Nonenzymatic Glycosylation of Serum IgG and Its Effect on Antibody Activity in Patients With Diabetes Mellitus

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SUMMARY

Susceptibility to infection is assumed to be increased in diabetic patients, although its mechanism is unknown. The purpose of this study was to determine whether glycosylation of circulating immunoglobulins is related to the decrease of antibody activity in diabetic patients. Thirty-five patients with type II (non-insulin-dependent) diabetes and 14 age-matched normal controls were examined. Nonenzymatic glycosylation of serum immunoglobulin G (IgG) in vivo was measured by two different techniques, colorimetry and affinity chromatography. The levels of glycosylated IgG were significantly higher in diabetic patients than in normal controls.

To evaluate the antibody activity of glycosylated IgG, anti-streptolysin O (ASO) titers after in vitro glycosylation of IgG and antibody titers before and after in vivo immunization with influenza vaccine were determined. IgG specific for streptolysin O purified by affinity chromatography decreased ASO titers after in vitro glycosylation. In diabetic patients, serum titers of hemagglutinin-inhibiting antibody against influenza viruses 4 wk after initial immunization were significantly lower than those in normal controls. These results indicate that serum IgG in diabetic patients was nonenzymatically glycosylated, and this modification in vivo might be associated with its functional alteration. *Diabetes* 36:822–28, 1987

Recent observations suggest that various proteins in diabetic patients, including hemoglobin (1), albumin (2), collagen (3), crystalline lens (4), erythrocyte membrane proteins (5), lipoproteins (6), and other proteins (7,8), are nonenzymatically glycosylated in vivo. This postsynthetic modification of proteins by nonenzymatic glycosylation has opened new approaches that may contribute to clarifying the pathogenesis of long-term and progressive complications in diabetic patients. It is postulated that glycosylation of proteins in diabetics results in functional alterations and thus induces the development of various complications in patients with diabetes mellitus. Serum immunoglobulin G (IgG) was chosen in this study to elucidate the relationship between glycosylation and functional alteration of protein molecules in diabetic patients.

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MATERIALS AND METHODS

Thirty-five patients with type II (non-insulin-dependent) diabetes without renal failure or infections and 14 age-matched healthy adults were examined in this study. Thirty-seven percent of the diabetics and 28% of the controls were women. The ages of the patients ranged from 28 to 74 yr (52.4 ± 12.9, mean ± SD), and that of the controls ranged from 29 to 66 yr (48.2 ± 12.6) ($P > .1$). No patient with annual immunizations of influenza vaccines was included in this study. Serum IgG levels were measured by laser nephelometry (9). Mean values of serum IgG were 1225 ± 126.9 mg/dl in diabetic patients and 1239 ± 65.7 mg/dl in control subjects ($P > .1$).

Purification of serum IgG. Purified IgG fractions were obtained from serum samples by affinity chromatography on immobilized Cibacron Blue F3-GA (Bio-Rad, Richmond, CA) (10). Briefly, the serum sample was dialyzed for 12 h against the starting buffer solution (0.02 M K_2HPO_4 and 0.02% NaN_3, pH 8.0) and then applied to the affinity column. Five milliliters of initial fractions were obtained and eluted with three bed volumes of the starting buffer. The sample solution was concentrated to 2.0 ml with MINICON-B (Amicon, Danvers, MA), and the concentration of IgG was quantitated by the protein-dye method (11). The purity of the fractionated IgG was confirmed by immunoelectrophoresis.

Glycosylated IgG and albumin. Glycosylated IgG was determined by colorimetry (12) and affinity chromatography

(13). The colorimetric technique was based on the quantitation of nonenzymatically bound glucose released as 5-(hydroxymethyl)-2-furaldehyde (HMF). In this procedure, 0.5 ml of IgG samples, previously purified by affinity chromatography, were mixed with 0.5 ml of 1 M oxalic acid in screw-capped tubes. These tubes were tightly sealed and incubated at 100°C for 5 h. The contents of the tubes were allowed to cool to room temperature, and protein was precipitated by addition of 0.5 ml of 40% trichloroacetic acid and then centrifuged at 3000 rpm for 40 min at room temperature. Then 1.0 ml of supernatant was incubated with 0.5 ml of 0.05 M thiobarbituric acid at 37°C for 30 min. The absorbance at 443 nm was measured photometrically. Each sample was assayed in duplicate against its own $NaBH_4$-reduced blank, and glycosylated IgG was expressed as nanomole HMF per milligram protein with pure HMF (Sigma, St. Louis, MO) as a standard. An intra-assay C.V. of 9% and an interassay C.V. of 11% were obtained in this assay with IgG sample pools.

Affinity chromatography with the Glyc-Affin system (Isolab, Akron, OH) was employed for determination of glycosylated IgG and albumin. Briefly, 200 μl of IgG samples were applied to a glucose-elimination column, and free glucose was removed by preconditioned resin. After the sample was eluted by glycine buffer, the eluant was applied to a boronic acid–affinity column. Nonglycosylated and glycosylated fractions were eluted by glycine and sorbitol solution, respectively. The concentration of IgG in each fraction was determined by Glyc-Affin protein reagent. The absorbances at 620 nm of glycosylated and nonglycosylated fractions were measured, and percent glycosylated IgG was calculated by the formula

$$\text{percent glycosylated IgG} = [G/(G + 6N)] \times 100$$

where G is absorbance of the glycosylated fraction, and N is absorbance of the nonglycosylated fraction.

Glycosylated albumin was also measured by the Glyc-Affin system. Hemoglobin A_1 (HbA_1) was measured by the column chromatography method of Welch and Boucher (14).

Purification of specific IgG for streptolysin O. To determine the alteration in IgG function after glycosylation, specific IgG for streptolysin O was prepared by affinity column chromatography as follows. CNBr-activated Sepharose 4B (5.0 g) was swollen in 100 ml of 1 mM HCl and then washed three times with 0.1 M borate buffer (pH 8.5) containing 0.5 M NaCl. Streptolysin O (Nissui, Tokyo, Japan) containing 100 mg of protein was mixed with Sepharose 4B and stirred gently at room temperature for 2 h. Unbound protein was washed from the resin with 0.1 M borate buffer and reacted with 1 M ethanolamine at room temperature for 1 h. The resin was washed with 0.1 M acetic buffer (pH 8.0) and then equilibrated with phosphate-buffered saline (PBS; pH 7.2) containing 0.5 M NaCl. Streptolysin O–bound Sepharose and IgG solution were mixed gently for 1 h at room temperature, washed with PBS three times, and then applied to the column. This column was thoroughly washed with PBS to remove unbound IgG, and highly purified IgG was eluted with 0.05 M glycine/HCl buffer (pH 2.2). The eluate was neutralized by 0.5 M $NaHCO_3$ and dialyzed against PBS for 12 h. This product was concentrated by ultrafiltration.

In vitro glycosylation of purified IgG and assay of antistreptolysin O titer. Purified IgG specific for streptolysin O obtained by affinity chromatography was incubated in RPMI-1640 (Gibco, Grand Island, NY) with the addition of 0, 22.2, and 44.4 mM glucose in 5% CO_2 for up to 8 days. Anti–streptolysin O (ASO) titers were measured after IgG was dialyzed against PBS for 12 h and concentrated by ultrafiltration to 4.0 mg/ml. The determination of ASO titer was carried out by a modification of the method of Hodge and Swift (15) and expressed in Todd units. The measurement of in vitro glycosylated IgG was performed by affinity chromatography with the Glyc-Affin system.

Administration of influenza vaccines and assay of hemagglutinin-inhibiting (HI) titer. The influenza vaccine used in this study was a commercially available vaccine [influenza hemagglutinin (HA) vaccine, Takeda, Osaka, Japan], containing 1 ml of the viral strains A/Ishikawa/7/82 (H3N2), A/Kumamoto/37/79 (H1N1), and B/Singapore/222/79. The initial injection of HA vaccine (0.5 ml) was given subcutaneously, after obtaining consent for influenza immunization, in October and November 1983. Repeated immunization by HA vaccine (0.5 ml) was performed 4 wk after the initial injection. Serum samples were obtained on the day of immunization and during the 4th and 8th wk after the initial immunization.

Serum titers of HI antibody were determined against two viral strains, including A/Ishikawa/7/82 and B/Singapore/222/79. HI antibody was titrated according to standard lab-

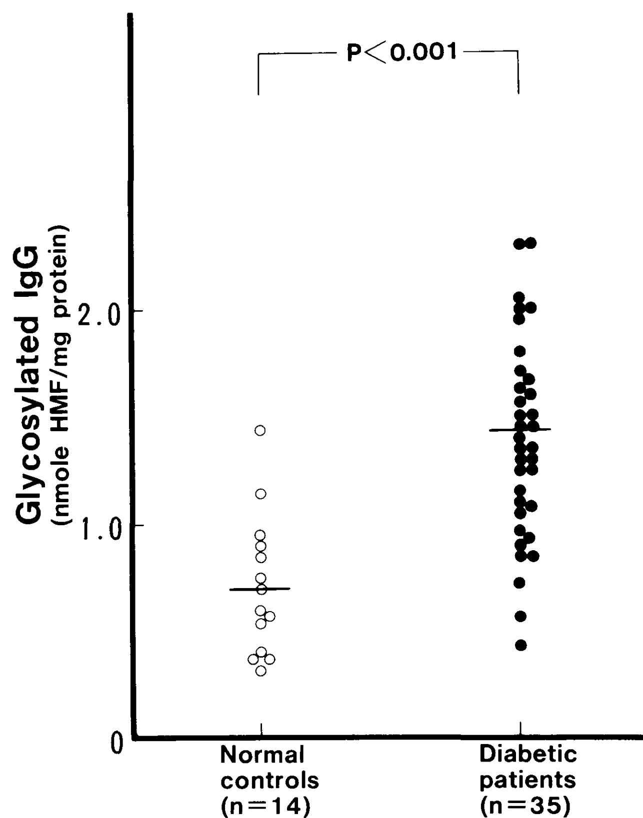

FIG. 1. Glycosylated IgG levels in normal controls and diabetic patients determined by colorimetric assay. *Horizontal lines* show mean values.

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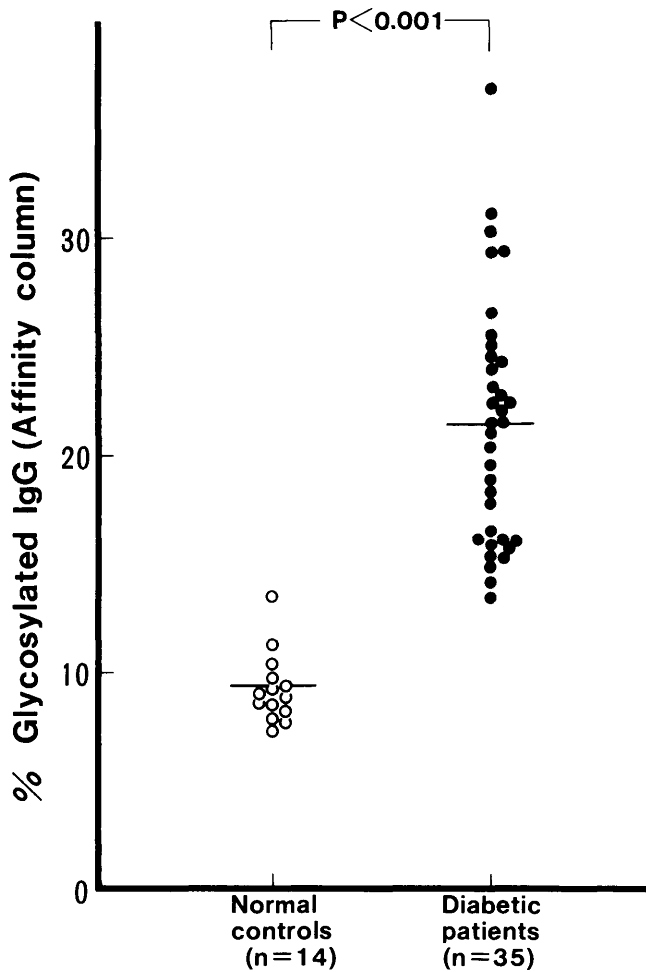

FIG. 2. Glycosylated IgG levels in normal controls and diabetic patients measured by affinity chromatography. *Horizontal lines* show mean values.

oratory techniques (16). HbA_1 was measured on the day of immunization and 8 wk after the initial immunization. Glycosylated IgG and albumin were determined on the day of immunization.

Statistical analysis. Data are expressed as means ± SD to show ranges of variation. Linear regression analysis and the Mann-Whitney *U* test were used for statistical analysis.

RESULTS

The concentration of purified IgG obtained by affinity chromatography with immobilized Cibacron Blue F3-GA ranged from 6.6 to 8.0 mg/ml. Glycosylated IgG determined by the colorimetric method showed significantly ($P < .001$) high levels in diabetic patients (1.43 ± 0.47 nmol HMF/mg protein) compared with those in normal controls (0.70 ± 0.31 nmol HMF/mg protein) (Fig. 1).

Glycosylated IgG was also measured by affinity chromatography (Fig. 2). The percentage of glycosylated IgG in diabetic patients (21.36 ± 5.43%) was significantly ($P <$ .001) higher than that in normal controls (9.27 ± 1.50%). There was a significant correlation ($P < .01$) between measurement of glycosylated IgG by colorimetry and by affinity chromatography (Fig. 3). Significant correlations were also observed between HbA_1 and percent glycosylated IgG ($r = .72$, $P < .01$), as well as between percent glycosylated IgG and percent glycosylated albumin ($r = .86$, $P < .001$) (Fig. 4). Furthermore, percent glycosylated IgG in diabetic patients showed a strong correlation with percent glycosylated albumin ($r = .70$) compared with HbA_1 ($r = .34$) when the diabetic data were plotted separately.

Table 1 shows the decreased ASO titers after in vitro glycosylation of IgG obtained from four healthy adults by affinity chromatography. The mean increase of percent glycosylated IgG after in vitro glycosylation for 8 days was from 9.2 to 24.3% in the incubation with 22.2 mM glucose and from 9.6 to 42.8% with 44.4 mM glucose. Approximately 5% of purified IgG specific for streptolysin O was obtained from the total IgG applied to the affinity column. ASO titers (Todd units) before incubation (day 0) were 220 ± 66.3, and significantly ($P < .02$) decreased ASO titers were observed 8 days after incubation, i.e., 95 ± 38.4 with 22.2 mM glucose and 60 ± 14.1 with 44.4 mM glucose.

Table 2 shows that HbA_1 levels in both diabetics and controls before immunization were not significantly different from those after immunization. Table 3 shows HI antibodies

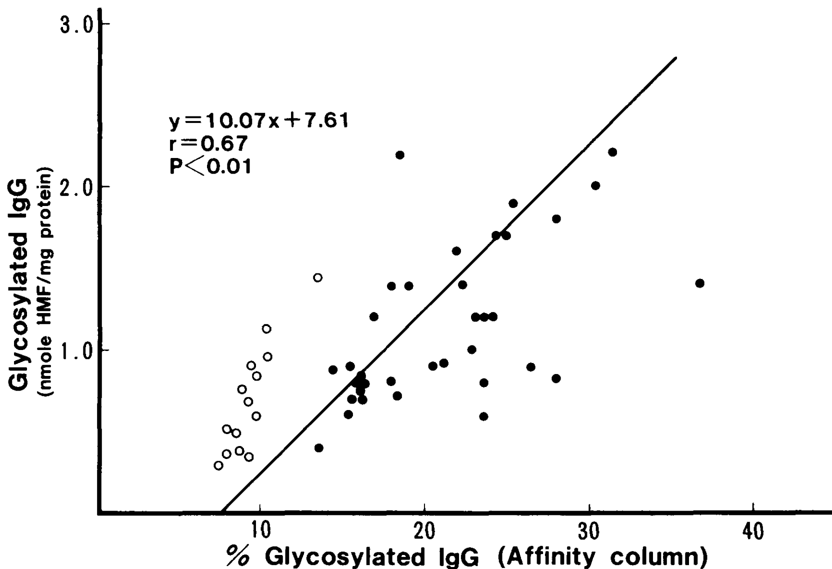

FIG. 3. Relationship between glycosylated IgG measured by colorimetric and affinity column chromatography in control (○) and diabetic (●) subjects. Significant correlation was observed between these 2 methods.

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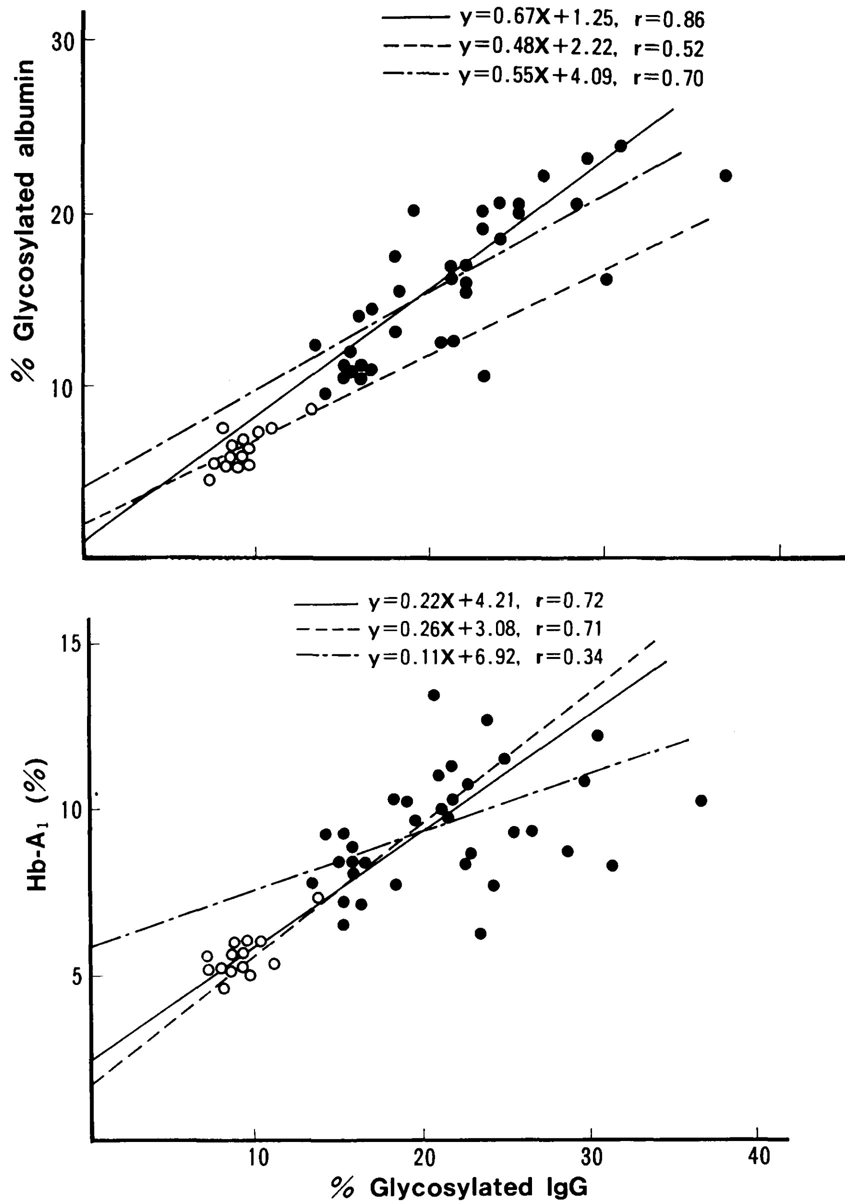

FIG. 4. Relationship between glycosylated IgG and HbA_1 or glycosylated albumin in control (○) and diabetic (●) subjects. Each regression line represents both control and diabetic subjects (*solid line*), controls (*dashed line*), and diabetics (*dashed and dotted line*).

determined against two viral strains. In diabetic patients, HI titers 4 wk after the initial immunization were significantly ($P < .05$) lower in both strains than were those in normal controls. However, there was no significant difference in HI titers between diabetics and controls 8 wk after the initial immunization.

Therefore, diabetic patients were classified into three different groups according to the ratio of the rise in their HI titers 4 wk after the immunization (Fig. 5): low-response (≤2×), moderate-response (4×), and high-response (≥8×) groups. The levels of glycosylated IgG and albumin in the low-response group were significantly ($P < .01$) higher than those in the high- and moderate-response groups. However, no significant difference was observed in the levels of HbA_1.

TABLE 1
Anti-streptolysin O titers after in vitro glycosylation of IgG from 4 healthy adults

	Days of incubation			
Glucose addition	0	2	4	8
0 mM	220 ± 66.3	180 ± 34.6	180 ± 66.3	160 ± 56.5
22.2 mM	240 ± 56.5	140 ± 34.6	120 ± 40.0	95 ± 38.4*
44.4 mM	220 ± 66.3	160 ± 56.5	90 ± 41.2*	60 ± 14.1*

Values are expressed as means ± SD in Todd units.
*$P < .05$ compared with preincubation titer (day 0).

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TABLE 2
HbA_1 levels before and after immunization

	Age (yr)	HbA_1 (%) Wk 0 (preimmunization)	HbA_1 (%) Wk 8 (postimmunization)	Serum IgG* (mg/dl)
Diabetics (n = 35)	52.4 ± 12.9	9.4 ± 1.6	9.3 ± 1.8	1225 ± 126.9
Controls (n = 14)	48.2 ± 12.6	5.6 ± 0.6	5.4 ± 0.4	1239 ± 65.7

Values are means ± SD.
*Measurement of serum IgG was determined on day of immunization.

DISCUSSION

Recently, a number of studies have demonstrated nonenzymatic glycosylation of various serum proteins in diabetic patients. However, these observations have not clearly proven any relationship between nonenzymatic glycosylation and functional alteration of these proteins and their relation to diabetic complications (17). The functional impairment of serum or tissue proteins after glycosylation is presumed to be an initial modification that may lead to local and/or systemic manifestations of diabetes mellitus.

In this study, serum IgG was used to evaluate functional alteration of glycosylated protein. It is generally assumed that serum IgG may play an important role in the host defense mechanism in diabetic patients (18). Because serum levels of immunoglobulins are essential for detecting immunologic function, age-matched healthy adults were selected as controls. Nonenzymatic glycosylation of serum IgG was demonstrated by two different assay techniques, colorimetry for the chemical assay and affinity chromatography. There was a slight but not significant difference in the amount of glycosylated IgG determined by these two techniques.

To evaluate antibody activity of glycosylated IgG, ASO titers and influenza immunization were employed on the basis of the recommended tests of immune function proposed by the World Health Organization scientific group (19). ASO titers were determined as naturally occurring antibodies, and active immunization was carried out with influenza vaccine.

Immunoglobulin G obtained from four healthy adults showed significantly decreased ASO titers after in vitro glycosylation. This result confirms the recent observation by Dolhofer et al. (20), who reported that human serum IgG led to a marked decrease in biological activity, as determined in a microcomplement-fixation test for varicella-zoster virus and lues-spirochete.

There was no significant influence of immunization on carbohydrate metabolism in either the diabetics or controls, as reflected by the levels of HbA_1 before and after immunization.

In this procedure, influenza vaccine was given in two 0.5-ml doses each at 4-wk intervals to detect primary and secondary immune response after immunization. Feery et al. (21) reported that those with a history of annual or repeated vaccination have higher initial and final titers than do individuals without such experience. Therefore, diabetic and control subjects immunized in this study were not regular vaccinees and had received no vaccine in recent years.

The significant correlation between HbA_1 and glycosylated IgG indicates that high values of glycosylated IgG are proportional to the degree of hyperglycemia. Many authors have reported that the measurement of glycosylated serum proteins provides an intermediate index of hyperglycemia (22,23). I observed that glycosylated IgG has a more marked correlation with glycosylated albumin than with HbA_1, especially when the diabetic data were plotted separately. This result suggests that glycosylated IgG reflects a shorter duration of hyperglycemia because the half-life of IgG is shorter than that of HbA_1 (24).

There was a significant elevation in the serum levels of glycosylated IgG and albumin in patients with a low response to influenza vaccines, although the levels of HbA_1 in these patients were not significantly different. This seems to indicate that the alteration in the primary immune response is related to the short-term glycemic control before immunization. Despite the low response 4 wk after initial immunization in diabetic patients, the HI titers after 8 wk showed no significant difference between diabetics and controls. Note, however, that the booster immunization caused an increase of HI titers after 8 wk in diabetic patients as well as in controls. The secondary immune response, in contrast to the primary immune response, may not be significantly impaired in these patients. It might be assumed that sufficient

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TABLE 3
Hemagglutinin-inhibiting antibodies before and after immunization

Type of vaccine	Preimmunization	Postimmunization Wk 4	Postimmunization Wk 8
A/Ishikawa/7/82			
Controls (n = 14)	45.0 ± 33.9	184 ± 86.3	243 ± 153
Diabetics (n = 35)	44.8 ± 28.4	152 ± 102*	260 ± 185
B/Singapore/222/79			
Controls (n = 14)	44.8 ± 34.1	148 ± 73.9	204 ± 127
Diabetics (n = 35)	48.0 ± 32.0	119 ± 79.8*	196 ± 134

Values are means ± SD.
*P < .05 compared with controls.

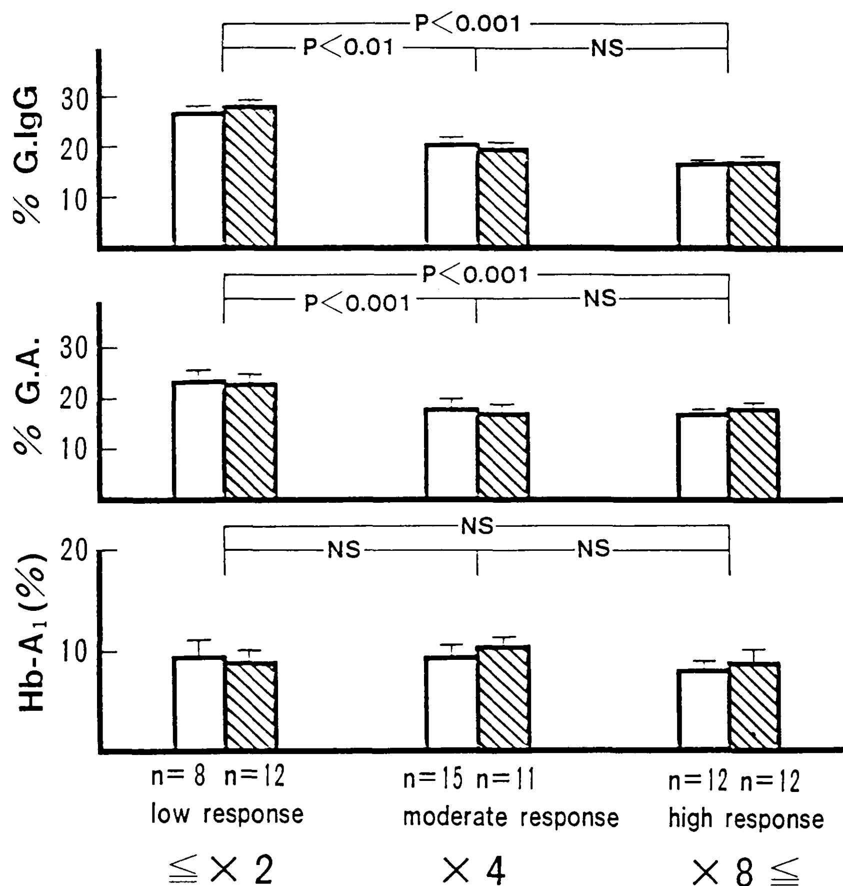

FIG. 5. HbA$_1$, glycosylated albumin (%GA), and glycosylated IgG (%GlgG) in three diabetic groups classified according to antibody increase 4 wk after influenza immunization with A/Ishikawa/7/82 (*open bars*) or B/Singapore/222/79 (*hatched bars*). Levels of GlgG and GA in low-response group were significantly higher than those in moderate- and high-response groups.

amounts of IgG to prevent influenza infection were obtained after the secondary immune response because the plateau levels of antibody production are much greater in the secondary response (25). Feery et al. (21) noted that diabetic patients under good glycemic control responded normally to single doses of influenza vaccine, whereas poorly controlled diabetics or patients with hypoproteinemia showed a retarded antibody response (26).

However, Kunin et al. (27) observed no apparent relation between the antibody response to the influenza virus vaccine and the levels of γ-globulin in patients with nephrosis. Therefore, the antibody response to influenza immunization seems to be related to the quality of serum IgG. Further experiments will be needed for evaluation of the glycosylated site of IgG and its functional aberration.

In this study, serum IgG in diabetic patients was nonenzymatically glycosylated, and it is speculated that this modification might be associated with the impairment of IgG function, such as in vitro decreased ASO titers and retarded primary immune response in vivo after immunization. The results also suggest that a booster immunization is recommended for those patients who show a low antibody response after the initial immunization against influenza virus. Because serum IgG is the major immunoglobulin class with an abundance of biological and immunological properties, this alteration of serum IgG in vivo after glycosylation might be responsible for the impairment of the host-defense mechanism in poorly controlled diabetic patients.

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