Determination of Insulin Specific IgE in Serum of Diabetic Patients by Solid-Phase Radioimmunoassay

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Summary. A solid-phase assay system for quantitative measurement of insulin specific IgE has been developed. Insulin specific IgE and IgG are bound to insulin covalently coupled to Sepharose particles. After a washing procedure which removes unbound immunoglobulins, ^{125}I -anti-human IgE-rabbit globulin is added to the Sepharose to determine the amount of bound IgE. The use of standardized ^{125}I -anti-human IgE-globulin permits quantitation against a calibration curve of IgE and expression as units/ml. No cross-reactivity of IgG was found. Insulin specific IgE was determined in the sera of diabetic patients. Patients treated with porcine or mixed species purified insulin (monocomponent MC) did not differ significantly from a non-diabetic control group, whereas serum samples taken from patients treated with crystallized insulin preparations showed a significantly higher level of insulin specific IgE ($p < 0.05$). Twenty-four patients with generalized insulin allergy and eight patients with immunological insulin resistance also had considerably higher values of IgE antibodies than the control group ($p < 0.001$ and $p < 0.005$, respectively). No correlation was found between the concentration of insulin specific IgE and IgG in individual sera and the level of insulin specific IgE was independent of the total IgE. In all cases of allergy elicited by purified insulin (monocomponent MC), it was ascertained that the diabetic patients in question had received less pure insulin during earlier treatment.

Key words: Insulin specific IgE antibodies (IgE_1), insulin allergy, insulin resistance, crystalline insulin, purified insulin (monocomponent MC).

A few reports have shown that insulin specific IgE (IgE_1) may be induced by treatment with crystalline insulin and is present not only in diabetic patients with allergic symptoms, but also in diabetics without allergy. Patterson et al. [1, 2] confirmed in 1969 in six

patients that spontaneous insulin allergy was caused by antibodies of the IgE class. Kumar et al. [3, 4], using a paper radioimmunosorbent technique, investigated insulin treated diabetics and could not detect IgE_1 titres in non-diabetic controls, whereas detectable IgE_1 was found in all the diabetics. Twelve patients with generalized allergy and resistance had significantly higher titres. Nakagawa et al. [5] described a modification of Wide's radio-allergosorbent test [6] and found higher values in six patients with insulin allergy than in normal persons or insulin treated diabetics without insulin allergy. Velcovsky et al. [7] used a direct method as well as a method calculating the IgE_1 as the difference between the total IgE determined by Pharmacia's paper radioimmunosorbent test and the residual IgE after removal of IgE_1 . This technique allowed the results to be given in U/ml IgE, thus facilitating the comparison of results obtained in different laboratories.

The aims of the present study were to establish a routine method for the determination of IgE_1 , to compare the IgE_1 levels in groups of diabetics treated with insulin of different purity, and finally to investigate the IgE_1 levels in patients with insulin allergy and resistance.

Patients and Methods

Patients

Sera from the following groups of patients were analysed: Control group and Type 1 (insulin-dependent) diabetic patients treated with insulin of different purity and with no overt local or systemic allergy to insulin: 25 non-diabetic subjects, 20 Type 1 (insulin-dependent) diabetic patients treated for 1 year with four to five times crystallized (porcine and bovine) insulin preparations, and 20 Type 1 (insulin-dependent) diabetic patients treated exclusively with porcine purified insulin (monocomponent MC) for 2 years.

Insulin allergic and resistant patients: (a) 24 patients who have had episodes of generalized allergic reactions to insulin, ranging from urticaria and local oedema to anaphylactic reactions, (b) 17 patients where the only complaint was redness at the injection site and which rapidly disappeared, (c) eight immune-type insulin resistant patients with a daily insulin requirement > 150 U.

Table 1. Insulin specific IgE (IgE_I) in non-diabetic subjects and Type 1 diabetic patients

	IgE _I (U/ml)
Non-diabetics (<i>n</i> = 25)	0.6 ± 0.1
Type 1 diabetic patients	
Treated 1 year with four to five times crystallized insulin preparations (<i>n</i> = 20)	1.2 ± 0.2
Treated exclusively with purified porcine insulin (monocomponent MC) for 2 years (<i>n</i> = 20)	0.4 ± 0.1
Treated exclusively with mixed purified porcine and bovine insulin (mono- component MC) for 2 years (<i>n</i> = 15)	0.3 ± 0.1

Results expressed as mean ± SEM

Methods

Insulin was coupled to Sepharose particles (Pharmacia Sepharose 4B) and characterized according to Heding [8]. The Sepharose-insulin particles were suspended in a phosphate buffer (0.04 mol/l, pH 7.4) containing electrophoretically pure human albumin (1 g/l, Behringwerke, Marburg FGR), thiomersal (0.2 g/l, British Drug Houses, Poole, UK), and NaCl (6 g/l). This buffer is referred to as NaFAM. The final concentration of Sepharose-insulin was 1 mg bound insulin/ml. All standards and samples were diluted in NaFAM and run in duplicate. Sepharose-insulin suspensions (500 µl) and sera samples (500 µl) were shaken overnight at 4 °C whereby total binding of both insulin specific IgG (IgG_I) and IgE_I occurred. After washing three times with 2 ml NaFAM followed each time by centrifugation (2000 × *g* for 10 min), the Sepharose-insulin antibody complex was incubated overnight with 100 µl of ¹²⁵I-anti-IgE globulin solution (4.2 mCi/mg, 0.16 µg/ml, Pharmacia, Uppsala, Sweden). With each series of samples, ¹²⁵I-anti-IgE globulin (100 µl) was added to a range of Pharmacia standards (0–50 U/ml IgE, 100 µl) to obtain a calibration curve.

After a further washing procedure (3 × 2 ml NaFAM) for both samples and standards, the radioactivity was counted in a gamma well counter and the results were calculated from the calibration curve and expressed in U/ml of IgE.

Possible interference in the assay by IgG was investigated by determining the IgE_I in (a) 10 patients with IgG_I > 0.5 mU/ml and (b) 10 patients with IgG_I < 0.02 mU/ml. The insulin binding to IgG was determined by the immuno-electrophoretic method of Christiansen [9]. Total IgE was determined by the Pharmacia PRIST method.

Results

The detection limit of the assay, defined as the smallest amount of IgE which can be distinguished from zero with 99% confidence limits, was 0.7 U/ml calculated from 10 calibration curves. The within assay variation expressed as a constant standard deviation (SD) was 0.4 U/ml (range 0–20 U/ml, *n* = 20), and the between assay variation had a SD of 0.5 U/ml (range 5–10 U/ml, *n* = 20).

Multiple estimations on a serum with high IgE_I content at varying dilutions (range 1:10–1:100) gave values of IgE_I ranging from 10.2–14.4, mean 12.0 ± 1.4 U/ml (± SD, *n* = 6). The non-specific binding to Sepharose without insulin was 0.9 ± 0.4% bound radioactivity (mean ± SD) and corresponding values

for non-specific binding to an IgE-free serum (Pharmacia) was 1.5 ± 0.6% bound radioactivity.

In Type 1 diabetic patients with IgG_I > 0.5 mU/ml, IgE_I was found to be 0.2 ± 0.1 U/ml (mean ± SD, *n* = 10), and in a similar group of diabetics with IgG_I < 0.02 mU/ml, the values were 0.3 ± 0.3 U/ml (mean ± SD, *n* = 10). Since there is no significant difference between these two groups IgG_I does not interfere in the assay in the concentrations found here.

In five serum samples without IgG_I, recovery experiments showed 84 ± 3% at the level of 9.3 U/ml IgE_I and 84 ± 3% at the level of 13.6 U/ml IgE_I. In five serum samples containing IgG_I (0.5–2.2 mU/ml), recovery experiments gave identical results. In five serum samples with IgG_I ranging from 5.7–23.9 mU/ml, the recovery was only 51 ± 30% at the level 9.3 U/ml IgE_I and 44 ± 31% at the level 13.6 U/ml IgE_I. Using ample amounts of Sepharose for these determinations the recovery rate was about 85%; if the IgG_I level is > 2.0 mU/ml somewhat more Sepharose-insulin suspension than indicated in the method description is necessary to ensure complete antibody binding.

Table 1 gives the results of the IgE_I analyses of sera from Type 1 diabetic patients treated with insulin of different purity. Sera from Type 1 diabetic patients treated at the same time with 5 × crystallized insulin preparations show a significantly higher level of IgE_I than non-diabetic subjects (*p* < 0.05) and diabetics treated with purified insulin (monocomponent MC) (*p* < 0.001).

The insulin-allergic patients have elevated levels of IgE_I (Fig. 1) ranging from 1.0–18.4 U/ml (7.1 ± 1.2; mean ± SEM) in the group with manifest symptoms (*n* = 24) compared with both the non-diabetic subjects and the diabetic patients treated with purified insulin (monocomponent MC) (*p* < 0.001). The group where the clinical complaints were limited to local redness did not show significantly higher IgE_I levels than the non-diabetic subjects and diabetics treated with purified insulin (monocomponent MC). In the insulin resistant group, one patient had an IgE_I level of 18 U/ml, the other eight insulin-resistant patients showed a significantly higher level of IgE_I than the non-diabetic subjects and the diabetics treated with purified insulin (monocomponent MC) (*p* < 0.005). The *p*-values are calculated according to the Wilcoxon-Mann-Whitney rank sum test. Figure 2 shows the ratios of $\frac{\text{IgE}_I}{\text{IgG}_I}$ obtained in the various groups of patients.

We have excluded patients with IgG_I < 0.034 mU/ml because levels below this limit do not represent significant binding relative to that found in normal subjects [9]. Only eight out of the 20 diabetics treated with purified insulin (monocomponent MC) had IgG_I values > 0.034. No statistically significant

Fig. 1. The distribution of immunoreactive IgE (IgE_I, U/ml) in the investigated groups of Type 1 (insulin-dependent) diabetic patients and control subjects. MC = monocomponent insulin

Fig. 2. Ratio of immunoreactive IgE/immunoreactive IgG (IgE_I/IgG_I) in the investigated groups of Type 1 (insulin-dependent) diabetic patients. Only patients with IgG_I < 0.034 are included. There is no significant difference between the various groups investigated. MC = monocomponent insulin

difference can be shown between the ratio of $\frac{\text{IgE}_I}{\text{IgG}_I}$ in the different groups (5% level). No correlation can be shown between the total IgE and the IgE_I.

Discussion

The solid-phase radioimmunoassay used to measure IgE_I appears to be specific, sensitive and not subject to interference by IgG_I. Besides allowing immediate comparison of intra-laboratory results, the inclusion of an IgE calibration curve allows the results to be expressed in U/ml, which is not possible with the methods of Kumar et al. [3, 4] and Nakagawa et al. [5]. Recently, Hamilton et al. [10] have described a method similar to ours, demonstrating high IgE_I in three allergic patients and in one of three patients with insulin resistance. Their results were reported in ng = U/ml, determining the IgE_I level as the difference of IgE measured before and after removal of IgE_I from the serum sample. Quantitation of the IgE_I in this way is rather time consuming in a routine assay. An advantage of our method is that it allows calibration in routine assays. Kumar [3] reported low ratios of $\frac{\text{IgE}_I}{\text{IgG}_I}$ in insulin allergic patients compared with insulin resistant diabetic and Type 1 diabetic patients without these complications. We have not been able to verify this finding and thereby confirm Kumar's suggestion that the circulating IgG_I antibodies could act as

'blocking' antibodies and render some protection against IgE_I mediated reactions. This theory has also been supported by Revatelle and Ovary [11] and Halper and Metzger [12]. Witters et al. [13] contributed to the theory of IgG_I as 'blocking' antibodies, stating that they have demonstrated rising levels of IgG_I in some patients during desensitization.

Our results may explain the fact that Kumar [3] found a difference between Type 1 diabetic patients without allergy and normal subjects, whereas Nakagawa et al. [5] found the two groups identical. Nakagawa's Type 1 diabetic patients were treated with purified insulin (monocomponent MC). Our results show no difference between the non-diabetic control group and patients treated with purified insulin (monocomponent MC). Kumar's patients may have received crystalline insulin and therefore showed the same difference from the normal control group as we too have demonstrated between diabetic patients treated with crystalline insulin and normal subjects (Table 1).

The fact that all patients with generalized allergy had significantly higher values suggests the usefulness of our method as a diagnostic aid in vitro for the choice of treatment. This is supported by the fact that one of the allergic patients showed a decrease in IgE_I after changing insulin (NPH → Lente MC), resulting in disappearance of the clinical allergic symptoms, a finding similar to the case described by Nakagawa et al. [5]. In another allergic patient the gradual decrease

in IgE_I after desensitization was again paralleled by the disappearance of clinical symptoms. Kumar [3] has also demonstrated the same response after desensitization. Our data confirm Kumar's [3] and Velcovsky et al.'s [7] earlier observations that the level of IgE_I is not correlated to the degree of severity of the allergic manifestations. One patient had severe allergic reactions in spite of low IgE_I levels (1.2 U/ml), whereas another patient, whose IgE_I levels were considerably increased (16.8 U/ml), only showed moderate allergic reactions.

Velcovsky et al. [7] have shown that there is no correlation between the content of total IgE and IgE_I, a result we can confirm. Thus the assay for total IgE, still widely used, is, in fact, irrelevant in demonstrating the presence of insulin allergy.

Leslie [14] reported a case of generalized allergic reaction to MC-insulin, where his patient had been treated with insulin for a short period 11 years earlier. Our patient material confirms that allergic reactions are elicited even by purified insulin (monocomponent MC), but only in patients with a history of previous, perhaps only very brief, insulin treatment. Six of our patients developed allergy to purified insulin (monocomponent MC); they all had a history of earlier discontinued conventional insulin therapy.

Kurtz and Nabarro [15], Grünekle [16] and Kahn and Rosenthal [17] support our conclusion by their statements that the institution of insulin therapy needs very careful thought, so as to avoid instances of discontinued treatment leading to an unwanted booster effect on resumed therapy. Grünekle [16], Andreani et al. [18], Reisner et al. [19], Federlin et al. [20] and Vinik et al. [21] confirm the importance of using the purest insulin available in order to avoid allergic reactions created by a possible adjuvant effect of the impurities. Alberti and Natrass [22] came to the same conclusion, advocating the use of insulin preparations of low antigenicity.

In conclusion, our results confirm the likelihood that the use of highly purified insulins, by minimizing the risk of inducing insulin antibody formation of the IgE type, will not be associated with allergic problems (provided the patients are not already immunized).

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