NOTE

Protamine-containing insulin but not analog insulin and duration of insulin use are risk factors for the production of insulin autoantibodies in insulin-treated patients with diabetes mellitus

Hidenao Nishimura^{1), 2)}, Katsumi Iizuka^{1), 3)} and Jun Takeda¹⁾

¹⁾ Department of Diabetes and Endocrinology, Graduate School of Medicine, Gifu University, Gifu 501-1194, Japan

²⁾ Department of Pharmacy, Hashima City Hospital, Hashima 501-6206, Japan

³⁾ Gifu University Hospital Center for Nutritional Support and Infection Control, Gifu 501-1194, Japan

Abstract. Insulin autoantibodies can be produced by insulin injections but rarely cause severe side effects such as glucose instability and insulin allergy. We study the characteristics of insulin autoantibody-positive diabetic patients with a medical history of insulin therapy using single and multiple (adjusted for age, sex, type of diabetes) logistic regression analyses. Associations between insulin autoantibodies and age, sex, type of diabetes, HbA1c, and serum creatinine were not significant, but the association between insulin autoantibodies and duration of insulin use was significant. Unadjusted and adjusted odds ratios were 1.08 (1.02–1.14) and 1.07 (1.01–1.14), respectively. Unadjusted and adjusted odds ratios for protamine-containing insulin were 3.08 (1.49–6.34) and 4.27 (1.90–9.58), respectively. The adjusted odds ratios for premixed biphasic insulin and intermediate-acting insulin were 2.21 (1.03–4.73) and 2.35 (1.01–5.49), respectively. Associations between insulin autoantibodies and any insulin analog were not significant. These results suggest that protamine-containing insulin and duration of insulin use are risk factors for the production of insulin autoantibodies. If patients with poorly controlled diabetes have a history of protamine-containing insulin therapy over a long time, the appearance of insulin autoantibodies should be monitored.

Key words: Insulin autoantibodies, Glucose instability, Insulin allergy, Protamine, Diabetes mellitus

DIABETES MELLITUS (DM) is an important social problem that carries a considerable economic burden for healthcare systems [1]. Insulin therapy is used to protect pancreatic beta cells from glucose toxicity [2]. However, insulin therapy often has severe side effects, such as hypoglycemia and gain in body weight [3]. Insulin allergy and glucose instability owing to the production of insulin autoantibodies are severe side effects of insulin therapies [4–6].

Previously, animal insulin and contaminants in the insulin-preparation process were major causes of insulin autoantibodies [6]. During the 1980s, cessation of the use of animal insulin, and the use of recombinant human insulin, decreased the prevalence of insu-

lin autoantibodies [5–6]. However, glucose instability caused by insulin autoantibodies has been reported occasionally even after cessation of the use of animal insulin [4, 7–11]. Previously, we reported a patient with glucose instability and insulin allergy owing to insulin autoantibodies [4].

Several risk factors for insulin autoantibodies have been suggested [4]. The insulin additive protamine is thought to produce insulin autoantibodies [7–11]. Protamine is a cationic polypeptide isolated from the sperm of salmon and other fish species [9–14]. Protamine produces autoantibodies against the protamine–heparin complex [12–14]. However, an association between insulin autoantibodies and protamine has not been fully confirmed.

In the present study, we undertook a case–control study to clarify the risk factors for insulin autoantibodies. We examined the associations between insulin autoantibodies and patient background using logistic regression tests adjusted for age, sex and type of DM.

Submitted Dec. 26, 2013; Accepted Mar. 5, 2014 as EJ13-0544
Released online in J-STAGE as advance publication Mar. 28, 2014

Correspondence to: Katsumi Iizuka, Department of Diabetes and Endocrinology, Graduate School of Medicine, Gifu University, Gifu 501-1194, Japan. E-mail: kiizuka@gifu-u.ac.jp

Materials and Methods

Study design and background

This was a hospital-based, retrospective study of patients with DM diagnosed according to criteria set by the Japan Diabetes Association [15]. This study (number 24–395) was approved by the Research Ethics Committee of Gifu University (Gifu, Japan). From April 2009 to December 2011, levels of insulin autoantibodies were measured in 147 patients at the Department of Diabetes and Endocrinology at Gifu University Hospital. All patients had a history of receiving insulin therapy. Levels of insulin autoantibodies were measured using an Insulin Antibody kit (Yamasa Corporation, Chiba, Japan). If the titer of insulin autoantibodies was $>0.4\%$, insulin autoantibodies were diagnosed as “positive” (case): this was seen in 101 patients (Table 1). If the titer of insulin autoantibodies was $0–0.4\%$, insulin autoantibody was diagnosed as “negative” (control): this was seen in 46 patients. For this case–control study, we listed patient backgrounds in terms of age, sex, type of DM, duration of insulin use, duration of DM, serum creatinine, serum HbA1c at the same time that insulin autoantibodies were measured. Moreover, we checked the types of insulin that patients have ever used. The types of insulin injections were categorized as “protamine”, “intermediate-acting”, “premixed”, “rapid-acting”, “regular”, and “long-acting” insulin. The number of cases for protamine-containing insulin ($n=91$) comprised intermediate-acting type only ($n=29$), premixed type only ($n=44$), and both types ($n=18$). The numbers for rapid-acting insulin ($n=105$) comprised insulin aspart only ($n=68$), insulin lispro only ($n=16$), insulin glulisine only ($n=6$), insulin aspart and insulin lispro ($n=12$), insulin aspart and glulisine ($n=2$), and insulin aspart, insulin lispro, and insulin glulisine ($n=1$). The numbers for long-acting insulin ($n=85$) comprised insulin glargine only ($n=50$), insulin detemir only ($n=28$), and both insulin glargine and insulin detemir ($n=7$). The numbers for total human insulin ($n=97$) comprised regular type only ($n=17$), intermediate-acting and premixed type only ($n=55$), and both ($n=25$). The numbers for total insulin aspart comprised rapid-acting type only ($n=65$), premixed type only ($n=11$), and both ($n=18$). The numbers for human insulin lispro ($n=35$) comprised rapid-acting type only ($n=26$), premixed type only ($n=6$), and both ($n=3$).

Statistical analyses

Continuous variables are expressed as the arithmetic mean (standard deviation (SD)). Categorical data are expressed as actual frequencies and percentages. The following covariates were incorporated into logistic analyses: presence or absence of insulin autoantibodies, age, sex, type of DM (type-1 diabetes mellitus (T1DM) or type-2 diabetes mellitus (T2DM)), presence or absence of several types of insulin injections or oral hypoglycemic drugs, and laboratory data (HbA1C and creatinine). Single and multiple logistic regression analyses were carried out using StatView-J 5.0 for Windows (SAS Institute, Cary, NC, USA). Odds ratios (ORs) and 95% confidence intervals (95% CI) adjusted and unadjusted for sex, age, and type of DM (T1DM or T2DM) were calculated with logistic regression analyses. $p<0.05$ was considered significant [equal to OR (95% CI) does not contain 1.0].

Results

Duration of insulin use and protamine-containing insulin were risk factors for the production of insulin autoantibodies in insulin-treated patients with DM

To clarify the association between the production of insulin autoantibodies and patient background, we undertook logistic regression analyses unadjusted and adjusted for age, sex, and type of DM. The associations between the production of insulin autoantibodies and age, sex and type of DM were not significant ($p>0.05$) (Table 1). HbA1c (%) and serum levels of creatinine in insulin autoantibody-positive and autoantibody-negative patients were similar, and associations between insulin autoantibodies and these parameters were not significant (Table 1). The durations of DM in insulin autoantibody-positive and autoantibody-negative patients were similar, but the duration of insulin use in insulin autoantibody-positive patients was much longer (9.14 ± 7.14 vs. 5.77 ± 7.53 years, respectively) (Table 1). Moreover, the unadjusted and adjusted ORs (95% CI) for the duration of insulin use were 1.08 (1.02–1.14) and 1.07 (1.01–1.14), respectively. Therefore, the duration of insulin use was a risk factor for the production of insulin autoantibodies.

Next, we examined the association between the production of insulin autoantibodies and the type of injected insulin. The association between the production of insulin autoantibodies and protamine-containing insulin was significant; the unadjusted and adjusted ORs

Table 1 Patient background characteristics and use of insulin autoantibodies

	Antibody (+) ^a n=101	Antibody (-) ^b n=46	Total	<i>P</i>	Unadjusted odds ratio (95% CI)	<i>P</i>	Adjusted odds ratio (95% CI) ^c
Age (years)	56.80 (16.26)	57.11 (14.41)	56.90 (15.71)	0.91	1.00 (0.98–1.02)	0.62	1.01 (0.98–1.03)
Sex							
Male (n)	45	24	69	0.39	1.36 (0.68–2.73)	0.68	1.17 (0.56–2.42)
Female (n)	56	22	78				
Type of diabetes							
T1DM (n)	32	9	41	0.13	0.52 (0.23–1.22)	0.16	0.51 (0.20–1.300)
T2DM (n)	69	37	106				
HbA1c (%)	8.34 (1.72)	8.19 (2.13)	8.29 (1.86)	0.65	1.05 (0.86–1.27)	0.65	1.05 (0.85–1.29)
Serum creatinine (mg/dL)	0.79 (0.45)	0.81 (0.37)	0.80 (0.43)	0.84	0.92 (0.42–2.05)	0.84	1.07 (0.43–2.69)
Duration of disease (years)	15.55 (9.15)	15.48 (10.98)	15.53 (9.76)	0.97	1.00 (0.97–1.04)	0.97	1.01 (0.96–1.05)
Duration of insulin use (years)	9.14 (7.14)	5.77 (7.53)	8.09 (7.43)	0.013	1.08 (1.02–1.14)	0.013	1.07 (1.01–1.14)
Protamine							
Yes (n)	71	20	91	0.002	3.08 (1.49–6.34)	0	4.27 (1.90–9.58)
No (n)	30	26	56				
Intermediate-acting							
Yes (n)	48	14	62	0.054	2.07 (0.99–4.34)	0.041	2.21 (1.03–4.73)
No (n)	53	32	85				
Premixed							
Yes (n)	37	10	47	0.076	2.08 (0.93–4.68)	0.048	2.35 (1.01–5.49)
No (n)	64	36	100				
Rapid-acting							
Yes (n)	74	31	105	0.46	1.33 (0.62–2.83)	0.38	1.21 (0.56–2.63)
No (n)	27	15	42				
Regular							
Yes (n)	30	12	42	0.65	1.20 (0.55–2.62)	0.67	1.19 (0.54–2.63)
No (n)	71	34	105				
Long-acting							
Yes (n)	61	24	85	0.26	1.40 (0.69–2.83)	0.51	1.28 (0.62–2.63)
No (n)	40	22	62				

^a, titer of insulin autoantibodies was >0.4%; ^b, titer of insulin autoantibodies was 0–0.4%; ^c, Odds ratios (ORs) and 95% confidence intervals (95% CI) adjusted for sex, age, and type of DM (T1DM or T2DM).

(95% CI) were 3.08 (1.49–6.34) and 4.27 (1.90–9.58), respectively (Table 1). Similarly, associations between the production of insulin autoantibodies and intermediate-acting or premixed insulin were partially significant ($p<0.05$). The association between the production of insulin autoantibodies and rapid-acting insulin, regular insulin, and long-acting insulin were not significant (Table 1). Thus, protamine-containing insulin was a risk factor for the production of insulin autoantibodies.

Effect of insulin and insulin analogs on the production of insulin autoantibodies

Next, we ascertained if human insulin and insulin analogs could affect the production of insulin autoantibodies. The association between the production of insulin autoantibodies and total human insulin (regular + intermediate-acting + premixed) was significant ($p<0.05$) (Table 2). However, the association between the production of insulin autoantibodies and regular-

type human insulin was not significant. By contrast, the association between the production of insulin antibodies and protamine-containing human insulin (intermediate acting + premixed) was significant ($p<0.05$). Thus, human insulin itself was not a risk factor for the production of insulin autoantibodies.

The association between the production of insulin autoantibodies and total insulin aspart (rapid acting + premixed) was significant ($p<0.05$). The association between the production of insulin autoantibodies and rapid-acting insulin aspart was not significant, but the association between the production of insulin autoantibodies and premixed insulin aspart was significant ($p<0.05$). Thus, insulin aspart itself was not a risk factor for the production of insulin autoantibodies.

The association between the production of insulin autoantibodies and total insulin lyspro, rapid-acting insulin lyspro, and premixed insulin lyspro, long-acting insulin glargine, long-acting insulin detemir, and rapid-

Table 2 Association between insulin autoantibodies and human insulin or insulin analogs

	Antibody (+) ^a n=101	Antibody (-) ^b n=46	Total	<i>p</i>	Unadjusted odds ratio (95% CI)	<i>p</i>	Adjusted odds ratio (95% CI) ^c
Total Human Insulin							
Yes (n)	72	25	97	0.046	2.09 (1.01–4.30)	0.049	2.12 (1.00–4.49)
No (n)	29	21	50				
Regular							
Yes (n)	30	12	42	0.65	1.20 (0.55–2.62)	0.67	1.19 (0.54–2.63)
No (n)	71	34	105				
Premixed + Intermediate-acting							
Yes (n)	62	18	80	0.013	2.47 (1.21–5.06)	0.007	2.89 (1.34–6.24)
No (n)	39	28	67				
Total Insulin Aspart							
Yes (n)	72	22	94	0.0068	2.71 (1.32–5.57)	0.0078	2.75 (1.31–5.74)
No (n)	29	24	53				
Rapid-acting							
Yes (n)	62	21	83	0.076	1.89 (0.94–3.83)	0.1	1.82 (0.89–3.72)
No (n)	39	25	64				
Premixed							
Yes (n)	25	4	29	0.03	3.45 (1.13–10.60)	0.017	4.04 (1.29–12.70)
No (n)	76	42	118				
Total Insulin Lyspro							
Yes (n)	26	9	35	0.42	1.43 (0.61–3.35)	0.57	1.31 (0.52–3.30)
No (n)	75	37	112				
Rapid-acting							
Yes (n)	21	8	29	0.63	1.25 (0.51–3.07)	0.88	1.082 (0.39–3.00)
No (n)	80	38	118				
Premixed							
Yes (n)	7	2	9	0.55	1.64 (0.33–8.21)	0.16	1.68 (0.33–8.65)
No (n)	94	44	138				
Insulin Glargine							
Yes (n)	38	19	57	0.67	0.86 (0.42–1.75)	0.34	0.68 (0.32–1.48)
No (n)	63	27	90				
Insulin Detemir							
Yes (n)	28	7	35	0.1	2.14 (0.86–5.34)	0.089	2.24 (0.89–5.65)
No (n)	73	39	112				
Insulin Glulisine							
Yes (n)	4	5	9	0.12	0.34 (0.086–1.32)	0.16	0.36 (0.089–1.49)
No (n)	97	41	138				

^a, titer of insulin autoantibodies was >0.4%; ^b, titer of insulin autoantibodies was 0–0.4%; ^c, Odds ratios (ORs) and 95% confidence intervals (95% CI) adjusted for sex, age, and type of DM (T1DM or T2DM).

acting insulin glulisine, was not significant (Table 2). Thus, no type of insulin analog was significantly associated with the production of insulin autoantibodies.

Discussion

Our case-control study shows that the duration of insulin use and protamine-containing insulin were risk factors for the production of insulin autoantibodies.

The duration of insulin use was a significant (albeit weak) risk factor for the production of insulin autoantibodies. It has been reported that the titers of insulin autoantibodies are highest within 4–8 months after

administration of an insulin injection and persist in insulin-treated patients with T1DM and T2DM [5]. A reason for this observation could be that, as the duration of insulin use increases, the percentage of insulin autoantibody-positive patients also increases. The duration of insulin use in insulin autoantibody-positive patients in our study was significantly longer than that for insulin autoantibody-negative patients (9.14±7.14 years vs. 5.77±7.53 years, $p=0.0005$); whereas the duration of DM was similar (15.55±9.15 years vs. 15.48±10.98 years, $p=0.97$). Therefore, duration of insulin use was a risk factor of the production of insulin autoantibodies.

Our results show that protamine-containing insulin

was a risk factor for the production of insulin autoantibodies. In agreement with our study, some studies have reported that insulin NPH or protamine promote the production of insulin or protamine autoantibodies [5, 9–12, 16]. We suggest three possible mechanisms for how protamine produces insulin autoantibodies. First, protamine may induce a conformational change in insulin (or an insulin analog) and consequently produce insulin autoantibodies [5]. Second, allergic reactions may result from immunity to salmon protamine in premixed or intermediate-acting insulin, as well as immunological cross-reactions between human protamine and salmon protamine [17]. Third, the amount of protamine may be dependent upon the appearance of insulin autoantibodies. Premixed human insulin and insulin aspart caused the production of significant quantities of insulin autoantibodies, whereas premixed insulin lispro did not. In this regard, the protamine content of insulin NPH (1 mg in 3 mL) and insulin aspart mix (1.0 mg in 3 mL) was much greater than that of insulin lispro (0.84 mg in 3 mL). As none of the insulin analogs produced significant amounts of insulin autoantibodies (Table 2), the appearance of insulin autoantibodies might be dependent upon the amount of protamine. Further research is needed to clarify the relationship between the amount of protamine and the production of insulin autoantibodies. Accordingly, protamine-containing insulin should be used with caution.

Whether an insulin analog itself can produce insulin autoantibodies is controversial. Some authors have reported that insulin analogs can increase the risk of the production of insulin antibodies [18], whereas other scholars have reported that insulin or insulin analogs do not differ with respect to immunogenicity [8]. Compatible with the latter notion, we show insulin aspart, insulin lispro, and insulin glulisine did not affect the production of insulin autoantibodies (Table 2). None of the insulin analogs tested was risk factors for insulin autoantibodies. Therefore, instead of using intermediated-acting insulin and premix biphasic insulin without caution, protamine-free insulin injections should be considered, such as: (i) long-acting insu-

lin plus other hypoglycemic agents (e.g., metformin, dipeptidyl peptide-IV inhibitors, and α -glucosidase inhibitors, (ii) long-acting insulin plus rapid-acting or regular insulin, (iii) insulin degludec/insulin aspart (IDegAsp), and (iv) long-acting insulin plus a GLP-1 receptor agonist [19–22].

This study had limitations. It was not a randomized study. We measured the production of insulin autoantibodies in insulin-treated patients with DM and collected data only from our hospital in Japan. We selected patients (i) with a history of insulin therapy and (ii) in whom insulin autoantibodies could be measured. We also monitored the effect of the production of insulin autoantibodies when glucose instability was observed. Patients in the insulin autoantibody-positive and autoantibody-negative groups showed poor glycemic control ($8.34 \pm 1.72\%$ and $8.19 \pm 2.13\%$, respectively). We were mindful that our study had selection bias. To further investigate the effects of protamine-containing insulin on the production of insulin autoantibodies, a prospective study (such as cohort study) is required.

In conclusion, protamine-containing insulin and duration of insulin use were risk factors for the production of insulin autoantibodies. Careful consideration of the use of protamine-containing insulin to help prevent the production of insulin autoantibodies is important. New insulin therapies such as basal supported oral therapy (BOT) or IDegAsp maybe lower the risk of the production of insulin autoantibodies.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (Iizuka K (22590973), Takeda J (25670432)).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

References

1. Reaven GM (1988) Banting lecture 1988. Role of insulin resistance in human disease. *Diabetes* 37: 1595–1607.
2. Pennartz C, Schenker N, Menge BA, Schmidt WE, Nauck MA, et al. (2011) Chronic reduction of fasting glycemia with insulin glargine improves first- and second-phase insulin secretion in patients with type 2 diabetes. *Diabetes Care* 34: 2048–2053.

3. Seaquist ER, Anderson J, Childs B, Cryer P, Dagogo-Jack S, et al. (2013) Hypoglycemia and diabetes: a report of a workgroup of the American Diabetes Association and the Endocrine Society. *Diabetes Care* 36: 1384-1395.
4. Iizuka K, Tomita R, Horikawa Y, Takeda J (2012) A case of glycemic instability and insulin allergy due to anti-insulin antibodies in a patient with type 2 diabetes. *Diabetol Int* 3: 233-238.
5. Fineberg SE, Kawabata TT, Finco-Kent D, Fountaine RJ, Finch GL, et al. (2007) Immunological responses to exogenous insulin. *Endocr Rev* 28: 625-652.
6. Schernthaner G (1993) Immunogenicity and allergenic potential of animal and human insulins. *Diabetes Care* 16 Suppl 3: 155-165.
7. Ellerhorst JA, Comstock JP, Nell LJ (1990) Protamine antibody production in diabetic subjects treated with NPH insulin. *Am J Med Sci* 299: 298-301.
8. Mianowska B, Szadkowska A, Pietrzak I, Zmysłowska A, Wegner O, et al. (2011) Immunogenicity of different brands of human insulin and rapid-acting insulin analogs in insulin-naïve children with type 1 diabetes. *Pediatr Diabetes* 12: 78-84.
9. Nell LJ, Thomas JW (1988) Frequency and specificity of protamine antibodies in diabetic and control subjects. *Diabetes* 37: 172-176.
10. Kurtz AB, Gray RS, Markanday S, Nabarro JD (1983) Circulating IgG antibody to protamine in patients treated with protamine-insulins. *Diabetologia* 25: 322-324.
11. Gregory R, Tattersall RB (1993) Bovine and human NPH insulins as T cell immunogens. *Diabetes Res Clin Pract* 20: 139-146.
12. Bakchoul T, Zöllner H, Amiral J, Panzer S, Selleng S, et al. (2013) Anti-protamine-heparin antibodies: incidence, clinical relevance, and pathogenesis. *Blood* 121: 2821-2827.
13. Lee GM, Welsby IJ, Phillips-Bute B, Ortel TL, Arepally GM (2013) High incidence of antibodies to protamine and protamine/heparin complexes in patients undergoing cardiopulmonary bypass. *Blood* 121: 2828-2835.
14. Singla A, Sullivan MJ, Lee G, Bartholomew J, Kapadia S, et al. (2013) Protamine-induced immune thrombocytopenia. *Transfusion* 53: 2158-2163.
15. Seino Y, Nanjo K, Tajima N, Kadowaki T, Kashiwagi A, et al. (2010) Report of the Committee on the classification and diagnostic criteria of diabetes mellitus. The Committee of the Japan Diabetes Society on the diagnostic criteria of diabetes mellitus. *Diabetol Int* 1: 2-20.
16. Sánchez MB, Paolillo M, Chacón RS, Camejo M (1982) Protamine as a cause of generalised allergic reactions to NPH insulin. *Lancet* 1: 1243.
17. Samuel T (1977) Antibodies reacting with salmon and human protamines in sera from infertile men and from vasectomized men and monkeys. *Clin Exp Immunol* 30: 181-187.
18. Yanai H, Adachi H, Hamasaki H (2011) Diabetic keto-sis caused by the insulin analog aspart-induced anti-insulin antibody: successful treatment with the newest insulin analog glulisine. *Diabetes Care* 34: e108.
19. Hirsch IB, Bode B, Courreges JP, Dykiel P, Franek E, et al. (2012) Insulin degludec/insulin aspart administered once daily at any meal, with insulin aspart at other meals versus a standard basal-bolus regimen in patients with type 1 diabetes: a 26-week, phase 3, randomized, open-label, treat-to-target trial. *Diabetes Care* 35: 2174-2181.
20. Forst T, Larbig M, Hohberg C, Forst S, Diessel S, et al. (2010) Adding insulin glargine vs. NPH insulin to metformin results in a more efficient postprandial beta-cell protection in individuals with type 2 diabetes. *Diabetes Obes Metab* 12: 437-441.
21. Mori Y, Taniguchi Y, Miyazaki S, Yokoyama J, Utsunomiya K (2013) Effects of add-on treatment with sitagliptin on narrowing the range of glucose fluctuations in Japanese type 2 diabetes patients receiving insulin therapy. *Diabetes Technol Ther* 15: 237-240.
22. Riddle MC, Forst T, Aronson R, Sauque-Reyna L, Souhami E, et al. (2013) Adding once-daily lixisenatide for type 2 diabetes inadequately controlled with newly initiated and continuously titrated basal insulin glargine: a 24-week, randomized, placebo-controlled study (GetGoal-Duo 1). *Diabetes Care* 36: 2497-2503.