Antibodies to Covalent Aggregates of Insulin in Blood of Insulin-Using Diabetic Patients

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SUMMARY

A covalent aggregate twice the size of insulin accounts for ~28% of total circulating insulin immunoreactivity in type I diabetic patients. These aggregates are probably covalent dimers of insulin and should contain unique epitopes distinct from the parent molecule. Therapeutic insulin contains a similar material and is the source of the circulating aggregate. Anti-aggregate antibodies were detected by binding-inhibition techniques in 9 of 29 long-term diabetic patients. These antibodies were directed against structures distinct from those of the parent molecule insulin monomer. All antibody-positive patients were men whose blood also contained antibodies to insulin monomer. We conclude that the blood of ~30% of insulin-using diabetic patients contains antibodies directed against epitopes unique to the insulin aggregates. Because insulin monomer and aggregates probably share a common primary amino acid sequence, the anti-aggregate antibodies are probably directed against conformational determinants. Further work is needed to determine whether such aggregates promote or accentuate the development of anti-insulin antibodies in certain genetically predisposed individuals. *Diabetes* 36:838–41, 1987

The blood of insulin-dependent (type I) diabetic patients treated with conventionally purified insulins contains various antibodies rarely seen in the nondiabetic population. Some of these antibodies are directed against insulin and various pancreatic products including proinsulin, somatostatin, glucagon, vasoactive intestinal polypeptide, and pancreatic polypeptide (1). It has been proposed that the development of these antibodies is associated with the therapeutic use of conventionally purified insulin (1–3). Insulins used to treat these patients contain trace amounts of noninsulin pancreatic peptides that may act to immunize the patient. Alternatively, anti-insulin antibodies found in untreated type I diabetic patients may be markers of the autoimmune process initiating pancreatic destruction (4–6).

In addition to trace amounts of other peptides in previously marketed insulin preparations, ~2% of the protein content of all contemporary commercial insulin products contains a covalent aggregate twice the size of insulin (7,8). The structure of the aggregate is thought to be one or more end-to-end or end-to-side dimers (9,10). Recent work has shown that a large percentage (28 ± 1.3%, n = 50) of insulin immunoreactivity (8,11) in the blood of insulin-using diabetic patients is comprised of a substance with physicochemical similarities to the aggregate (8). Delayed clearance relative to insulin monomer partially accounts for the relatively larger proportions of the aggregate in the blood than in the insulin bottle (12).

We determined if antibodies directed against the insulin aggregate could be identified in the blood of insulin-using diabetic patients. If so, such antibodies would provide evidence that the aggregates contain antigenic determinants differing from monomeric insulin. These findings would encourage further investigations into their role in the formation of anti-insulin antibodies in patients such as those treated solely with human and highly purified pancreatic insulins.

SUBJECTS AND METHODS

After informed consent was obtained, blood was taken from 16 men and 13 women during routine clinic visits. The mean age ± SE was 43.3 ± 2.7 yr, and the duration of diabetes was 16.7 ± 2.1 yr. Serum was separated and held at −20°C for later analyses. Three individuals were insulin-using, non-insulin-dependent diabetics (NIDDM). At the time of phlebotomy, 11 used beef-pork preparations, 4 used purified

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pork, and the remainder used biosynthetic human insulin. The patients using biosynthetic insulin had previously used conventional beef-pork insulin preparations.

A14-[^{125}I]monoradioiodinated and crystalline biosynthetic human insulins were the gifts of Dr. B. Frank (Lilly, Indianapolis, IN). Purified porcine dimers (b component; covalent aggregates; >99% dimers, <1% proinsulin-like material and monomers) were supplied by Dr. R. Chance (Lilly). ^{125}I-labeled aggregates were produced by a modification of the chloramine-T technique (13).

The presence of anti-insulin and anti-aggregate antibodies in serum was sought by a modification of the methods of Fineberg et al. (14). In preparation for analysis, prebound antigen was removed from serum by acid-charcoal extraction. Antibodies to insulin aggregates were detected by incubating diluted (final concn 1:12) aliquots of deinsulinized serum incubated with buffer alone (7.0 mM barbital-sodium, 0.5% bovine serum albumin buffer) and excess amounts of insulin monomer (8.4 μg/ml). After 2 h incubation at 37°C, ^{125}I-aggregates were added (20,000 cpm; sp act 362 μCi/μg). The mixture was held at 4°C overnight before the addition of 12.5% (final concn) polyethylene glycol (M_r 8000) to precipitate globulins and the associated tracer. The addition of large amounts of native monomer was used to block binding sites in cross-reacting antibodies. Nonspecific binding included the amount of binding that could not be blocked by excess amounts of aggregates (4.2 μg/ml).

All serum samples were simultaneously tested with tracer amounts of ^{125}I-labeled insulin tracer (sp act 311 μCi/μg, replacing radioiodinated aggregate) and 4.2 μg/ml insulin aggregates. In this instance, nonspecific binding was assumed to be the percentage of radioactivity precipitable in the presence of 8.4 μg/ml insulin monomer (99.1% monomeric, 0.9% aggregates).

The serum pool made from the blood of eight normal volunteers served as a control for each experiment. Tracer precipitable among these controls in replicate in each assay served as a basis for comparison: sera from diabetic volunteers binding a percentage of tracer >3SD above the replicate measures of the controls were considered antibody positive.

Additional binding-inhibition experiments were performed on sera from six diabetic volunteers, of whom only four were positive for the presence of anti-aggregate antibodies. Diluted (1:12), deinsulinized sera were incubated with combinations of ^{125}I-insulin (20,000 counts/tube) or ^{125}I-aggregates (20,000 counts/tube) with 1.04×10^6 to 5.6×10^2 pM of aggregates or 2.05×10^6 to 9.75×10^2 pM of insulin monomer.

RESULTS

Sera from 26 of the 29 patients were judged to possess antibodies that specifically bound insulin monomer. Anti-aggregate antibodies were present in 9 (31%), all of whom were positive for anti-insulin antibodies. Figure 1 shows the range of responses. A positive assay was conservatively defined as one in which specific binding of ^{125}I-aggregates was at least 3 times the standard deviation of the mean of replicate measures of binding in the normal serum pool. When the cutoff for positivity was considered to be 2SDs above the mean, all patient sera were positive for anti-insulin antibodies, and 11 contained anti-aggregate antibody. Note that percent binding of insulin monomer tracer was generally several times that of aggregate binding.

Among the diabetic group, anti-aggregate-antibody–positive individuals were all males (Yates corrected χ^2; P = .038). Three used biosynthetic human insulin, two used purified pork insulin, and four used beef-pork insulin. Two additional responders were detected when the criteria for positivity was extended to include subjects with binding between 2 and 3SDs above the normal pool. One of the low-level responders was female. No correlation was apparent between a positive anti-aggregate antibody response and insulin dose, age of the patient, duration of diabetes, or a clinical tendency toward metabolic instability. None of the sera from the three patients with NIDDM were positive for anti-aggregate antibody.

The antibodies were further characterized by inhibition-binding experiments in which quantities (shown along the abscissa of Fig. 2) of unlabeled aggregate or insulin monomer were incubated in the presence of deinsulinized serum and radioiodinated monomer or aggregate. Figure 2*A* displays the data from a typical experiment expressed as the percent specifically bound along the vertical axis over the amount of unlabeled protein added along the horizontal scale. Antibody binding to insulin tracer was totally extinguished by the addition of large amounts of unlabeled insulin or aggregate. On the other hand, a different pattern was observed when the experiment was performed with ^{125}I-aggregates. Specific binding was totally eliminated by the addition of large amounts of unlabeled aggregate, whereas 10% of aggregate tracer remained bound in the presence of excess amounts of native insulin monomer. This pattern

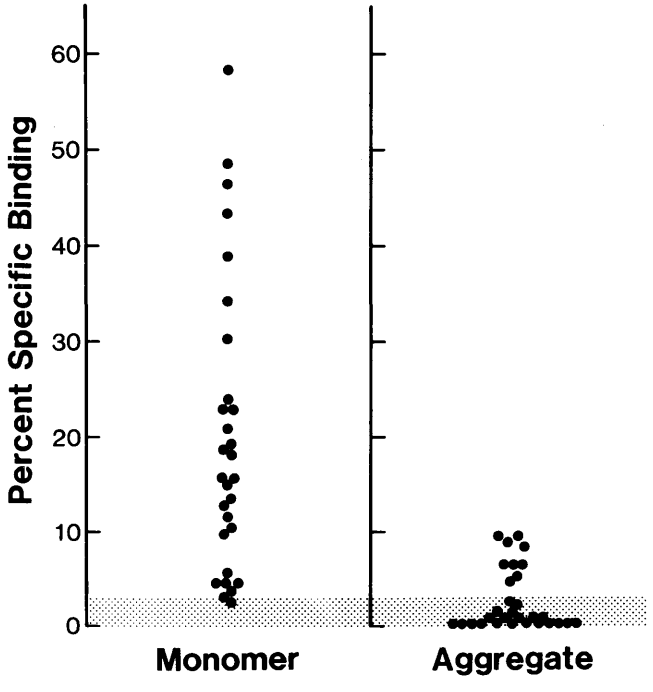

FIG. 1. Percent bound and frequency of anti-aggregate (*right*) and anti-insulin (*left*) antibodies among 29 diabetic volunteers. Positive response was considered > 3SD of normal controls. Twenty-six of 29 diabetic patients had antibodies to insulin. Nine also had antibodies to 12,000-M_r aggregate.

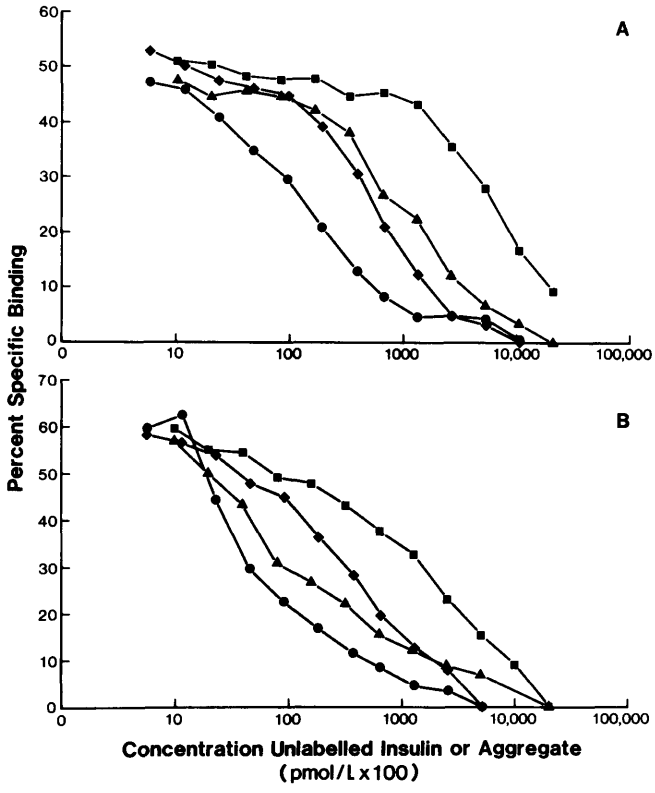

FIG. 2. Antibody binding in presence of increasing amounts of "cold" protein. Combinations of radiolabeled insulin and aggregates and nonradioactive homologous proteins were tested. *A*: results when serum from aggregate-antibody–positive subject was used; *B*: results of same procedures when serum from aggregate-antibody–negative subject was tested. ▲, Radiolabeled insulin incubated with nonradioactive insulin; ●, radiolabeled insulin incubated with nonradioactive aggregates; ■, radiolabeled aggregates incubated with nonradioactive insulin; ♦, radiolabeled aggregates incubated with nonradioactive aggregates.

was found in the four sera that were positive for anti-aggregate antibodies.

Figure 2*B* displays the other pattern found when sera from anti-aggregate-antibody–negative patients were used. The addition of native aggregate or monomer completely eliminated binding to either radioiodinated tracer, indicating that all the antibodies detected were directed against sites common to both insulin monomer and aggregate.

Note that the addition of unlabeled aggregate to serum in both cases inhibited relatively more tracer binding than molar-equivalent amounts of insulin (Fig. 2). Twice as much insulin was finally required to totally extinguish binding (1×10^6 vs. 2×10^6 pM). Thus, the aggregate appeared to have twice the number of binding sites as insulin monomer for antigenic determinants common to both.

For the entire group, there was no correlation between the percent monomer and aggregate tracer binding ($r = .35$, $P > .05$), indicating there were clearly individuals whose sera specifically bound large amounts of ^{125}I-insulin but no ^{125}I-aggregate tracer (in the presence of large amounts of nonradioactive insulin). However, when the statistical analysis was limited to sera positive for anti-aggregate antibody, a highly significant correlation existed between the amount of monomer and aggregate bound ($r = .77$, $P < .01$; Fig. 3).

DISCUSSION

These studies resulted from our work characterizing insulin immunoreactivity in the blood of type I diabetic patients. When it became evident that a rather large proportion of circulating insulin was a covalent structure about twice the size of insulin, we hypothesized that this material may promote or in some way enhance the immune response to insulin. For example, an unsettling observation in patients treated solely with human insulin has been the appearance of anti-insulin antibodies (15). Although this may be a manifestation of the autoimmune β-cell–destructive process in some patients, it may also be related to the injection of structurally related aggregates in the therapeutic insulin preparations that augment the response to native insulin.

The presence of aggregate-specific antibodies in ~30% of insulin-using diabetic patients demonstrates that these antibodies occur frequently and, as we had postulated, constitute a population clearly distinct from those directed against insulin. The finding of the antibody only among male patients was an unexpected observation that will require confirmation by testing a larger population. We see no reason why this observation should be sex associated.

The antibodies directed against the aggregates were less common and bound less tracer than the anti-insulin anti-

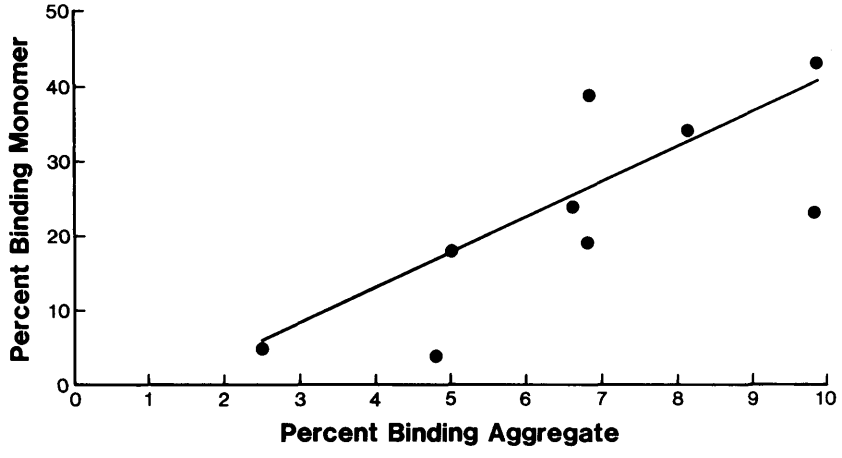

FIG. 3. Values of tracer binding of insulin monomer (*vertical axis*) were plotted against tracer binding of aggregate (*horizontal axis*) among individual sera judged to contain anti-aggregate antibodies. There was significant correlation ($r = .77$, $P < .01$) between the 2 parameters.

bodies. This observation is in contrast to the binding activities of anti-proinsulin antibodies in similar patients (14). In this instance, proinsulin, which also represents a minor constituent of some therapeutic insulins, is bound to a greater extent than insulin. Further characterization of the binding kinetics of the anti-aggregate antibody will be necessary to determine whether the decreased binding of tracer alone is due to lower affinity and/or concentration than the anti-insulin antibodies.

The correlation between the tracer binding of aggregate and monomer only among patients who were sera positive for anti-aggregate antibody raises the possibility that the aggregate may promote antibody formation against the monomer. Repeated testing over time in newly diagnosed individuals may be necessary to determine if this hypothesis is valid. By analogy, a similar situation has been shown to occur in mice injected with insulin and proinsulin in which proinsulin augments the antibody response to insulin (16).

The correlation between the degree of monomer and aggregate tracer binding suggests that certain individuals possess the genetic potential to react to both antigens. Similar to the response to insulin, it follows that the response to the aggregate will be controlled by the genes of the major histocompatibility complex. In humans, mice, and guinea pigs, I-region genes control T-lymphocyte responses to insulin (17–20). In vitro studies with human T-lymphocytes have shown that more epitopes can be recognized than would be anticipated from studies of inbred animals and that many of these epitopes are not related to amino acid exchanges (19–21). Additionally, anti-insulin antibodies have been demonstrated in subjects treated with human insulin (15) or in type I diabetic patients before therapy (4,5,22). Because complex, conformational determinants are probably recognized in humans, it is reasonable to speculate that epitopes specific to the aggregate are conformational and related to the tertiary aggregate structure. If the aggregate-specific response is directed at fewer antigenic epitopes and is found in a more restricted population of patients, an HLA linkage, if present, can probably be defined.

In conclusion, antibodies to insulin aggregates frequently occur among insulin users. Whether the aggregates promote the formation of anti-insulin antibodies in certain individuals needs to be resolved, and whether the injection and accumulation of insulin aggregates constitutes a benign or unhealthy event must be determined.

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