Circulating Glucagon Antibodies in Children Who Have Insulin-dependent Diabetes Mellitus

Clinical Significance and Characterization

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SUMMARY

A substance present in the sera of diabetic children that interferes with the radioimmunoassay for glucagon was found in six of 66 children who were participating in an inpatient study of diabetic control. Detailed studies documented unequivocally that this glucagon-binding substance is a specific antibody to glucagon and is located in the immunoglobulins. In a survey of diabetic children in the outpatient diabetes clinic and in a diabetes summer camp, antibodies to glucagon were found in about 12% of those evaluated. However, no children who had had diabetes for less than three years were found to have antibodies, and there appeared to be an increase with increasing duration of disease of up to greater than 20% at eight years' duration. The presence of glucagon antibodies may be of pathologic significance in that the patients have a greater tendency to develop hypoglycemia than do diabetic children without glucagon antibodies. DIABETES *28*:294–299, April 1979.

Antibodies to exogenously administered insulin can be demonstrated in essentially all diabetic patients within weeks of the initiation of insulin treatment.[1] Insulin antibodies affect the time course of insulin action, thus altering the metabolic response of the patient.[2–7] There have been few previous reports relating to the finding of antibodies to glucagon in the sera of patients with insulin-requiring diabetes mellitus, and these studies have differed widely in the frequency and possible significance of the observations.[5–8]

In a study of the concentration of glucagon in the plasma of children with diabetes mellitus in our laboratory, a glucagon-binding substance (GBS) was detected in an occasional specimen. This observation led us to a systematic study of the presence of this substance in the sera of children with insulin-requiring diabetes. The study led to the documentation that this substance is a specific antibody to glucagon. The purposes of this report are to present evidence characterizing the nature of this antibody, to define the frequency of the presence of glucagon antibodies in children with diabetes mellitus, and to suggest that glucagon binding may alter the biochemical and clinical course of these patients.

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MATERIALS AND METHODS

PATIENT POPULATION

Sixty-six adolescents who had had diabetes mellitus for 6 to 12 years participated in an inpatient evaluation of the endocrine, metabolic, and vascular alterations associated with long-standing diabetes. It was from this group of patients that the initial observations on glucagon binding were made. Utilizing the charcoal technique for glucagon assay, an antibody-like, interfering substance will produce a spuriously low glucagon value. Six patients in the group of 66 studied were found to have a GBS that interfered with the assay. A screening test for the detection of this interfering substance was developed and was applied to plasma from diabetic and normal children. Outpatient studies were carried out on a total of 215 children with insulin-requiring diabetes and 50 nondiabetic control children matched for age and sex. Children with diabetes mellitus varied in age from 6 to 17 years and duration of diabetes from six months to 15 yr. The group included 116 boys and 99 girls.

MATERIALS AND METHODS

All venous specimens were collected into cold EDTA test tubes containing 2000 U of Trasylol per 4 ml of whole blood. Specimens were promptly centrifuged and the plasma separated. Aliquots were taken for glucose determination as measured by a glucose-oxidase method. The remainder of the specimen was frozen at −70°C

until ready for additional assays. Glucagon was assayed by a previously described method.[9] The antiserum used was K-30, obtained from Unger. The radioglucagon was obtained from Nuclear Medical Laboratories in Dallas, Texas. It had a mean specific activity of 400 mCi/μg of protein. At electrophoresis, 97.2% remained at the origin. Total and free glucagon were assayed by our modification of the Nakagawa method for total and free insulin.[10] Insulin was assayed by the charcoal technique of Herbert et al.[11]

DETECTION OF GLUCAGON-BINDING SUBSTANCE (GBS)

The plasma of normal individuals contains no specific glucagon-binding antibodies. There is a low level of nonspecific binding present when normal plasma or buffer is mixed with I^{125}-glucagon. Glucagon-binding substance (GBS) was considered to be positive when the percentage of radioactive glucagon bound by the plasma was higher than the mean plus three standard deviations of the percent radioactivity bound by plasma from the 50 nondiabetic control children. This criteria (that is, plasma binding of 12% or less of the radioactive glucagon) includes 99% of the control population.

The assay is carried out as follows: 200 μl of the subject's plasma is incubated with 15 pg of I^{125}-glucagon brought to a final volume of 0.6 ml with 0.2% glycine, 0.25% albumin, 1% normal lamb serum, and pH 8.8 buffer. After four days of incubation at 4°C, dextran-charcoal is added, which binds free radioactive glucagon. Separation by centrifugation of the charcoal-bound free glucagon leaves I^{125}-glucagon bound to antibody and nonspecific binding in the supernatant. The bound and free fractions are counted for radioactivity in a Packard autogamma isotope counter.

STUDIES ON GBS SPECIFICITY

The specificity of GBS binding was tested by utilizing plasma from a patient with positive GBS in an assay system with I^{125}-glucagon. A constant mixture of I^{125}-glucagon and GBS serum was added to a series of tubes containing increasing amounts of unlabeled glucagon, insulin, growth hormone, and thyroxine.

Isolation and Identification of GBS. Studies utilizing paper electrophoresis of serum proteins were carried out on plasma from diabetic children with and without GBS. 300 μl of plasma was incubated with 90 pg of I^{125}-glucagon. As a control, 300 μl of glycine buffer and 90 pg of I^{125}-glucagon were also incubated. After four days of incubation, paper electrophoresis was carried out utilizing a Spinco electrophoretic cell. The strips were dried at 100°C and stained with bromophenol blue to localize the serum proteins. The strips were cut into segments of 2 mm in length and then counted in the gamma counter.

Gamma globulin was isolated from normal and positive GBS plasma by ion-exchange chromatography using DEAE #40 packed in 120 × 2 cm glass columns, and eluted with phosphate buffer at pH 1. Five-milliliter fractions were collected in a fraction collector and protein concentration was determined by ultraviolet absorption. The fractions containing the protein peaks were dialyzed against distilled water and lyophilized. IgG was reconstituted in glycine buffer, pH 8, at a 1% dilution. Purity was determined by gel immunodiffusion in Ouchterlony plates. In separate experiments, 200 μl of diluted IgG was incubated with 15 pg of I^{125}-glucagon in a set of tubes containing increasing amounts of unlabeled glucagon. After four days of incubation at 4°C, free and bound radioactivity were separated using the dextran-charcoal method described above and counted.

Glucagon Assay of Commercial Insulin. Several vials of different types of commercial insulin were assayed for the presence of glucagon utilizing the standard glucagon assay.

TABLE 1
Diabetic patients screened for glucagon antibodies

Group	No. of patients	No. of positives	Percent
Outpatient	85	11	12.9
Inpatient	50	6	12.0
Summer camp	70	7	10.0
Total	205	24	11.6

CLINICAL STUDIES

Sixty-six children with diabetes mellitus participated in the inpatient evaluation of hormonal, metabolic, and vascular alterations of long-standing diabetes mellitus. In all cases, blood specimens were obtained every 30 min from 2000 until 0800 the following morning from an indwelling heparin lock. The specimens were analyzed for glucose, glucagon, and total and free insulin. The blood glucose variation overnight in the six patients found to have glucagon antibodies were compared statistically with the remaining 60 diabetic patients. These patients were then further arbitrarily classified in the following way. Those children with a mean overnight glucose concentration of 150 mg/100 ml or less (25 determinations) were classified as good control (N = 21); those children with mean overnight glucose concentrations between 151 and 250 mg/100 ml were considered in fair control (N = 24); while those children with a mean overnight glucose concentration in excess of 250 mg/100 ml were considered to be in poor metabolic control (N = 15). These three subcategories were then also compared statistically with the glucose variation in the six children found to have circulating antibodies to glucagon.

RESULTS

The results of the screening studies for the presence of glucagon antibodies in our patients with diabetes mellitus is presented in Table 1. Of a total of 215 children studied, 24, or 11.6%, were found to have antibodies to glucagon. The presence of glucagon antibodies did not correlate with age or sex of the patients. The frequency of glucagon antibodies as related to the duration of diabetes is presented in Figure 1. No patients who had had diabetes for less than three years were found to have glucagon antibodies. However, there appears to be an increasing prevalence of glucagon antibodies with increasing prevalence of glucagon antibodies with increasing duration of disease, with about 25% of those patients who had had

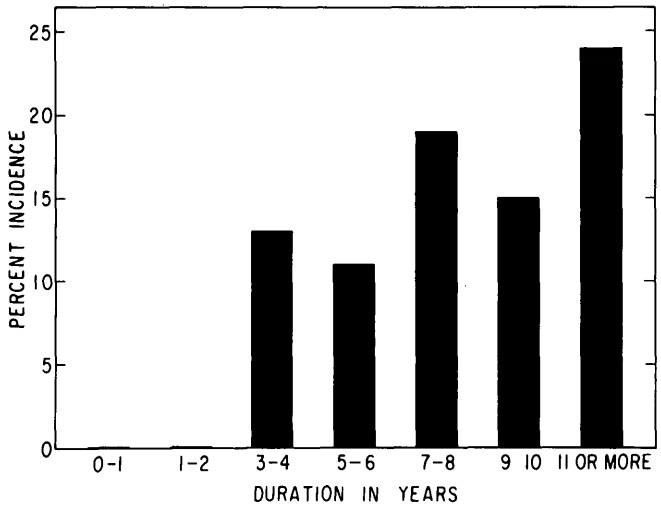


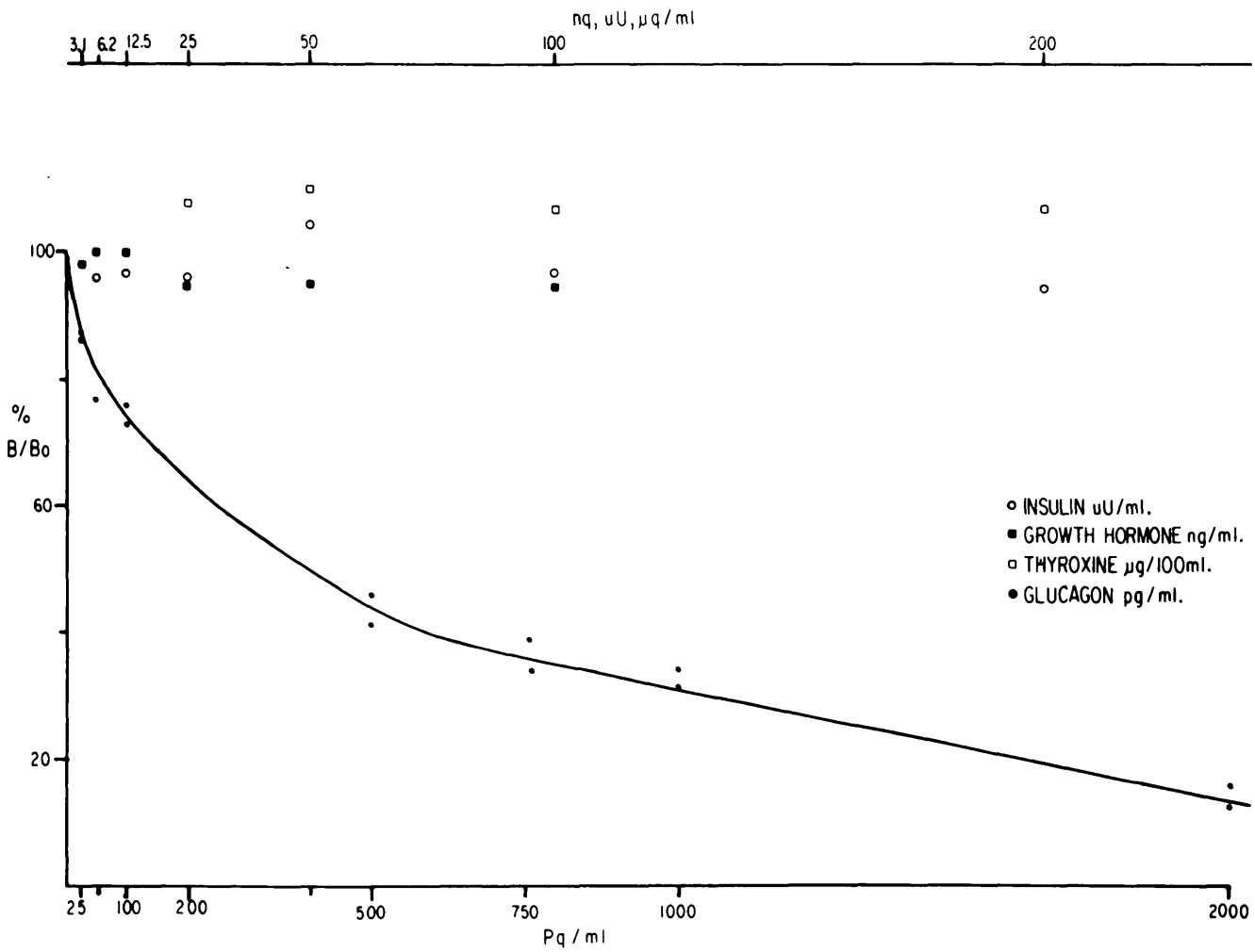
FIGURE 1. The frequency of glucagon antibodies in 204 children with diabetes mellitus. The percentage of patients with positive glucagon antibodies is based on duration of diabetes.

diabetes in excess of eight years being found to have antibodies to glucagon.

A history of prior glucagon administration was present in only three of the 24 children found to have glucagon antibodies. Two of these children had had a single glucagon injection for the treatment of hypoglycemia, while the other child had been given glucagon for hypoglycemic reactions on two occasions. Six of the 24 children had a history of mild, frequent, hypoglycemic reactions for eight months to three years before the evaluation and three had a history of severe hypoglycemia requiring hospitalization. None of these patients had ketoacidosis after initial diagnosis. The insulin dose in this group of patients was appropriate for age and body weight (0.97 ± 0.18 U/kg) and was not statistically different from that of the group of patients without glucagon antibodies (1.09 ± 0.33 U/kg). Further, the rate of weight gain was not consistent with over-insulinization.

In Figure 2 the results of incubation of a single GBS-positive plasma with radioactive glucagon and a variety of hormones are presented. There is displacement of radioactive glucagon when amounts of cold glucagon are increased while no evidence of displacement occurs when amounts of cold insulin, growth hormone, or thyroxine are increased, documenting that the GBS is specific for glucagon. The maximum glucagon binding of the six GBS-positive sera varied from 15 to 70% of radioactivity at a dilution of 1:3. The most potent of the GBS-positive plasma bound 35% of the glucagon radioactivity at a

FIGURE 2. Displacement of radioglucagon by several hormones. No evidence of displacement of radioglucagon is seen after concentrations of growth hormone, insulin, or thyroxine were increased. However, the addition of cold glucagon results in displacement of the radioactive tag of radioglucagon.



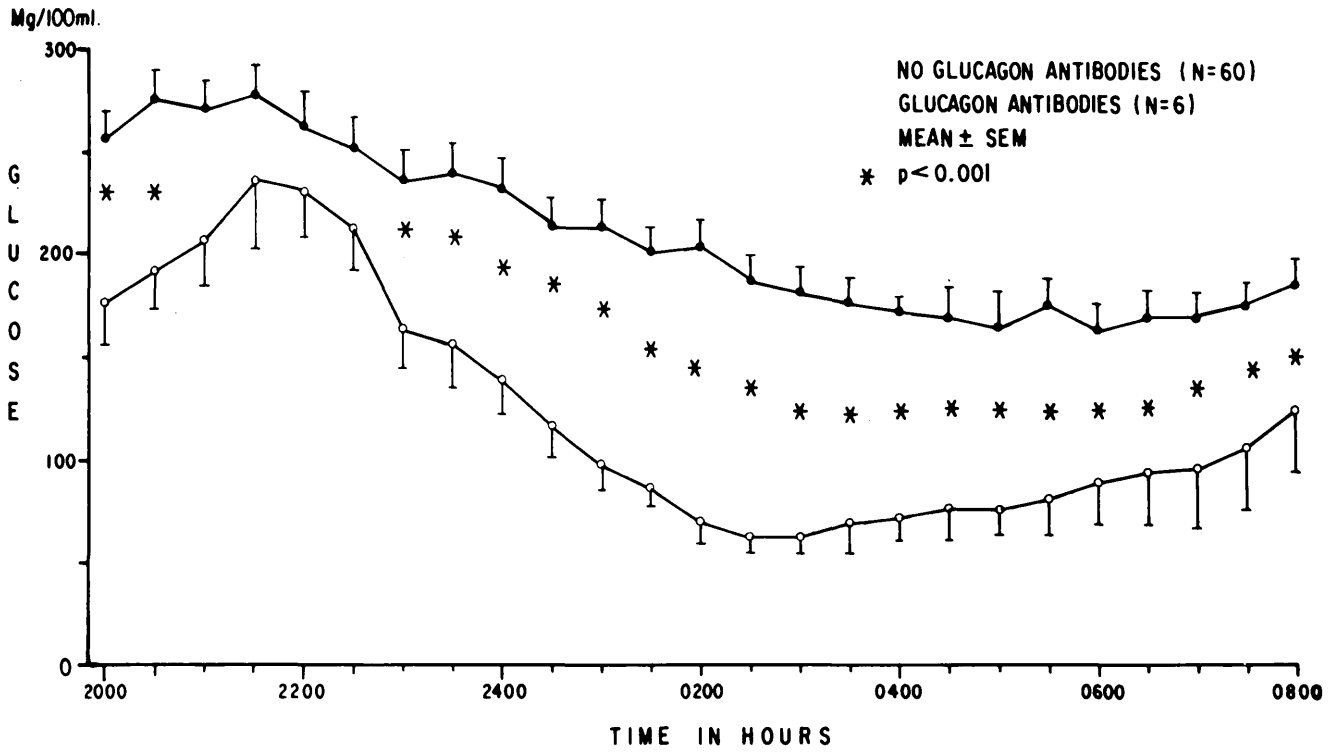


FIGURE 3. Mean glucose variations overnight in 60 diabetic children who had no glucagon antibodies and in six children who had antibodies to glucagon. Blood specimens were obtained every 30 min from 2000 until 0800. The values are means ± SEM.

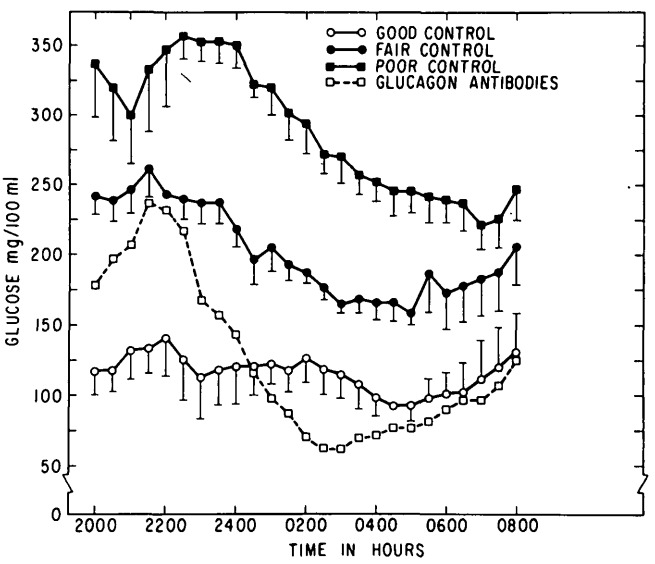
plasma dilution of 1:75. Further localization of radioactive glucagon was obtained by paper electrophoresis of plasma. Electrophoresis of the I^{125} glucagon added to normal human plasma or buffer demonstrated that the radioactivity was retained at the origin. However, when I^{125} glucagon was added to GBS-positive sera the radioactivity moved to the right of origin in the area of the immunoglobulins. Forty-five percent of the radioactivity was found bound to IgG and 25% bound to IgA.

Immuno gel diffusion of the IgG obtained by an ion exchange chromatography from plasma of children with positive GBS demonstrated precipitation bands in Ouchterlony plates only when reacting with specific anti-IgG antibody. No bands of precipitation were observed when reacting with specific anti-IgA, anti-IgM or anti-IgE. Radial electrophoresis in Mancini plates resulted in a single sharp band. This IgG diluted up to simulate its original plasma concentration bound 25–36% of the radioactive glucagon, which was displaced from the binding sites by increasing amounts of unlabeled glucagon. The IgG isolated from normal plasma did not bind radioglucagon.

In Figure 3 is presented the mean glucose concentration overnight in those six children found to have glucagon antibodies compared with the 60 diabetic children without glucagon antibodies. The glucose values are statistically lower in the glucagon antibody group throughout the course of the night. In Figure 4 is presented a similar comparison but with the nonglucagon antibody–containing patients subdivided into the categories of glucose control. The mean insulin dose per kilogram per day does not differ significantly among the four groups. Despite this, mean glucose concentrations differ significantly between the good, fair, and poor groups over most of the period of observation. The glucose response curve of the children with glucagon antibodies is appreciably different. There is an initial rise in mean glucose concentration after the evening snack, followed by a sharp decline to a nadir of 50 mg/100 ml at 0230. The individual nadirs varied from 12 mg/100 ml to 50 mg/100 ml and occurred between 0200 and 0700.

The nocturnal profile of the plasma concentration of

FIGURE 4. Mean glucose variations overnight in 66 children with diabetes mellitus. Blood specimens were obtained every 30 min from 2000 until 0800. The values are means ± SEM, and the patients were classified according to the described criteria as in good control (N = 21), fair control (N = 24), or poor control (N = 15). The nocturnal glucose variations in the six children with glucagon antibodies are presented for comparison.



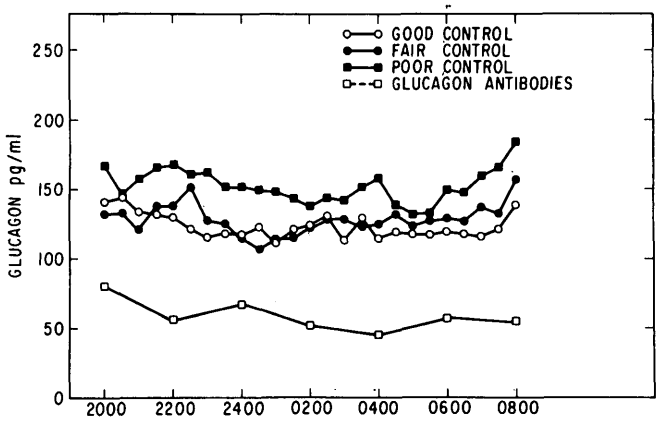


FIGURE 5. Variations in glucagon concentration overnight. Blood specimens were obtained every 30 min from 2000 until 0800, and the patients were classified as previously described: good control (N = 21), fair control (N = 24), or poor control (N = 15). There are no statistical differences between the mean glucagon concentrations in these three groups of patients. In those six patients who had glucagon antibodies the free glucagon concentrations are presented. These mean values are statistically lower than in those individuals who had no glucagon antibodies. Free glucagon did not differ appreciably from total glucagon in the nonglucagon antibody sera.

glucagon is presented in Figure 5. It is important to note that despite the wide and highly significant differences in glucose concentration between those patients considered in good, fair, and poor metabolic control, there are no differences in mean glucagon concentration. The "free glucagon" concentration is presented for those children with antibodies to glucagon. The values are significantly lower than in those patients without antibodies. The concentration remains constant overnight despite the marked fall in plasma glucose.

GLUCAGON IN COMMERCIAL INSULINS

The assay of glucagon in currently available commercial U.S. insulin preparations documented that glucagon was present in all specimens assayed, varying in concentration from 400 to 700 pg/ml.

DISCUSSION

The importance of glucagon in the modulation of energy metabolism in the normal individual and particularly its place in the pathophysiology of diabetes mellitus is a subject of much current dispute.[12,13] Our observations may provide further insight into the action of glucagon in children with diabetes mellitus.

Our studies have unequivocally documented the presence of a glucagon-binding substance in the plasma of some children with diabetes mellitus. This material is a highly specific antibody for glucagon, located in the gamma globulins. Glucagon antibodies have not been detected in nondiabetics or in insulin-requiring diabetics of short duration while there appears to be an increasing frequency with increasing duration of treatment, reaching 25% in those receiving insulin longer than eight years. Although it is possible that the development of glucagon antibodies may represent an autoimmune process that includes the alpha cells, it appears much more likely to be a result of exogenous immunization from the physiologically unimportant quantities of glucagon present in commercial insulin.

The previous studies of the presence of glucagon-binding antibodies in the sera of patients with diabetes mellitus gave results quite different than our own. In the initial study by Thomsen, no evidence of glucagon binding was found in the study of the sera obtained from 100 diabetics treated over 10 yr with insulin.[5] Stahl et al. studied 72 sera from juvenile diabetics and found evidence of glucagon binding in only one specimen.[6] Cresto et al. detected antibodies to glucagon in three of 85 insulin-requiring diabetics.[7] This combined experience gives a frequency of only 1.6% of insulin-requiring diabetic patients with antibodies to glucagon. This is to be compared with our experience of 11.6%. The difference in results probably can be explained by the somewhat different method used.

Our experience strongly suggests that the development of antibodies to glucagon may alter the clinical course of diabetes mellitus. A history of frequent hypoglycemia was common in the patients who were found to have antibodies to glucagon on the outpatient, screening studies, while nocturnal hypoglycemia was documented in all the patients. The absence of a significant difference in the concentration of glucagon overnight in the diabetic children who had widely differing degrees of glucose control suggests to us that glucagon is of little importance in the hyperglycemia of diabetes mellitus in the insulin-treated patient. However, it appears that glucagon is important as a counter-regulatory mechanism in the prevention of hypoglycemia. The relative deficiency of free glucagon in our patients who had glucagon antibodies increases the likelihood of hypoglycemia. This thesis has received considerable support from the recent report that the injection of glucagon antibodies into normal animals results in an acute fall in glucose concentrations.[14,15]

Our observations add yet another factor—the development of antibodies to glucagon—to the complex of problems already surrounding the care of the patient with insulin-deficient diabetes mellitus. It remains to be determined what effect this may have on the long-term course of the disease.

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REFERENCES

[1] Berson, S. A., and Yalow, R. S.: The present status of insulin antagonist in plasma. Diabetes *13*:247–59, 1964.

[2] Villalpando, S., and Drash, A.: Total and free plasma insulin levels in children with long-standing diabetes mellitus. Diabetes *24* (Suppl. 2):447, 1975. Abstract.

[3] Drash, A., and Villalpando, S.: Endocrine aspects of metabolic

control in juvenile diabetes. *In* Pediatric and Adolescent Endocrinology. Volume II: Balance of Diabetes in Juveniles, Part I: Medical Aspects. Laron, Z., Ed. Basel, Switzerland, S. Karger, A. G., Publishers, 1976.

[4] Kuzuya, H., Bli, P., Horwitz, D., Steiner, D., and Rubenstein, A.: Determination of free and total insulin and C-peptide in insulin-treated diabetics. Diabetes *26*:22, 1977.

[5] Thomsen, H. G.: Insulin treatment does not induce glucagon antibodies. Horm. Metab. Res. *3*:57, 1971.

[6] Stahl, M., Nars, P., Herz, G., and Baumann, J.: Glucagon antibodies after long-term insulin treatment in a diabetic child. Horm. Metab. Res. *4*:224, 1972.

[7] Cresto, J., Lavine, R., Perrino, P., Recant, L., August, G., and Hung, W.: Glucagon antibodies in diabetic patients. Lancet *1*:1165, 1974.

[8] Jaspan, J. B., and Rubenstein, A. H.: Circulating glucagon: plasma profiles and metabolism in health and disease. Diabetes *26*: 887–904, 1977.

[9] Aguilar-Parada, E., Eisentraut, A., and Unger, R. H.: Pancreatic glucagon secretion in normal and diabetic subjects. Am. J. Med. Sci. *257*:415–19, 1969.

[10] Nakagawa, S., Nakagawa, H., Sasaki, T., Yoshino, K., Yu, Y., Shinosaki, K., Aoki, S., and Mashimo, K.: A simple method for the determination of serum free insulin levels in insulin-treated patients. Diabetes *22*:590–600, 1973.

[11] Herbert, W., Lou, K. S., Gottlieb, C. W., and Bleicher, S. J.: Coated charcoal immunoassay of insulin. J. Clin. Invest. *39*:1157, 1965.

[12] Sherwin, R., Wahren, J., and Felig, P.: Evanescent effects of hypo and hyper glucagonemia on blood glucose homeostasis. Metabolism *25* (Suppl. 1):1381, 1976.

[13] Bennett, P., Aronoff, S., and Unger, R. H.: Evidence for an insulin-independent alpha-cell abnormality in human diabetes. Metabolism *25* (Suppl. 1):1527, 1976.

[14] Dunbar, J., Walsh, M., and Foa, P.: Effects of somatostatin, insulin and antiglucagon serum in depancreatized rats. Program of the Endocrine Society, Abstract 63, 1976, p. 88.

[15] Kumar, D., Miller, L. V., Lee, G. A., Rangel, M. A., and Shv, M.: Effects of anti-glucagon antibodies on blood glucose. Program of the American Federation for Clinical Research (Western section). Clin. Res. *26*:129A, 1978.