor: Dolhofer et al. (2) found a marked decrease in complement-fixing capacity of both human and rabbit antisera that had been incubated with high concentrations of glucose in vitro. More recently, Kaneshige (3) reported evidence to suggest that glycated human immunoglobulins against streptolysin and influenza virus were functionally impaired.

We, however, found no evidence to support impairment of antigen–antibody binding with goat polyclonal and mouse monoclonal antibodies (4). In an effort to resolve the apparent discrepancy between our findings and those of Dolhofer et al. (2) and Kaneshige (3), we extended our studies to human immunoglobulins. We glycated human anti-rubella IgG and IgM, as well as antibodies against Varicella zoster, streptococcal exoenzyme, heterophile antibodies associated with infectious mononucleosis, and human lymphocytotoxic antibodies (HLA). We also separated human immunoglobulins from diabetic patients into glycated and nonglycated fractions, normalized the fractions for immunoglobulin content, and compared their immunoreactivity for rubella and hepatitis antibodies. Despite the broad spectrum of antibodies examined, the use of both whole serum and purified immunoglobulins, and the use of diverse assays based on antigen–antibody binding, complement fixation, cell agglutination, and sensitive complement-mediated cytotoxicity, we were unable to detect (with one exception) any evidence of immunoglobulin impairment resulting from nonenzymic glycation.

Materials and Methods

Pooled and individual sera from nondiabetic patients were glycated by incubation with glucose (480 mmol/L) for five days at ambient temperature in 66 mmol/L sodium-potassium phosphate buffer, pH 8.2, containing sodium azide, 1 g/L. As previously reported (4) and reconfirmed in the present studies, this assures that 80–90% of the immunoglobulins are sufficiently glycated to be retained on "GlycoGel" boronate affinity support (Pierce Chemical Co., Rockford, IL 61105). Likewise, we glycated human IgG anti-rubella antibodies that had been previously purified by affinity separation on beads coated with human rubella virus ("Rubzyme" beads; Abbott Laboratories, North Chicago, IL 60064). Adsorption and bead washing were done in phosphate-buffered saline and elution was with pH 3.0 glycine buffer (80 mmol/L). Eluted fractions were concentrated by ultrafiltration in a Centricon 30 (Amicon, Danvers, MA 01923). Human sera previously typed for HLA antibodies were glycated in the same manner. After glycation, glucose was removed by dialysis. Control aliquots were treated in the same manner, except that glucose was not included in the incubation. Immunological activity of both treated (glycated) and untreated (control) aliquots was evaluated before and after incubation.

Sera from 22 diabetic patients (glycated hemoglobin >15%) were divided into three pools and separated into glycated and nonglycated fractions on columns of GlycoGel as previously described (5, 6); the immunoglobulin content was estimated by turbidimetry with antibodies and protocols from Atlantic Antibodies (Scarborough, ME 04074), and the immunoglobulin concentration of the nonglycated fraction was normalized to that of the glycated fraction.

Rubella antibodies were measured by the Rubzyme quantitative immunoassay. Complement fixation was performed by the micro adaptation of the standardized Labo-

ratory Branch Complement Fixation (LBCF) test (7). Antibodies to streptococcal exoantigens were titrated with "Streptozyme SIZ" (Wampole Laboratories, Cranbury, NJ 08512). Heterophile antibodies associated with mononucleosis were titrated with "Mono-Diff" (Wampole). HLA antibodies were measured by both a complement-dependent lymphocytotoxicity test and an antiglobulin-augmented complement-dependent lymphocytotoxicity test as described by Rodey and Fuller (8). Anti-hepatitis A total antigen, anti-hepatitis B surface antigen, and hepatitis B core antibodies were assessed with the appropriate kits from Abbott Laboratories.

Results

The Abbott Rubzyme assay is based on antigen-antibody binding; as shown in Table 1, there are no indications that glycated immunoglobulins against rubella are impaired in their ability to bind rubella antigen. This is true for both IgG and IgM, whether whole serum or purified antibodies are used. In fact, there is a slight bias in the data to suggest that glycated antibodies may have enhanced immunoreactivity.

Complement-fixation evaluations (Table 2) of 16 individual sera for Varicella zoster antibodies did not yield any indication that glycation interfered with the complement-fixing properties of immunoglobulins against this virus. Here too, several sera suggest that glycation may enhance immunoreactivity. Further, no impairment of complement-fixing activity could be noted, even when the antigen concentration was varied (Figure 1). At antigen dilutions >1:64, glycated antibodies appear to have enhanced reactivity.

Two distinct assays based on cell agglutination, one for anti-streptococcal antigens and the other for anti-mononucleosis-associated antigens, revealed no impairment of antibody function by glycation (Table 3).

A very sensitive complement-mediated cytotoxicity assay for HLA antigens likewise did not reveal any impairment of immunoglobulin function, except in two cases when cross-reactive target cells were used (Table 4). The

<table>
<thead>
<tr>
<th>Table 1. Rubzyme Index for IgG and IgM of Glycated and Nonglycated Aliquots of Pooled Human Sera and Purified Human Anti-Rubella IgG</th>
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</thead>
<tbody>
<tr>
<td>Rubzyme Indexb</td>
</tr>
<tr>
<td>Poola (n = 10)</td>
</tr>
<tr>
<td>A (IgG)</td>
</tr>
<tr>
<td>B (IgG)</td>
</tr>
<tr>
<td>C (IgG)</td>
</tr>
<tr>
<td>D (IgG)</td>
</tr>
<tr>
<td>E (IgM)</td>
</tr>
<tr>
<td>Pure anti-rubella IgG</td>
</tr>
<tr>
<td>IgG (n = 6)</td>
</tr>
</tbody>
</table>

* Poools created from samples with approximately the same pre-incubation values for Rubzyme index, sufficiently glycated to retain 80–90% of Ig on boronate affinity support; glycation of pure anti-rubella IgG assessed on affinity support as described earlier (4). "Size" refers to the number of samples in each pool. "n" to number of replicates.

b Ratio of response (measured as absorbance) of sample to response of low positive control. The higher the value, the greater the concentration of anti-rubella antibody.
relatively minor effects noted are remarkable for their slightness in view of the extremely high and nonphysiological concentrations of glucose used.

Pooled sera from diabetic patients, separated into Glyco-Gel-bound (glycated) and unbound (nonglycated) fractions and tested for rubella and hepatitis antibodies by antigen-antibody binding (Table 5), did not reveal any suggestion that immunoglobulins glycated in vivo were impaired. Again, a slight enhancement of immunoreactivity was suggested.

Discussion

To our knowledge, besides our earlier report (4), only two published reports have examined the effects of glycation on immunoglobulin function, and these two reports appear to be at odds with our own observations.

Dolhofer et al. (2) reported a decrease in complement-fixation properties for human and rabbit IgG incubated with relatively low concentrations of glucose for several days. Their results are now surprising to us, because we were unable to detect any notable impairment of immunoglobulin function either with a complement-fixation assay for Varicella zoster (also used by Dolhofer et al.) or even with an extraordinarily sensitive complement-mediated assay (HLA). Although we did observe an apparent impairment of function with one antisemur when cross-reacting cells were used, the effect was unique and not pronounced. On the basis of the very extensive glycation attained in our experiments and the unambiguous results of Dolhofer et al., we expected a more pronounced and more generalized effect.

Although Dolhofer et al. state that they used a micro complement-fixation test, apparently they were actually using a macro (milliliter quantities) test. Most laboratories performing complement fixation for serological diagnosis use the micro LBCF procedure, which yields semiquantitative results of twofold serial dilutions. The method used by Dolhofer et al. involved a spectrophotometric determination of hemolytic activity. These methods may not be directly comparable. As shown in Figure 1, it is possible to derive curves similar to those of Dolhofer et al. by varying the antigen concentration. We observed no difference in complement activity between glycated and nonglycated specimens for a twofold dilution increment, whereas Dolhofer et al. observed a 50% decrease in hemolytic activity. Although a 50% decrease may potentially be less than that needed to observe a twofold dilution decrease, we find their conclusion of a significant decrease in complement activity to be tenuous, because significant changes in titer are usually measured by fourfold increments. Also, complement-fixation procedures are performed with excess complement, to ensure complete reactions in vitro: what relation this may have to in vivo activity is not known. Standardized serologic complement-fixation tests with whole sera indicate no detectable effect of glycation on the complement-fixing activity.

The report of Kaneshige (3) appears flawed to us for several reasons. The data reported in his Table 1 show a significant decrease in anti-streptolysin O titer, even when the incubation was done without glucose. In fact, when his glycated samples are compared with nonglycated controls (rather than pre-incubation controls), the titers are well within 2 SD of each other. The incubation conditions described make no mention of microbial preservatives or protease inhibitors, and the results appear to us to be more consistent with degradation of proteins than the effects of glycation. The immunization data are significantly different when controls and diabetics are compared, but the
Table 4. HLA Titers for Glycated and Nonglycated Alliquote of Antisera of Defined Specificities

<table>
<thead>
<tr>
<th>HLA specificities</th>
<th>Nonglycated</th>
<th>Glycated</th>
<th>Nonglycated</th>
<th>Glycated</th>
<th>Nonglycated</th>
<th>Glycated</th>
<th>Nonglycated</th>
<th>Glycated</th>
</tr>
</thead>
</table>

*Brackets indicate target cell type. Data in parentheses are pre-incubation values. Most determinations were performed in two sets of triplicates. Samples were sufficiently glycated to retain 80–90% of Ig on borate affinity support; nonglycated samples retained 3 to 7%. CDC, complement-dependent lymphocytotoxicity test. AHG, antiglobulin-augmented CDC test.

Table 5. Antibody Reactivity of In Vivo Glycated (G) and Nonglycated (N) Fractions Compared in Three Pooled Sera from Poorly Controlled Diabetic Patients

<table>
<thead>
<tr>
<th></th>
<th>Pool 1</th>
<th>Pool 2</th>
<th>Pool 3</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>G</td>
<td>N</td>
</tr>
<tr>
<td>Rubazyme*</td>
<td>4.20</td>
<td>4.26</td>
<td>2.14</td>
</tr>
<tr>
<td>HAVAB*</td>
<td>1.21</td>
<td>1.09</td>
<td>1.16</td>
</tr>
<tr>
<td>AntiHB*</td>
<td>1.14</td>
<td>1.09</td>
<td>1.16</td>
</tr>
<tr>
<td>HB core*</td>
<td>0.88</td>
<td>0.96</td>
<td>0.96</td>
</tr>
<tr>
<td>Glycated Ig, %</td>
<td>26</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>No. samples in pool</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Glycated Hgb, %</td>
<td>18</td>
<td>16</td>
<td>16</td>
</tr>
</tbody>
</table>

*Expressed as ratio of reactivity of nonglycated to glycated where the nonglycated response is set to 1; n = 3; CV = 3 to 7%.

In summary, our data—encompassing a relatively broad scope of human and animal antibodies, assessed by antigen–antibody binding, complement fixation, agglutination, and cytotoxicity—indicate that glycated antibodies are not immunologically impaired.

Both the present study and our previous study (4) were undertaken with the anticipation of finding clear evidence of immunoglobulin impairment with glycation. In our earlier report, involving animal immunoglobulins, we used physiological as well as physiologically incompatible concentrations of glucose to achieve glycation, and found that, regardless of the extent of glycation, no evidence of antigen–antibody binding impairment could be discerned. In the current study, we explored several antibodies and several alternative approaches in search of evidence to support the intuitive supposition that glycated antibodies may be impaired. The overall investigation was both broad and extensive, yet we found little evidence to suggest that glycated antibodies are impaired. Because we attained in vitro glycation to the extent that 80–90% of immunoglobulins were retained on boronate gel, whereas immunoglobulins from poorly controlled diabetics are retained to no more than 28%, it is very unlikely that immunoglobulins of diabetics are functionally impaired. Even antibodies glycated in vivo, when compared with their unglycated counterparts, did not reveal any evidence of impairment. This is quite consistent with clinical observations that diabetics improve promptly in immune competence on correction of the hyperglycemia (1, 9).

References