

Circulating IgG Antibody to Protamine in Patients Treated with Protamine-Insulins

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Summary. Sera from patients treated with different types of protamine-insulin were assayed for IgG antibody to protamine. A high prevalence of circulating antibody was found in patients treated with either bovine isophane insulin (26 out of 28 patients; 26 of whom also had antibodies to insulin), or bovine protamine zinc insulin (27 out of 30 patients; all 30 had antibodies to insulin). In sera from 24 patients treated with highly purified porcine isophane insulin, protamine antibody was detected in nine; circulating insulin-antibody was de-

tected in 12 patients, eight of whom had protamine-antibody; in the 12 patients with no detectable antibody to insulin, antibody to protamine was detected in only one ($\chi^2 = 8.7$, $p < 0.01$). This relationship between insulin and protamine antigenicity is of interest as it suggests that the protamine-insulin complex is itself immunogenic.

Key words: Isophane insulin, protamine zinc insulin, insulin-antibody, protamine, protamine-antibody.

Protamine has been used as a retardant of insulin absorption since the nineteen-thirties [1]. It is a basic polypeptide found in sperm and because of its charge it will bind acidic proteins; the usual source of the protamine used in insulin manufacture has been salmon or rainbow trout. Several different protamines can be found even in one species; a typical structure is the salmon-protamine salmine A1 with molecular weight of 4,000 daltons and containing 32 amino acids, of which 21 are arginine [2]. At neutral pH insulin and protamine form an insoluble ionically bonded complex which crystalizes, under suitable conditions, in the presence of zinc into needle-shaped crystals [3]. This preparation was initially called protamine-insulinate to indicate its salt-like nature [1]. In isophane insulins the usual molar ratio of protamine to insulin is about 1:6 (i.e. 0.3–0.4 mg protamine/100 U insulin) with more protamine being used in protamine zinc preparations (1–1.7 mg/100 U insulin). The protamine in protamine-insulin has been found to be immunogenic; in one report antibodies were detected by complement fixation [4], and in another by lymphocyte proliferation [5]. In this study we have looked for circulating IgG antibody to protamine in patients receiving different types of protamine-insulin.

Methods

Patient Groups

Serum was collected from patients who had been treated only with either conventional bovine isophane and soluble insulins ($n = 28$, age range 15–66 years) or with bovine protamine zinc and soluble insulins ($n = 30$, age range: 28–75 years). Serum was collected also from 24 patients treated only with highly purified porcine isophane and soluble insulins for one year ($n = 24$, age range 16–55 years, median 27 years and daily insulin dose 24–60 U, median 36 U).

No patient had a history of skin allergy or of resistance to insulin.

Assay Methods

Antibodies to both protamine and insulin were determined by measurement of the binding of the labelled antigen by serum and the result expressed as a percentage of the total labelled antigen.

Protamine (from salmon; obtained from Weddel Pharmaceuticals, Stockport, UK) was labelled with ¹²⁵I using the technique described by Bolton and Hunter [6]. Protamine (5 µg) was conjugated with 1 mCi of the N-succinimidyl ester of 3-(4-hydroxy, 5-(¹²⁵I) iodophenyl propionate (sp. act. 2000 Ci/mmol; Amersham International, Amersham, Bucks, UK). Separation of the products of iodination was performed on a Sephadex G50 F column (15 × 0.4 cm) in sodium phosphate buffer (0.05 mol/l, pH 7.5), containing gelatin (2.5 g/l). The first peak of labelled material contained the antigen. Analysis showed that 92% of the label was precipitated by trichloroacetic acid (10% v/v). Initial batches of labelled protamine were pre-

Table 1. The binding of labelled protamine and insulin by serum of patients treated with bovine protamine zinc insulin or bovine isophane insulin

Insulin	No of subjects	Age (years)	Duration (years)	Dose (U)	Protamine B (%)	Insulin B (%)
Protamine zinc insulin	30	50.1 ± 11.7 ^a	20.7 ± 10	57.3 ± 28	20.8 ± 10.1	27.8 ± 16.8 ^a
Isophane insulin	28	38.7 ± 16.1	18.8 ± 7.7	48.5 ± 16.1	18.1 ± 6.1	12.6 ± 10.2

Results are expressed as mean ± SD

^a Significant differences between the two groups of patients at $p < 0.01$

pared in our laboratory; subsequent batches, similarly prepared, were supplied by Nordisk, Gentofte, Denmark.

Serum (10 µl) was incubated for 16 h at 4 °C with labelled protamine (approximately 20,000 cpm) in buffer (200 µl), containing sodium phosphate (0.05 mol/l), pH 7.5, sodium chloride (9 g/l) and EDTA (0.01 mol/l). Rabbit anti-human IgG (100 µl/tube; A090 from Dakopatts, Copenhagen, Denmark) was then added and the incubation continued for 20–24 h at 4 °C. Phosphate buffered saline (1.5 ml/tube) containing cetyl trimethyl ammonium bromide (10 g/l) was then added and the tubes vortexed and then centrifuged at 2,500 rev/min for 30 min. The precipitates, containing the antibody-bound labelled protamine, were washed with 1.5 ml of buffer containing Triton X100 (0.5 g/l), centrifuged and finally washed with 1.5 ml of the the buffer containing cetyl trimethyl ammonium bromide before counting. Non-specific binding, which was determined by including sera from subjects who had never received insulin averaged 4.9 ± 0.7% (mean ± SD). Displacement curves were produced by the addition of unlabelled protamine at concentrations ≤ 20 ng/tube.

The method of determining insulin binding has been described [7]: a serum sample (20 µl) was used with a polyethylene glycol separation procedure. The labelled bovine insulin used (Novo Laboratories, Copenhagen, Denmark) had a specific activity of 25 µCi/µg. Porcine insulin labelled at the A14 position (spec. act. 250 µCi/µg) was prepared using the method described by Linde and Hansen [8]. Non-specific binding averaged 1.4 ± 0.2% (mean ± SD).

Antibody to insulin or protamine was considered to have been detected if the binding of labelled antigen exceeded the non-specific binding plus three standard deviations. Statistical analyses were with the chi-square test, Student's t-test and least squares regression.

Results

The binding of labelled insulin and protamine in the groups of patients treated with bovine isophane and protamine zinc insulins are shown in Table 1 together, with details of age, duration of diabetes and daily insulin dose. The group treated with protamine zinc insulin was significantly older and the insulin binding significantly higher. There was no difference in the prevalence or levels of protamine antibody. Of the 28 patients treated with bovine isophane and soluble insulins, antibody to protamine was detected in 26 and of the 30 patients treated with protamine zinc and soluble insulins antibody was detected in 27 patients. Insulin antibody was detected in 26 out of 28 treated with bovine isophane insulin and, of note, the two subjects in whom insulin-antibody was not detected did have protamine-antibody. All patients treated with protamine zinc insulin had antibody to insulin. In neither group was there any correlation between the levels of insulin binding and protamine binding (in both groups $r = 0.10$, NS); there was also no correlation of either age or duration of treatment with the levels of binding.

The prevalence of both insulin and protamine antibodies was much lower in the 24 patients treated for only one year with highly purified porcine soluble and isophane insulins. The binding of insulin by serum ranged from 1.2 to 47% (mean ± SD; 12.1 ± 12.3%) with antibody detected in 12, while protamine binding ranged from 40 to 43% (mean ± SD, 15.2 ± 12%) with antibody detected in nine. Sera from eight of the 12 subjects which bound insulin also bound protamine while the serum from only one subject did not bind insulin but did bind protamine ($\chi^2 = 8.7$, $p < 0.01$).

Two sera with high protamine-binding were studied in displacement assays. In both, labelled protamine was displaced by unlabelled protamine; 50% displacement occurred at concentrations of protamine of 25 and 40 ng/ml. The inclusion of protamine (5 µg/ml) completely blocked specific binding although insulin (125 U/l) did not displace the labelled protamine.

Discussion

This study shows a high prevalence of circulating antibodies to protamine and to insulin in patients on long-term treatment with bovine protamine zinc and bovine isophane insulins. These two groups of patients were not strictly comparable, the group treated with protamine zinc insulin being somewhat older; nevertheless, there was no significant difference in the prevalence or levels of antibodies to protamine. However, in the group treated for a short time with highly purified porcine isophane insulin, the prevalence of antibodies to protamine and to insulin was much lower. The latter group was not comparable to the other groups of patients; however, the dissociation of protamine and insulin immunogenicity is of interest in this group.

In comparison to insulin, little attention has been paid to the immunogenicity of protamine. It has been commented that skin allergy problems are more common with protamine-insulin [8, 9] than with other insulin preparations, and in a case report in which acute urticaria followed injection of NPH insulin, skin tests indicated allergy to protamine and not to insulin and desensitization with protamine was achieved [10]. In an investigation of protamine-antibody formation following vasectomy, one 'control' subject's serum was found by a complement fixation test, to be positive; this patient had been treated with protamine-insulin [4]. Ros-

enthal found that some protamine-insulin treated patients had responded immunologically to protamine as judged by the lymphocyte proliferation test [5].

The determination of protamine binding to antibodies in serum presented several methodological problems. The preparation of the labelled material gave rise to difficulties. Protamine is heterogeneous and does not contain tyrosine. Iodination was achieved by conjugation with the Bolton Hunter reagent. The products of this reaction included immunoreactive labelled protamine in relatively low yield (20%) together with lower molecular weight material that was not precipitated by trichloroacetic acid and was not immunoreactive. Because of its basic nature protamine associates with acidic proteins. The problem of separation of antibody-bound and unbound labelled protamine, in the presence of binding of labelled protamine to other proteins, was overcome by using a double antibody technique, which ensures that only IgG-bound label is precipitated when a cationic detergent included to block the non-specific binding of protamine to acidic proteins. When the non-specific polyethylene glycol separation method, as used for insulin, was tried with labelled protamine, 70% or more of the label was precipitated in control sera.

The antigenic determinants for porcine insulin could occur in physically or chemically altered insulin or in complexes such as those formed by protamine and insulin [11]. In animal studies the immune response genes have been closely associated with the immunological response to insulins of differing amino acid sequences [12]. In man the situation is less clear, possibly because most patients have been 'immunized' with multiple and varying insulin preparations. However, it does appear that HLA-B7 subjects produce less antibody whereas B15 subjects produce more [13]. Although much attention has been focussed on antibodies to insulin and hormonal contaminants, the implications of antibodies to protamine have been overlooked. The present finding that insulin and protamine immunogenicities are linked could indicate that a protamine-insulin complex forms a common antigen or that the individual antigens are genetically associated.

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