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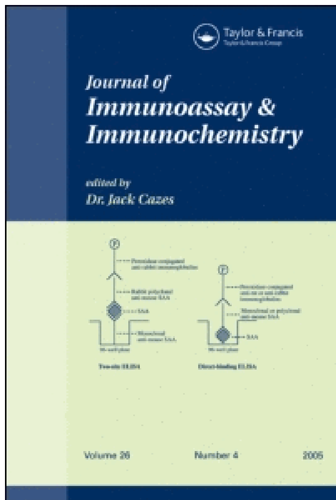

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C-PEPTIDE-MEASUREMENT: A SIMPLE METHOD FOR THE IMPROVEMENT OF SPECIFICITY

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ABSTRACT

Sera containing insulin antibodies and proinsulin give artifactual high readings in C-Peptide measurement. This problem can be circumvented by the removal of antibodies and proinsulin prior to C-Peptide assay. A method is described which enables the removal of antibodies and proinsulin from sera without affecting the C-Peptide determination. Furthermore, this method allows a semiquantitative estimation of proinsulin, provided there is enough cross reactivity of the C-Peptide antibody with proinsulin.

INTRODUCTION

The radioimmunological determination of C-Peptide allows measurement of residual B-cell function in insulin dependent diabetics (1). There are some studies which indicate a correlation between residual B-cell function and metabolic stability (2,3,4). Special tests have been developed for measurement of the residual B-cell function (5,6,7,8). However, we and other authors have sometimes found unexpected normal or even high C-Peptide fasting

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levels in insulin dependent maturity onset diabetics. These high levels, up to 22 ng/ml or 6.1 pMol/ml, could not be explained by high fasting blood sugar levels alone since in metabolically healthy subjects such high levels are never reached even after administration of large amounts of glucose (9,10).

In a previous study (11), we demonstrated that insulin antibodies and proinsulin both interfere with the radioimmunological determination of C-Peptide. As a result, high C-Peptide levels are artifactually found in fasting sera of insulin dependent diabetics. Most of the insulin dependent diabetics have circulating insulin antibodies (12), and the proinsulin portion of secreted material is generally high in diabetics (13,14). Therefore, it seems to be advisable to remove both insulin antibodies and proinsulin from serum samples in order to improve the specificity of the C-Peptide determination.

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A method for the removal of proinsulin from sera has been described by Heding et al (6), using insulin antibodies covalently bound to a solid phase. However, this method is restricted to sera not containing antibodies against insulin. Kuzuya et al (23) described a method which allows the removal of insulin antibodies and antibody bound proinsulin using precipitation with polyethylene glycol. This procedure however, does not eliminate free proinsulin which may be present during the remission phase in juvenile onset diabetes (9) or may occur by in vivo displacement of antibody bound proinsulin after insulin injection.

The aim of our study was therefore to develop a method for the removal of insulin antibodies and antibody complexes as well as proinsulin from sera of insulin dependent diabetics without affecting the C-Peptide determination.

MATERIAL AND METHODS

C-Peptide was measured according to Kaneko et al (16). Reagents were purchased from BYK-Mallinckrodt. Proinsulin was extracted from fresh human pancreas from the operating theatre according to Davoren et al (17). After chromatographic purification on Biogel P 30 in 1 mol/ acetic acid, the fractions containing proinsulin and intermediates were pooled and used as proinsulin standard. This standard was calibrated by L. Heding, Copenhagen, in several dilutions (13). Proinsulin standard dilutions were made by diluting the stock solution with charcoal extracted plasma.

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For the PEG-precipitation, 0.5 ml serum or standards dissolved in charcoal extracted plasma with or without added antibody was vortex mixed with 0.5 ml 24% Polyethyleneglycol 6000 (PEG 6000) in phosphate buffered saline and subsequently centrifuged. The clear supernatant was used for the C-Peptide determinations.

Insulin antibodies were raised in guinea pigs with porcine insulin coupled to bovine serum albumin by the carbodiimide method (18).

Preincubations were made by mixing 50 microliters of the appropriate antibody dilution with 450 microliters of the respective serum and incubating for 24 h at 4° Celsius.

RESULTS

Figure 1 shows the results of an experiment which was designed to demonstrate the cross reaction of proinsulin in the C-Peptide assay and the effect of insulin antibodies on the proinsulin dilution curve. At the 50% intercept a 50% cross reactivity of proinsulin in this assay can be calculated. After the addition of insulin antibodies, the proinsulin dilution curve becomes steeper, resulting in a higher reading from the C-Peptide curve.

After preincubation of the proinsulin dilutions with insulin antibody in excess (1:640) and subsequent precipitation with PEG no cross reactivity of proinsulin could be demonstrated in the C-Peptide assay. With insulin antibodies in higher dilution (1:6400) only low proinsulin concentrations could be bound and PEG-precipitated. At higher proinsulin concentrations, C-Peptide tracer displacement occurs again (Figure 2). Considering the 1:1 dilution of the standards by PEG, there is no loss of immunoreactivity after PEG treatment, nor does PEG affect the slope of the standard curves (Figure 1 vs. Figure 2).

The C-Peptide values of 37 sera from metabolically healthy subjects, ranging from 0.2 to 6.5 ng/ml before PEG precipitation, are

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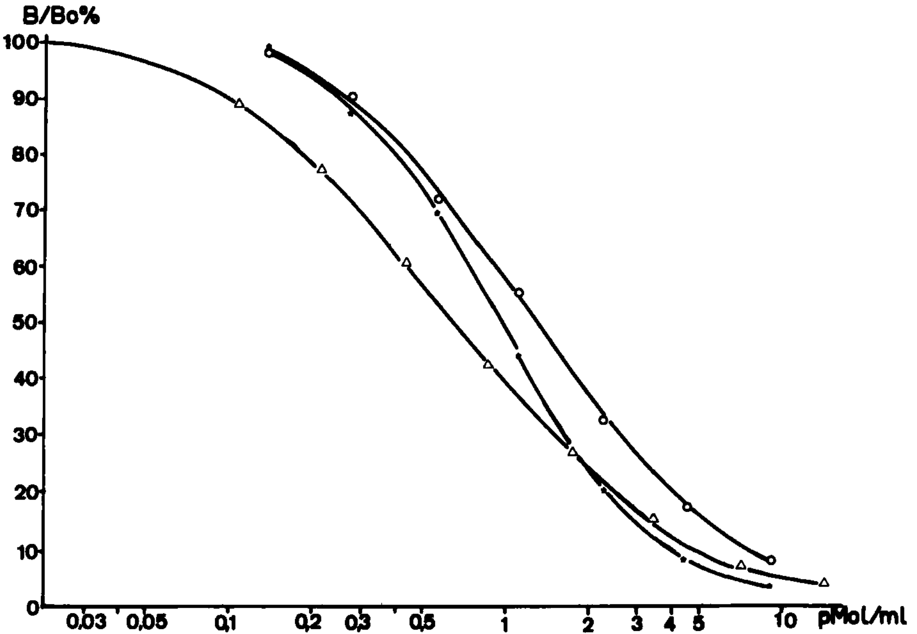

Fig. 1

Crossreaction of proinsulin (open circles) in comparison to C-Peptide (triangles) in the C-Peptide radioimmunoassay. Addition of insulin antibodies to the proinsulin curve (asterisks) causes a steeper dilution curve.

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highly correlated to those after PEG alone, indicating the good working of the measurement system for samples containing 12% PEG.

After preincubation with excess insulin antibodies and PEG-precipitation, 37 sera from metabolically healthy subjects before and after stimulation with various stimuli exhibited a decrease of C-Peptide values due to the removal of proinsulin. This decrease was found to be highly significant using the student's t-test with paired

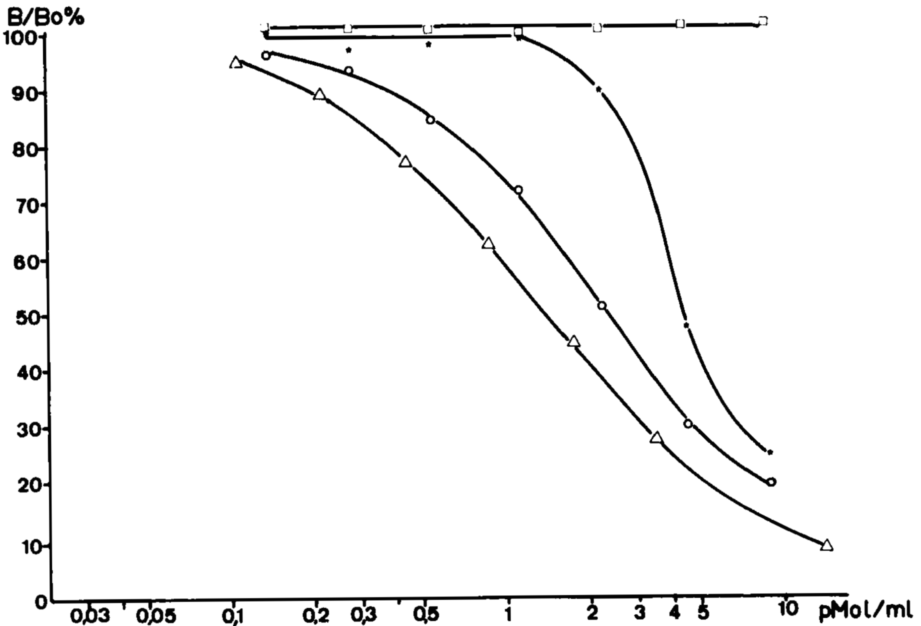

Fig. 2

The effect of the insulin antibody concentration . After preincubation with excess insulin antibodies (squares) all proinsulin has been removed. With low insulin antibody concentrations only low proinsulin concentrations have been removed (asterisks). Open circles and triangles show proinsulin and C-Peptide without preincubation with insulin antibodies.

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variables. The same experiment was performed with 24 sera from five patients with insulinoma. Calculating the contribution of proinsulin to the C-Peptide values from the difference before and after removal of proinsulin, and allowing for the assay cross reactivity, we estimated a contribution of about 20% proinsulin in the case of the metabolically healthy subjects and 36% in the case of the insulinomas.

After pretreatment with PEG along (Table 1), in sera from 17 insulin dependent patients we found mean C-Peptide concentrations of 2.29 ng/ml. After preincubation with excess insulin antibodies and subsequent PEG precipitation the mean C-Peptide value of these sera was 1.97 ng/ml. This difference is highly significant ($p < 0.002$) in the student's t-test with paired variables.

DISCUSSION

The proinsulin molecule contains the whole C-Peptide sequence as well as the entire insulin sequence. It therefore reacts with both the insulin antibodies and the C-Peptide antibodies to a substantial degree. In vitro, during C-Peptide determination these complexes will bind C-Peptide antibodies, resulting in double antibody complexes. There might be a precipitation of these complexes or only an impaired exchange of C-Peptide tracer molecules with this complex. The effect is in each case a lower counting rate in the radioimmunoassay, leading to a higher reading for C-Peptide. The extent of this artifact depends on the cross reactivity of the C-Peptide antibody with proinsulin as well as the concentration of proinsulin (11) and of circulating insulin antibodies.

The use of PEG for the precipitation of antibodies and antibody complexes has been described elsewhere (19,22,23). However, at higher concentrations, PEG sometimes precipitates higher molecular weight proteins. Proinsulin and C-Peptide seem to be small enough to remain soluble and fully immunoreactive at PEG concentrations of 12%.

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TABLE 1

C-Peptide Concentrations, μg/l, in sera from 17 Insulin Dependent Diabetics

Serum Number	PEG Precipitation A	Insulin Antibody and PEG Precipitation B	A - B
1	1.8	1.5	0.3
2	5.8	6.2	-0.4
3	0.4	0.4	0.0
4	2.7	2.5	0.2
5	4.9	4.5	0.4
6	3.9	2.9	1.0
7	2.1	1.6	0.5
8	2.9	2.3	0.6
9	1.8	1.4	0.4
10	1.5	1.0	0.5
11	1.6	1.2	0.4
12	2.7	2.2	0.5
13	3.9	3.1	0.8
14	0.6	0.5	0.1
15	0.8	0.7	0.1
16	1.0	1.0	0.0
17	0.5	0.5	0.0
Mean	2.29	1.97	0.32

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Therefore the C-Peptide assay artifact could be circumvented by binding all proinsulin to insulin antibodies and precipitating the formed complexes by PEG. The advantage of this approach is its applicability to sera containing insulin antibodies as well as free proinsulin.

Kuzuya et al (23) recommended the pretreatment of insulin antibody containing sera with PEG. In our experiments, we demonstrated, that preincubation with added excess insulin antibody and subsequent PEG-precipitation is superior to PEG alone, because considerable amounts of proinsulin became bound to the added insulin antibodies (Table 1). Two reasons may account for these findings: there are large variations in the antibody content of diabetic sera and in the proinsulin content as well. Furthermore, after exogenous insulin administration, there may be considerable in vivo displacement of antibody bound proinsulin. Therefore, for complete removal of proinsulin from sera it is essential to ensure complete binding of proinsulin to added insulin antibody before PEG-precipitation.

Furthermore, the described method may allow a semiquantitative estimate of proinsulin concentrations, provided the cross reactivity of the C-Peptide antibody with proinsulin is high enough to show sufficient sensitivity for proinsulin. For the exact measurement of proinsulin concentrations, however, methods as described elsewhere are more suitable (13,21).

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