Circulating Immune Complexes in the Serum of Diabetes Mellitus in Childhood by a Modified $^{125}\text{I}\text{-C}_{1q}$ Binding Test

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Synopsis

The $^{125}\text{I}\text{-C}_{1q}$ binding test for the detection of soluble immune complexes in native unheated human serum was applied to the study of sera from 52 patients with diabetes mellitus in childhood. This radiolabeled C_{1q} binding test is more sensitive and reproducible among the various methods proposed for the detection of immune complexes.

The $^{125}\text{I}\text{-C}_{1q}$ binding activity in 52 sera from diabetes mellitus in childhood was $9.47 \pm 0.36\%$ compared to $6.94 \pm 0.74\%$ in normal controls. $^{125}\text{I}\text{-C}_{1q}$ binding values in diabetes mellitus in childhood were significantly higher than normal controls. Slight high values were seen in 3 patients with positive anti-DNA-antibodies in diabetes mellitus in childhood.

$^{125}\text{I}\text{-C}_{1q}$ binding was not significantly increased in patients with positive antithyroid antibodies and insulin antibodies. There was no significant correlation between the duration of diabetes and $^{125}\text{I}\text{-C}_{1q}$ binding activity.

Recent investigations suggest that auto-immune processes may be responsible for the pathogenesis in insulin-dependent diabetics (IDD) (Nerup *et al.*, 1976; Irvine *et al.*, 1977).

The clinical association between IDD and autoimmune diseases and a high rate of occurrence of organ-specific antibodies in IDD have already been reported (Irvine *et al.*, 1970; Nerup *et al.*, 1973). Antibodies to pancreatic islet cells in IDD have been detected by using an indirect immunofluorescence technique (Irvine *et al.*, 1977; Lendrum *et al.*, 1975; Bottazzo *et al.*, 1974). The fact that this antibody was a complement fixing and of IgG class was demonstrated by Bottazzo (1974) and Lendrum (1975).

Various methods have been used for the

detection of soluble immune complexes in serum. For instance, biochemical methods such as chromatography and ultracentrifugation in sucrose density gradients were used for the detection. The radiolabeled C_{1q} has been known to react with sera containing immune complexes. This new method could be more sensitive and reproducible than previous methods (Creighton *et al.*, 1973). This C_{1q} binding test has been applied to the study of sera from patients with SLE or to the carriers of the hepatitis B antigen (Nydegger *et al.*, 1974). The purpose of the present study was to detect the immune complexes in the native unheated serum of diabetes mellitus in childhood by a modified $^{125}\text{I}\text{-C}_{1q}$ binding test which was reported by Zubler (1976) and to present evidence in support of the concept of immune complex diseases in some of those patients.

Materials and Methods

Patients studied

The patients were insulin-dependent diabetics who were attending Kinki region-summer camp of diabetic children in 1975 and 1976. They were 29 girls aged 3 to 15 years and 23 boys aged 8 to 14 years. 96 males and 176 females were chosen as normal controls. The age and sex matched control group consisted of 13 healthy children (5 boys and 8 girls) aged 0 to 15 years. These sera have been stored at -70°C .

Measurement of immune complexes

$\text{C}_{1\text{q}}$ was purified by a rapid purification method for the preparation of monospecific antisera (Yonemasu *et al.*, 1971).

The purification of $\text{C}_{1\text{q}}$ was ascertained by the immunoelectrophoresis and disc electrophoresis, and latexagglutination test. Radioiodination of $\text{C}_{1\text{q}}$ was performed by lactoperoxidase method of Heusser (1973) and was stored at -70°C . The radiolabeled $\text{C}_{1\text{q}}$ was diluted in 5 to 8 ml of Veronal Buffered Saline containing 1% bovine serum albumin and centrifuged at 18000g for 40 min at 4°C to discard aggregates of $\text{C}_{1\text{q}}$ before use (Tasaka *et al.*, 1978).

The $^{125}\text{I}-\text{C}_{1\text{q}}$ binding test was performed according to the modified method by Zubler (1976). First, $50\ \mu\text{l}$ of serum sample was mixed with 0.2M EDTA (pH 7.5) $100\ \mu\text{l}$ and incubated for 30 min at 37°C . The next step, $50\ \mu\text{l}$ of $^{125}\text{I}-\text{C}_{1\text{q}}$ and 3% polyethylene glycol (PEG) $1000\ \mu\text{l}$ were added. The mixture was stored for 60 min at 0°C and then centrifuged at 1500g for 20 min at 4°C .

The supernates were discarded and radioactivity in the precipitates was measured by a gamma counter. Results were expressed as percent of $^{125}\text{I}-\text{C}_{1\text{q}}$ precipitated as compared with the radioactivity pre-

cipitated in a trichloroacetic acid "TCA control" tube in which 1 ml 15% TCA was added to $150\ \mu\text{l}$ of normal human serum mixed with $50\ \mu\text{l}$ of $^{125}\text{I}-\text{C}_{1\text{q}}$. All tests were done in duplicate.

Autoantibodies

Circulating antibodies to thyroglobulin and to microsome of thyroid epithelial cells were determined by the tanned red cell hemagglutination method, using a commercially prepared reagents (Thyroid test, Microsome test-Fuji Zoki Co. Tokyo). Antithyroglobulin and antimicrosomal antibody titres were regarded as positive if hemagglutination occurred in 1:160 dilution of sera. For titration v-shaped wells of plastic agglutination trays (Cooke Instruments) were used because the settling pattern of the cells in the cup could be observed from the bottom. Anti-DNA-antibodies were tested with Anti-DNA kits from The Radio Chemical Center-Kaken Kagaku Co. Tokyo.

Insulin-antibody was detected by a slight modification of the method of Wright (1966). Insulin-antibody titres were regarded as positive when B corrected/F values were higher than 0.0375.

Fig. 1. $^{125}\text{I}-\text{C}_{1\text{q}}$ binding test in normal controls.

Fig. 2. $^{125}\text{I}-\text{C}_{1\text{q}}$ binding test in normal controls and diabetes mellitus in childhood.

Results

This modified $^{125}\text{I-C}_{1q}$ binding test was applied to the study of normal controls consisting of 96 males and 176 females in each 10-year age group over 1 to 100 years. In females, $^{125}\text{I-C}_{1q}$ binding activity ($^{125}\text{I-C}_{1q}$ BA) showed a progressive increase to the age of 40 years and a decrease over the age of 40 years. In males, maximum values of $^{125}\text{I-C}_{1q}$ BA were found in the latter half of 20 years and 60 years. The $^{125}\text{I-C}_{1q}$ BA in 96 males was 9.21 ± 0.54 compared to 10.15 ± 0.48 in 176 females. (Fig. 1)

52 insulin-dependent diabetes mellitus in childhood showed significantly higher values when compared to the values obtained from 13 normal controls (0-15 years old). The mean value ($\pm$ SE) for the diabetics

group was $9.47 \pm 0.36\%$ $^{125}\text{I-C}_{1q}$ BA. The mean $\pm$ SE of the normal controls was $6.94 \pm 0.74\%$. (Fig. 2)

3 patients with positive anti-DNA-antibodies in diabetes mellitus in childhood showed 8.56, 10.8, 11.6% $^{125}\text{I-C}_{1q}$ BA. (Fig. 3)

No significantly increased $^{125}\text{I-C}_{1q}$ binding activity was seen in patients with positive antithyroid antibodies and insulin antibodies. There was no significant difference of $^{125}\text{I-C}_{1q}$ precipitation between diabetics with antithyroid antibodies and those without antithyroid antibodies. (Fig. 4)

$^{125}\text{I-C}_{1q}$ BA in diabetics with antiinsulin antibodies and those without antiinsulin antibodies was much the same. (Fig. 5)

Table 1 illustrates $^{125}\text{I-C}_{1q}$ BA in diabetics according to the duration of diabetes. There was no significant correlation between the duration of diabetes and $^{125}\text{I-C}_{1q}$ BA.

Fig. 3. $^{125}\text{I-C}_{1q}$ binding test in diabetes mellitus in childhood with and without anti-DNA-antibody.

Fig. 4. $^{125}\text{I-C}_{1q}$ binding test in diabetes mellitus in childhood with and without antithyroid antibodies.

Discussion

Fig. 5. $^{125}\text{I-C}_{1q}$ binding test in diabetes mellitus in childhood with and without anti-insulin-antibody.

Table 1. $^{125}\text{I-C}_{1q}$ binding test in diabetes mellitus in childhood according to the duration of diabetes.

Duration	No. tested	C_{1q} BA (mean $\pm$ SE)
<1 year	12	9.89 ± 0.69
1-3 years	5	9.31 ± 1.05
4-6	11	8.79 ± 0.53
6-10	11	8.14 ± 0.60
>10 years	3	9.70 ± 1.39

Although the pathogenesis and etiology of diabetes mellitus remain undetermined, evidence accumulated in recent years suggest that an autoimmune mechanism is responsible for insulin-dependent diabetes (Nerup *et al.*, 1976; Irvine *et al.*, 1977). Increased clinical association of diabetes mellitus with Hashimoto's thyroiditis, Addison's disease and pernicious anemia, and increased frequencies of organ-specific antibodies such as thyroid, gastric parietal cells support that autoimmune mechanism is in pathogenesis of insulin-dependent diabetes.

Antibodies to pancreatic islet cells were found to be of complementary fixing and of IgG class using an indirect immunofluorescence method (Bottazzo *et al.*, 1974; Lendrum *et al.*, 1975).

The detection of circulating immune complexes has been performed by a great variety of methods such as chromatographic separation, ultracentrifugation based on biochemical methods, platelet aggregation test, Raji cell method, ADCC inhibition test and $^{125}\text{I-C}_{1q}$ binding test. The advantage of this $^{125}\text{I-C}_{1q}$ binding test is that it is possible to treat about three hundred specimen in duplicate in half a day by using a small amount of C_{1q} . On the contrary, the disadvantage is that it is necessary to use a considerable accommodation of treating radioisotope and the activity of C_{1q} has a tendency to diminish. As heparinized blood showed extremely high values of $^{125}\text{C-C}_{1q}$ BA, we excluded heparinized specimen. The condition of preservation of serum samples is very important. Only fresh and stored samples at -70°C must be used in this test. And heat-inactivated serum has been found to reduce the C_{1q} binding activity of immune complexes mixed with native serum. So we mixed $50\ \mu\text{l}$ of serum sample with $0.2\ \text{M}$ EDTA (pH 7.5) $100\ \mu\text{l}$ in order to prevent the

integration of $^{125}\text{I-C}_{1q}$ into the intrinsic C_{qrs} complex by Zubler's modified method. PEG was used at a lower concentration (final concentration 2.5%) in order to precipitate only the soluble antigen-antibody complexes. Using an aggregated human-globulins (agg HGG), we have already reported that agg HGG between the concentration of 0.5 γ/ml and that of 3 mg/ml were detectable for positive controls and the C_{1q} BA of the agg HGG increased nearly linearly (Tasaka *et al.*, 1978). And in 44 patients with SLE, $^{125}\text{I-C}_{1q}$ BA correlated with the titres of antinuclear factors, positive LE test and in nephrotic syndrome (membranous type), C_{1q} BA showed high values. C_{1q} BA in normal females between 30 and 49 years was significantly different from that in normal age matched males ($p < 0.01$). This difference is very important, compared with the fact that various autoimmune diseases are found frequently in females aged 30 to 50 years. Using a Raji cell assay, Irvine *et al.* (1977) revealed the significant prevalence of soluble immune complexes in newly diagnosed IDD with the positive islet cell antibody. And they have already clarified that islet cell antibody was most common in newly diagnosed IDD of recent onset and the prevalence of the humoral islet cell antibody was strongly dependent on the duration of the diabetics. In our experiments, significantly higher values of $^{125}\text{I-C}_{1q}$ BA were also found compared to normal aged matched control group, and, no significant correlation between the duration of diabetes and $^{125}\text{I-C}_{1q}$ BA was found. The reason for this discrepancy is not clear, but various including procedures for detecting immune complexes might be responsible. Several human-globulins (agg HGG), we have already reported that agg HGG between the concentration of 0.5 γ/ml to 3 mg/ml were detectable for positive controls and C_{1q} BA of the agg HGG increased nearly linearly (Tasaka *et al.*, 1978). And in 44 patients

with SLE, $^{125}\text{I-C}_{1q}$ BA correlated with the titres of antinuclear factors, positive LE test and in nephrotic syndrome (membranous type), C_{1q} BA showed high values. C_{1q} BA in normal females between 30 and 49 years was significantly different from that of normal age matched males ($p < 0.01$).

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References

- Bottazzo, G. F., A. F. Christensen and D. Doniach (1974). *Lancet* 2, 1279.
- Creighton, W. D., P. H. Lambert and P. A. Miescher (1973). *J. Immunol.* 111, 1219.
- Faulk, W. P., E. J. Tonsovic and H. H. Fundenberg (1970). *Amer. J. Med.* 49, 133.
- Faulk, W. P., J. H. Karam and H. H. Fundenberg (1971). *J. Immunol.* 106, 1112.
- Heusser, C., M. Boesman, J. H. Nordin and H. Isliker (1973). *J. Immunol.* 110, 820.
- Irvine, W. J., B. F. Clarke, L. Scarth, D. R. Cullen and L. J. P. Duncan (1970). *Lancet* 2, 163.
- Irvine, W. J., C. J. McCallum, R. S. Gray, C. J. Campbell, L. J. P. Duncan, J. W. Farquhar, H. Vaughan and P. J. Morris (1977). *Diabetes* 26, 138.
- Irvine, W. J., S. F. Al-khateeb, U. Dimario, C. M. Feek, R. S. Gray, B. Edmond and L. J. P. Duncan (1977). *Clin. Exp. Immunol.* 30, 16.

- Lendrum R., G. Walker and D. R. Gamble (1975). *Lancet* 1, 880.
- Nerup, J. and C. Binder (1973). *Acta Endocrinol.* 72, 279.
- Nerup, J., O. O. Anderson, M. Christy, P. Platz, L. Ryder, M. Thomsen and A. Sveigaard (1976). *Acta Endocrinol.* 83 (supple 205), 167.
- Nydegger, U. E., P. H. Lambert, H. Gerber and P. A. Miescher (1974). *J. Clin. Invest.* 54, 297.
- Tasaka, K., I. Takahashi, H. Yoshida, K. Inoue, Y. Nakai and Y. Hamajima (1978). *The Saishin Igaku* 33, 1312. (In Japanese)
- Wright, P. H. and W. J. Malaisse (1966). *Diabetologia* 2, 178.
- Yonemasu, K. and R. M. Stroud (1971). *J. Immunol.* 106, 304.
- Zubler, P. H., G. Lange, P. H. Lambert and P. A. Miescher (1976). *J. Immunol.* 116, 232.
- Zubler, R. H. and P. H. Lambert (1977). *Ann. Rheum. Dis.* 1, 27.