THE IMMUNOGENIC PROPERTIES OF HIGHLY PURIFIED INSULIN PREPARATIONS. 
THE CLINICAL IMPORTANCE OF INSULIN-BINDING ANTIBODIES

By

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ABSTRACT

Twenty-four diabetic patients were treated with porcine protamine-insulin (NPH-insulin) containing 7–13 mmol proinsulin per mol insulin and 27 diabetic patients were treated with porcine protamine-insulin (HP-insulin) containing 0.36 mmol proinsulin per mol insulin. 75% of the patients treated with NPH-insulin and 15% of the patients treated with HP-insulin formed detectable insulin-binding antibodies. The difference in the antibody titre in the two groups was significant. As a group, patients treated with HP-insulin did not have a significant rise in the plasma insulin-binding capacity when compared to pre-treatment values. When comparing patients with antibodies and patients without detectable antibodies no difference in the degree of regulation could be demonstrated between the two groups. Young patients with antibodies had a higher insulin requirement per kg per day than patients without detectable antibodies. Among patients in remission those without detectable antibodies had a longer remission period than those with antibodies. Apart from the difference in antibody formation and hence a different distribution in the groups compared, the patients treated with NPH-insulin and HP-insulin did not differ with regard to the degree of regulation, the insulin requirement or the duration of the remission period.

Commonly available recrystallized insulin preparations contain 1–2 per cent proinsulin (Steiner et al. 1968; Chance & Ellis 1969). The molecular weight of proinsulin is about 150 per cent of that of insulin (Frank & Veros 1968), and while the insulins generally used differ from human insulin in only one amino
acid, porcine insulin, or three amino acids, bovine insulin, the porcine and bovine C-peptide differ from each other and from human C-peptide in the length of the chain and in about 30 per cent of the amino acid positions (Chance et al. 1968; Schmidt & Arens 1968; Steiner et al. 1969; Oyer et al. 1970).

Usually insulin treated patients are given 10–30 µg proinsulin a day. In spite of these small amounts, the size order and the great difference in amino acid sequence of the proinsulin seems to be important for antibody formation in insulin treated diabetics (Ortved Andersen 1973b; Kumar & Miller 1973).

Treatment of diabetics with insulin preparations purified with special reference to the removal of high molecular derivatives results in formation of less antibodies reacting with insulin as compared to the antibody formation after treatment with common available insulin preparations (Schlichtkrull 1970; Fankhauser & Michl 1971; Andreani et al. 1972; Ortved Andersen 1973c).

The purpose of this investigation has been an attempt to evaluate the significance of insulin antibodies for the insulin dose, the regulation of diabetes and the remission period.

MATERIALS AND METHODS

Patients

Twenty-four diabetic patients, 10 women and 14 men, aged 7–64 years (mean: 28 years) started treatment in December 1967 – February 1970 with porcine insulin as a neutral crystal suspension of protamine-insulin (NPH-insulin).

Twenty-seven diabetic patients, 8 women and 19 men, aged 7–69 years (mean: 30 years) started treatment in September 1971 – April 1972 with highly purified porcine insulin as a neutral crystal suspension of protamine-insulin (HP-insulin).

None of the patients had previously been treated with insulin. None of the patients exhibited symptoms of late diabetic complications. One patient treated with NPH-insulin was overweight. Two patients treated with HP-insulin were allergic to penicillin. Apart from these, none of the patients had symptoms or signs of any other endocrine or immunological disease.

Insulin

Porcine insulin as a neutral crystal suspension of protamine-insulin (NPH-insulin) was produced by Nordisk Insulinlaboratorium, Copenhagen, according to the normal procedures which have remained unchanged for the last 8 years. Chromatographic highly purified porcine insulin, from which the high-molecular-weight derivatives had been removed almost completely, was manufactured as a neutral crystal suspension of protamine-insulin (HP-insulin) and kindly supplied by Nordisk Insulinlaboratorium, Copenhagen.

Using the double antibody method with the pre-precipitation technique of Hales &
Randle (1963) as described by Brunfeldt & Jørgensen (1967), the content of proinsulin and intermediate forms were estimated in the porcine insulin preparations used. In the assay highly specific human anti-porcine proinsulin gammaglobulins were used (Ortved Andersen 1973b).

As precipitating antisera rabbit anti-human IgA and IgG-serum supplied by Brostex A/S, Copenhagen, were used. Porcine proinsulin (batch No. PISGR 28A) was kindly supplied as a gift from lic. pharm. E. Pedersen, the Research Laboratory, Nordisk Insulinlaboratorium and Steno Memorial Hospital. The content of proinsulin in NPH-insulin was estimated to be 7–13 mmol proinsulin per mol insulin. The content of proinsulin in the HP-insulin was 0.36 mmol proinsulin per mol insulin.

**Control**

At 4–5 weekly intervals in the first year and later on with an average of 6 weekly intervals the patients reported for control in the out-patient clinic. The directions for treatment were approximately unchanged during the treatment period. The personnel of the clinic were ignorant of the results of the antibody determinations. Blood samples for antibody determinations were withdrawn previous to and several times during the insulin treatment.

**Blood sugar determination**

Blood sugar determinations were carried out according to the method of Hagedorn et al. (1946) and Bierens De Haan & Roth (1969).

**Determination of plasma insulin-binding capacity**

The insulin-binding capacity (IBC) of plasma was determined according to Ortved Andersen et al. (1972). The lower detection limit (LDL), determined as the upper 95% limit for the binding capacity of porcine insulin in poorly regulated non-treated diabetic patients, was found to be 32 μU/ml. In controls and in well-regulated diabetic patients treated for less than 14 days this was 27 μU/ml.

<table>
<thead>
<tr>
<th>Point</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post-prandial blood glucose (mg/100 ml)</td>
<td>≤ 200</td>
<td>200–250</td>
<td>250–300</td>
<td>&gt; 300</td>
</tr>
<tr>
<td>Amount of glucose in urine (g/24 h)</td>
<td>≤ 10</td>
<td>10–20</td>
<td>20–40</td>
<td>&gt; 40</td>
</tr>
<tr>
<td>Glucose concentration in morning urine (per cent)</td>
<td>0</td>
<td>≤ 1</td>
<td>1–2</td>
<td>&gt; 2</td>
</tr>
<tr>
<td>Ketone bodies in urine</td>
<td>0</td>
<td></td>
<td>+</td>
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</tr>
</tbody>
</table>
Evaluation of regulation

The regulation of the diabetes was evaluated by means of a point system including the following four parameters (Table 1):

1: Blood glucose concentration 2 h after the morning meal.
2: Amount of glucose 24 h urine.
3: Glucose concentration in morning urine.
4: The occurrence of ketone bodies in the urine.

The mean value of the four point values was taken as an expression of the degree of regulation for that particular day.

Remission

Remission in the diabetic state was defined as a reduction in the insulin requirement after discharge of the patient from hospital to a dose \( \leq 0.30 \) units per kg per day for more than two months. Relapse was defined as a persistent increased requirement > 50 per cent of the dose per kg per day.

RESULTS

Antibody formation

Plasma was examined on an average 5 times within the first year of treatment. Ortved Andersen (1972) showed that the maximum binding capacity was reached after treatment for 9–12 months.

Fig. 1 shows the IBC of plasma from the diabetic patients previous to insulin treatment and after treatment for 6–9 months with HP-insulin and NPH-insulin respectively. Of the patients treated with HP-insulin, 4 out of 27 (15 %) formed detectable antibodies, and 3 patients had a borderline IBC. Of the four patients with detectable antibodies, one patient (titre: 134 \( \mu \)U/ml) was allergic to penicillin.

Of the patients treated with NPH-insulin 18 out of 24 (75 %) formed detectable antibodies.

In the group of patients treated with HP-insulin the Wilcoxon test revealed no significant difference in the IBC before and after treatment for 6–9 months with insulin \( (P > 0.1) \).

When comparing the groups treated for 6–9 months with HP-insulin and NPH-insulin respectively, the Wilcoxon test revealed a significant difference in the IBC \( (P < 0.01) \).

Of the 51 patients, 2 patients treated with NPH-insulin and 2 patients treated with HP-insulin attended the out-patient clinic too infrequently, so that a reasonable expression of the degree of regulation and of the insulin requirement could not be obtained. One patient had renal glucosuria; hence the criteria for the calculation of the degree of regulation in this patient would give a
bias. As the criteria for the degree of regulation were the same as used in the insulin prescription the "insulin requirement" too, would be biased.

The remaining 46 patients have been registered in the first 1½ years of treatment and only 38 patients in the period 1½–2 years.

In 3 patients it was impossible to decide if they had detectable antibodies or not in the first ½ year of treatment. The same was the case for the 2 patients in the treatment periods ½–1 year and 1–1½ years and for 3 patients in the treatment period 1½–2 years.

Regulation

In 22 patients treated with NPH-insulin and in 24 patients treated with HP-insulin the degree of regulation was calculated on an average 37 times
in 1659 days and 14 times in 537 days respectively, i.e. the patients were registered on an average of 45 and 38 days respectively.

In Fig. 2 the indices of regulation (mean of the registered regulations in the period concerned) are indicated in 4 treatment periods. Mean value ± sd are shown for all, young and elderly patients with and without antibodies.

It is seen that young patients were best regulated in the first half year of treatment, yet the difference was insignificant. After that time no further deterioration was registered over a period of two years.

No difference was found in the regulation when the groups with and without detectable antibodies were compared. Patients treated with NPH-insulin and HP-insulin did not differ from each other.

**Fig. 2.**
The index of regulation in 4 treatment periods. Each column shows the mean value ± sd for all, young and elderly patients with or without detectable antibodies.
The insulin requirement per kg per day in 4 treatment periods. Each column shows the mean value $\pm$ SD for all, young and elderly patients with or without detectable antibodies.

**Insulin requirement**

The patients differed in age from 7–69 years, 15 patients were $\leq$ 15 years old. Therefore the insulin requirement has been calculated as the insulin requirement per kg per day.

Fig. 3 shows the insulin requirement per kg per day in 22 patients treated with NPH-insulin and in 24 patients treated with HP-insulin. Mean values $\pm$ SD are shown for all, young and elderly patients with and without detectable antibodies.

It is seen that the insulin requirement increases in the first two years of treatment. Furthermore it is seen that young patients with antibodies have a
significant higher insulin requirement than patients without detectable antibodies.

Apart from the difference in antibody formation and thus another distribution in the compared groups, the patients treated with NPH-insulin and HP-insulin did not differ in the insulin requirement.

**Fig. 4.**
The remission periods estimated on the insulin requirement per kg per day.

- ■: Remission period.
- ○: No detectable antibodies.
- ●: + antibodies.
- ←X→: Prolonged virus infection.
Remission

Among the patients treated with NPH-insulin or HP-insulin, 10 out of 24 (42 %) and 12 out of 27 (44 %) had a remission. The mean age (28 years) for patients with remission was not different from that of the total material.

Marked as a hatched area Fig. 4 gives a graphic presentation of the duration of the remission periods for each patient. The insulin requirement, time of antibody examination and the age of each patient are also indicated.

Four out of 12 patients without detectable antibodies (33 %) and 6 out of 10 patients with antibodies (60 %) relapsed within a year.

All patients younger than 16 years of age relapsed within a year. Excluding these children 20 per cent without detectable antibodies and 43 per cent with antibodies relapsed within a year.

Mean age for patients with remission was 28 years, 29 years for 12 patients without detectable antibodies and 27 years for 10 patients with antibodies.

The mean age for patients with relapse within a year was 24 years. This mean value was not different from that of patients without relapse within a year (Wilcoxon test: $P > 0.1$).

No pattern for a correlation between the occurrence of detectable antibodies and relapse could be found.

DISCUSSION

Deckert (1964) demonstrated the significance of contaminating impurities in the insulin preparations for insulin antibody formation. Ortved Andersen (1973b) and Kumar & Miller (1973) showed that common available insulin preparations containing 1–2 per cent proinsulin when given to diabetic patients gave rise to the formation of antibodies specific to proinsulin. The question was raised whether the proinsulin is of significance for the insulin antibody formation.

In this study a group of patients has been treated with an insulin preparation (HP-insulin) containing 3–5 per cent of the proinsulin content in common available insulin preparations. The insulin was administered in a form (crystal suspension) which is known to bring about a strong immunological stimulation (Ortved Andersen 1973a).

In accordance with Schlichtkrull (1970), Fankhauser & Michl (1971), Andreani et al. (1972) and Korp & Levett (1973) only a few patients treated with HP-insulin produced small amounts of insulin antibodies, and when compared as a group, the IBC was not significantly different from untreated patients.

Some patients had a small transient rise in the IBC, possibly because of tolerance or possibly because of transient IgM formation.
The significant lower antibody titre when compared to the antibody titre in patients treated with commonly available insulin preparations indicates that proinsulin might be responsible for the insulin antibody formation. This phenomenon is in agreement with the observed fall in antibody titre when the treatment of the diabetic is switched from conventional or monospecies to monocomponent insulin preparations (Andreani et al. 1972; Waldhäusl et al. 1972; Korp & Levett 1973).

When immunized with less than 1 μg proinsulin per day (HP-insulin or monocomponent insulin) the patients should develop low dose tolerance; however, a few patients developed small amounts of antibodies, possibly because of a high immunological reactivity (one patient was allergic to penicillin). Antibodies to pure insulin, however, can not be excluded in these few patients.

The clinical importance of the insulin antibodies is still not clear. In the introduction Orved Andersen (1972) mentioned that most investigators found high antibody titres in patients with insulin resistant diabetes whereas the correlation between the insulin dose and antibody titres in non-resistant diabetics was found to be less convincing. Korp & Levett (1973) found only small and inconstant alterations in the insulin requirement when they changed the treatment from monospecies to monocomponent insulin. Andreani et al. (1972) when changing the treatment from conventional to monospecies or monocomponent insulin, found the greatest reduction in insulin requirement when changing to monocomponent insulin. In this study young patients without detectable antibodies had a significant smaller insulin requirement per kg per day when compared to the requirement in young patients with antibodies.

In elderly patients no difference in the insulin requirement per kg per day could be demonstrated between the two groups.

The difference in the insulin requirement was only significant when the weight of the patients was taken into account.

As mentioned previously apart from the difference in antibody formation the patients treated with HP-insulin and NPH-insulin did not differ with regard to the insulin requirement, thus it must be concluded that patients treated with HP-insulin have a significant smaller requirement than patients treated with conventional monospecies insulin.

The fact that many investigators do not take the age and the weight of the patients into account may be responsible for some discrepancies in the results.

The study failed to demonstrate a correlation between the antibody titre and the degree of regulation. These observations are in agreement with Schlichtkrull et al. (1973) and Korp & Levett (1973). Orved Andersen (1972) found a correlation in young subjects and Waldhäusl et al. (1972) stated that a fall in antibody titre resulted in a better control. Dixon et al. (1972) found that diabetics with stable blood sugar had moderate amounts of low avid antibodies, while patients with labile blood sugar had small amounts of high avid
antibodies without capacity of buffering. This last mentioned result is not necessarily opposed to the observations of no correlation between antibody titre and degree of regulation. The stable and labile patients of Dixon et al. (1972) are not synonymous with good and poor control according to the definition used in this study.

At the start of the insulin treatment, especially in patients with ketoses a high insulin dose is necessary which can be reduced during the following days or weeks.

The cause of this primary high insulin need, is presumably a transient insulin antagonism, not related to remission.

Why some patients go into remission and why they relapse is still not cleared. A fluctuating state of action in the β-cells in the early phase of the diabetes might be an explanation. Antipancreatic cellular hypersensitivity which was demonstrated in patients with juvenile diabetes of short duration by Nerup et al. (1973) should be noted in this connection. The possible role of repeated virus infections should also be considered.

In the definition used in the present study only a reduction in the insulin requirement from a steady level for more than two weeks was accepted. The reduction should be such in amount and duration to indicate an endogenous insulin production. Relapse was defined as a steady enhanced requirement so as to exclude transient increased insulin need, as for instance determined by infections.

Menzel et al. (1973) showed, that human growth hormone seemed to be without significance for the relapse. Armin et al. (1960), Cunningham et al. (1963) and Logothetopoulos (1968) found transient hyperglycaemia in animals after intravenously or intraperitoneally injection of antibodies to heterologous or homologous insulin. Logothetopoulos (1968) too, found β-cell degranulation. They concluded the antibodies to exogenous insulin neutralized the animals’ endogenous insulin. Grodsky et al. (1966) were able to induce a diabetic-like state in rabbits immunized with bovine insulin. The islet of Langerhans revealed lymphocytic infiltration and β-cell degranulation. Karam et al. (1969) found evidence for a neutralizing cross-reaction between endogenous insulin and antibodies to exogenous insulin in a diabetic patient treated with bovine and porcine insulin preparations.

In accordance with these observations the present study shows, that in the group of patients with antibodies nearly twice as many relapsed within a year of treatment when compared to the group of patients without detectable antibodies. Even though no statistically difference could be revealed between the two groups, presumably because of the small number of patients, the observation point to the importance of antibodies for the relapse of β-cell insufficiency.
ACKNOWLEDGMENT

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ADDENDUM

Since the above mentioned investigation has been concluded, 29 patients has been treated with HP-insulin for more than one year. Three patients (10%) formed detectable antibodies.

Fifteen patients fulfilled the criteria for remission, among these 1 with antibodies and of the remaining 14 without detectable antibodies 5 (36%) relapsed within a year.

The two materials are comparable as to age, direction for treatment and control. If the materials are combined, 9 out of 26 patients without detectable antibodies (35%) and 7 out of 11 patients with antibodies (64%) relapsed within a year. Using a chi-square test no significant difference was found between the two groups: neither in the former material ($\chi^2$: 1.56) nor in the combined material ($\chi^2$: 2.65). The increased chi-square value, however, might suggest that a greater material would reveal a significant difference.

In the group of patients with antibodies not only a higher percentage of patients with relapse within a year was found, among patients with relapse within a year those with antibodies also had a shorter remission period than patients without antibodies, so the chi-square test will give a fallacy. Using Wilcoxon test, where all remission periods of more than one year were recorded as the same value, a significant difference was demonstrable between the two groups ($P < 0.05$).

REFERENCES

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