Decrease of Circulating Insulin Antibodies in Two Patients Treated with Continuous Subcutaneous Infusion of Human Insulin (recombinant DNA)

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Circulating insulin antibodies (CIA) were measured in two patients treated with continuous subcutaneous infusion (CSII) of human insulin (recombinant DNA). CIA were determined by a kinetic equilibrium assay after pretreatment of the serum to remove bound and free insulin. We observed that by changing from either purified bovine or purified porcine insulin to human insulin given by CSII, there is a gradual decrease in CIA detectable after 3 wk and more pronounced 3 mo after changing. These findings suggest that human insulin in combination with the improved metabolic control by CSII considerably reduces the antibody formation in patients with IDDM. DIABETES CARE 5 (SUPPL. 2): 171–174, 1982.

Circulating insulin antibodies (CIA) are present in most diabetic patients treated with bovine or porcine insulin. The generation of these immunoglobulins is most likely caused by the administration of foreign nonhuman protein with antigenic properties. The availability of human insulin (recombinant DNA) enabled us to study the effect of switching from animal insulin to human insulin on the level of CIA. The patients described in this study received insulin by continuous subcutaneous insulin infusion (CSII). Seven patients were started on CSII with purified porcine insulin in the period May 1981–November 1981. Three patients started CSII in the period January 1982–February 1982 and used human insulin. CIA were measured in plasma samples taken before and at several intervals during CSII. In this paper we report on initial findings after 3-mo CSII therapy with human insulin. The effect on CIA by switching from animal to human insulin should be differentiated from the possible effect of changing from intermittent to continuous subcutaneous insulin administration and of improved metabolic control.

MATERIALS AND METHODS

Patients. Ten patients were studied before and during CSII. Some clinical data, including duration of diabetes mellitus and type of insulin used before the start of CSII are summarized in Table 1.

All patients were insulin dependent: C-peptide levels, measured by a specific C-peptide radioimmunoassay (Novo Industri, Copenhagen, Denmark), before and 6 min after 1 mg glucagon intravenously were below the detection limit of 0.05 ng/ml.[1,2] The diabetes was complicated by retinopathy, as established by fluorescence angiography, and by peripheral neuropathy, as diagnosed by nerve conduction velocity measurements of the right median nerve. Informed consent for CSII treatment and the use of human insulin was obtained. All patients were admitted to the Academic Hospital, University of Utrecht, before and during the start of CSII. During hospitalization the patients received instructions in home glucose monitoring and in the practical use of the insulin infusion device. Two different pumps were used. Patients no. 1–4 used the Auto-Syringe AS 6C infuser; patients no. 5–10 used the Mill Hill 1001 HM infuser. The insulin in the Mill Hill 1001 HM infusion devices was mixed with Diluting Medium (Novo).

All patients had longstanding diabetes before CSII (Table 1) and had been treated with either purified porcine insulin or purified bovine insulin or both. During CSII patients no. 1–3 were treated with human insulin and patients no. 4–10 were treated with purified porcine insulin.

Methods. Serum samples for quantitative determination of glycosylated hemoglobin A_1 by agar gel electrophoresis[3] were taken 1 wk before, 3 wk after, and 3 mo after CSII. At the same time serum samples for CIA levels were taken.

Purified monocomponent bovine insulin (Novo) was used as a standard preparation. Commercially available ^{125}I bovine insulin (spec. activity > 50 μCi/ug) was obtained from The

TABLE 1
Summary of data on patients studied

Patient number	Sex	Age (yr)	Duration of diabetes before CSII (yr)	Type of insulin before CSII	Type of insulin during CSII	Circulating insulin antibodies before start of CSII
1(W.Y.)	M	29	24	PBI	human insulin	Subst.
2(E.D.)	M	48	12	PPI	human insulin	Subst.
3	F	42	10	PPI and PBI	human insulin	Neg.
4(B.A.)	F	23	14	PBI	PPI	Subst.
5	F	24	20	PPI	PPI	Int.
6	M	31	21	PPI	PPI	Neg.
7	M	45	30	PPI and PBI	PPI	Neg.
8	F	22	17	PPI	PPI	Neg.
9	F	36	22	PPI	PPI	Neg.
10	M	34	22	PPI and PBI	PPI	Neg.
	M/F = 1	mean: 33.4 range: 22–48	mean: 19.2 range: 10–30		human insulin/PPI = 3/7	

Abbreviations used: M, male; F, female; CSII, continuous subcutaneous insulin infusion; PBI, purified bovine insulin; PPI, purified porcine insulin; human insulin, human insulin of recombinant DNA origin; Subst., substantial level; Int., intermediate level; Neg., no evidence for circulating insulin antibodies.

Radio Chemical Centre, Amersham, U. K. The homogeneity of this preparation was checked by column chromatography. Bovine serum albumin was purchased from Povite, Poviet Products, Oss, The Netherlands, Dextran T70 from Pharmacia, Uppsala, Sweden, and charcoal (Aktivkohle reinst) from Merck, Darmstadt, West Germany. All other reagents were of pro analyse quality from British Drug House Ltd., Poole, U.K. Circulating insulin antibodies were determined by a kinetic method that differentiates between high- and low-affinity binding sites.

Removal of free and antibody-bound insulin. Prior to the assay, free and antibody-bound insulin were removed by the procedure of Dixon,[4] modified by substituting the original barbital-albumin buffer for a phosphate buffer (pH 7.5) with 0.25% bovine serum albumin (BSA).

Deinsulinization was accomplished by diluting the plasma with 4 parts (vol/vol) of 0.12 N HCl. After homogenization, 5 parts (vol/vol) of a dextran-coated charcoal suspension (0.25% Dextran T 70 and 2.5% charcoal in phosphate buffer without BSA) is added.

The mixture is vortex-mixed three times during a 15-min period and afterwards centrifuged at 4°C for 10 min at 2000 × *g*. The clear supernatant is adjusted to pH 7.5 by adding 4 parts (vol/vol) of 0.12 N NaOH. This supernatant is used for the CIA assay.

Kinetic binding assay for CIA. ^{125}I-insulin was diluted 1:10 (vol/vol) in phosphate-BSA buffer (0.02M NaH_2PO_4, pH = 7.5 with 0.25% BSA). Binding assays were performed with 4 × 10^{-11} M ^{125}I-insulin and increasing molar concentrations of cold bovine insulin. The total insulin concentration was varied between 10^{-10} M and 10^{-6} M. The incubation mixture consists of 50 µl tracer, 100 µl of cold insulin and 250 µl of deinsulinized diluted plasma. The assay was done in duplicate at 12 different insulin dilutions plus 2 blank incubations to estimate the nonspecific blank due to damage of the tracer. Incubation was carried out overnight at 0°C. After incubation, 200 µl of an ice-cold dextran-coated charcoal (DCC) suspension was added under continuous vortexing of the tubes. DCC treatment lasted 30 min at 0°C during which time the tubes were vortexed two times. Separation

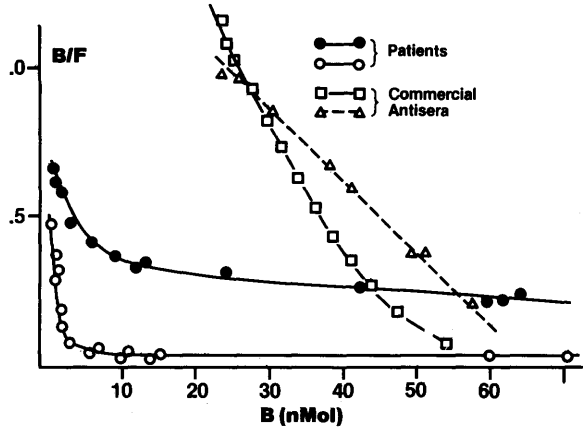

FIG. 1. *Scatchard plot of the binding data for two commercial antisera and two insulin-antibody–positive patients. Sera were pretreated to remove bound and free insulin at low pH. The pretreated sera were incubated with ^{125}I-bovine insulin and increasing amounts of unlabeled bovine insulin. Separation of bound and free hormone was done by dextran-coated-charcoal treatment (for details, see* MATERIALS AND METHODS*). B: bound hormone; F: free hormone; B is expressed as nmol per liter undiluted serum.*

TABLE 2
Circulating insulin antibodies (high affinity): The concentration of high-affinity binding sites, expressed as nmol per liter undiluted serum, for two patients (W.Y. and E.D.) who switched over from purified bovine insulin or purified porcine insulin to human insulin. The number of high-affinity sites were calculated from the Scatchard plots, given in Figures 2 and 3 for these patients.

	CSII		
Patients	1 wk before	3 wk after	3 mo after
1 W.Y.	28	13	3
2 E.D.	22	15	7.5

of bound and free was done by centrifugation at 3000 × g for 20 min at 4°C.

An aliquot of the supernatant was taken for counting. Aliquots of the ^{125}I-insulin stock solution were counted to measure total c.p.m. added to each tube. The percentage of damage of the tracer was calculated from the blank assays. This amount of radioactivity was subtracted from each bound fraction. From the bound (B) and the total radioactivity (T) the free fraction was calculated (F). The binding data were plotted according to Scatchard's equation.[5] B/F is expressed as a ratio; B in nmol per liter undiluted serum.

RESULTS

The method used for the CIA measurement in pre-extracted serum appeared to be very reliable. An almost identical approach was recently presented by Dr. E. Fineberg at the Symposium on Human Insulin of Recombinant DNA Origin, San Francisco, California, June, 1982.

This method has the advantage that antibodies can be characterized in high- and low-affinity binding sites, and their concentration can be measured in nmol per liter undiluted serum. To test the whole procedure, two commercial insulin antibodies, one from Wellcome and another that is used in our laboratory for the routine estimation of insulin, were taken through the whole procedure including the extraction step. The results are shown in Figure 1. The Scatchard plots for these antisera were almost straight lines indicating the presence of one homologous set of binding sites with high affinity (Kd ~ 1 nmol) for the tracer used. In the same figure two curves are shown for patients who had antibodies in their blood as shown by a routine method. These two curves show the presence of two populations of binding sites with different binding affinity (one high affinity: Kd ~ 1 nmol; the other low affinity: Kd > 10 nmol). When sera from healthy control persons without antibodies were taken through this procedure, we found an average B/F ratio lower than 0.03 without a negative slope in the curve (data not shown).

Substantial levels of circulating insulin antibodies before CSII were found in three patients only: nos. 1, 2, and 4. In patient no. 5, intermediate levels were found. In six patients who had been treated with either purified porcine or bovine insulin for many years (Table 1) no CIA levels could be

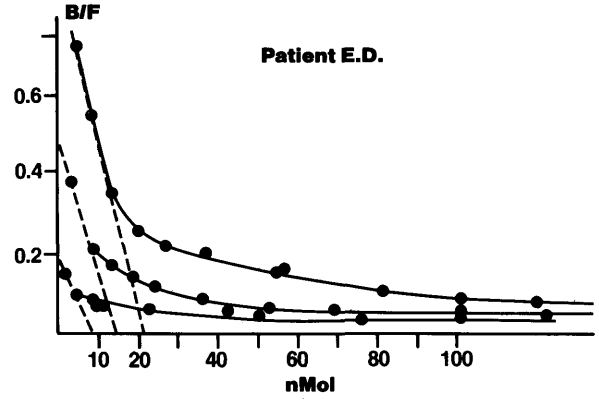

FIG. 3. Scatchard plot of the binding data of patient 2 (E.D.). See legend, Figure 2, for explanation.

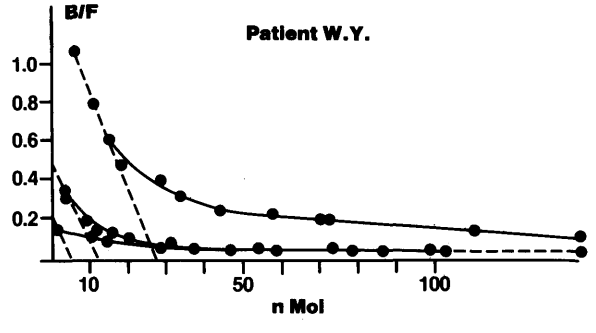

FIG. 2. Scatchard plot of the binding data of patient 1 (W.Y.). The upper curve is the data found in a serum sample taken before the switchover from purified porcine insulin to human insulin. The intermediate curve is the data found in a serum sample taken 3 wk after changing; the lower curve is the data from a serum sample taken 3 mo after changing.

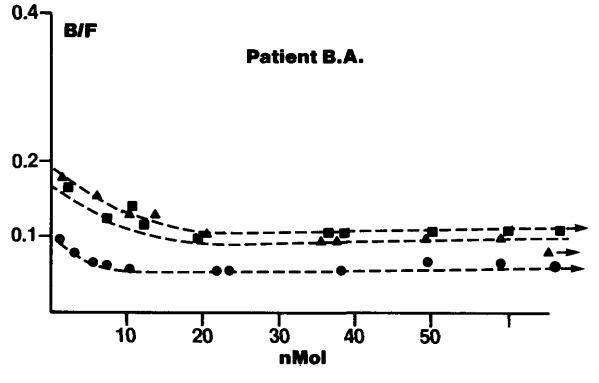

FIG. 4. Scatchard plot of the binding data of patient 4 (B.A.), who changed from purified bovine insulin to purified porcine insulin. For explanation, see legend to Figure 2.

TABLE 3
Total daily insulin dose

Patient	Before CSII (U/day)	3 mo after CSII (U/day)
1 W.Y.	66 PBI	60 human insulin
2 E.D.	76 PPI	65 human insulin
4 B.A.	64 PBI	62 PPI

Patient numbers correspond to numbers in Table 1.
CSII, continuous subcutaneous insulin infusion; PBI, purified bovine insulin; PPI, purified porcine insulin; human insulin, human insulin (recombinant DNA).

demonstrated before or after starting CSII. In patients no. 1 and 2, who were switched from animal insulin to human insulin, a striking decrease in high-affinity circulating insulin antibody level could be demonstrated 3 wk after switching (Table 2 and Figures 2 and 3). In patient no. 4, who was switched from purified bovine insulin to purified porcine insulin, antibody levels before switching were low with only a small contribution of high-affinity sites. After 3 mo of treatment there also was a distinct decrease (Figure 4) in the level of CIA.

As expected the total daily insulin dose decreased and glycosylated hemoglobin A_1 (nearly) normalized in all patients. Data are only given for the patients with substantial antibody levels (Tables 3 and 4). No improvement in fluorescence angiography or nerve conduction velocity could be demonstrated in any of the patients in the 3 mo of this study (data not given).

DISCUSSION

Substantial amounts of CIA were found in only 3 of the 10 patients under study. This is a rather low frequency in view of data from the literature. Although the ratio of high-affinity to low-affinity binding sites can be different for patients treated with either porcine or bovine insulin, CIA can be demonstrated in most diabetic patients treated with animal insulin.[7] Two factors should be considered with respect to our data.

First the patients had been treated for a long time with highly purified preparations of both bovine and/or porcine insulin, which are supposed to be less antigenic than less purified preparations. Second, our method for the measurement of CIA is able to distinguish specific binding by high- and low-affinity sites from aspecific binding in the serum. The method used for these studies is reliable as shown by the fact that insulin antibodies in two commercial antisera were detected adequately (Figure 1).[6,7]

TABLE 4
Glycosylated hemoglobin A_{1c} levels in three patients who participated in the switchover study

Patient	1 wk before CSII	3 mo after CSII
1 W.Y.	14.7	7.6
2 E.D.	7.8	8.1
4 B.A.	15.2	11.1

Normal hemoglobin A_{1c} is 4.5–8.5.

In the two patients a striking decrease in CIA on transfer to CSII with human insulin could be demonstrated. The estimations were done in one assay by a technician who did not know the origin of the samples. So the gradual decrease in CIA can neither be ascribed to large between-assay variations nor to bias by knowing the source of the samples. Since the half-life of insulin antibodies is supposed to be 4–6 wk, our observation indicates an acute change. This can only be explained by an absolute stop in antibody production or an enormously increased antibody clearance. Our studies do not permit favoring one of these possibilities. In discussing the mechanism of the change in CIA levels the effect of improved metabolic control should be differentiated from the switch to human insulin. From the available data no definite conclusion can be drawn, but it is tempting to hypothesize that human insulin is less antigenic than purified animal insulin. Thus far the long-term implication of the observation is not clear. No short-term effect on microangiopathic complications could be demonstrated, but the observation time was only 3 mo.

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