Immuneological Responses to Exogenous Insulin

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Regardless of purity and origin, therapeutic insulins continue to be immunogenic in humans. However, severe immunological complications occur rarely, and less severe events affect a small minority of patients. Insulin autoantibodies (IAs) may be detectable in insulin-naive individuals who have a high likelihood of developing type 1 diabetes or in patients who have had viral disorders, have been treated with various drugs, or have autoimmune disorders or paraneoplastic syndromes. This suggests that under certain circumstances, immune tolerance to insulin can be overcome. Factors that can lead to more or less susceptibility to humoral responses to exogenous insulin include the recipient’s immune response genes, age, the presence of sufficient circulating autologous insulin, and the site of insulin delivery. Little proof exists, however, that the development of insulin antibodies (IAs) to exogenous insulin therapy affects integrated glucose control, insulin dose requirements, and incidence of hypoglycemia, or contributes to β-cell failure or to long-term complications of diabetes. Studies in which pregnant women with diabetes were monitored for glycemic control argue against a connection between IAs and fetal risk. Although studies have shown increased levels of immune complexes in patients with diabetic microangiopathies, these immune complexes often do not contain insulin or IAs, and insulin administration does not contribute to their formation. The majority of studies have shown no relationship between IAs and diabetic angiopathies, including nephropathy, retinopathy, and neuropathy. With the advent of novel insulin formulations and delivery systems, such as insulin pumps and inhaled insulin, examination of these issues is increasingly relevant. (Endocrine Reviews 28: 625–652, 2007)

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Despite experience from more than three-quarters of a century, uncertainties about the significance of the immune response to therapeutic insulins remain. Such issues are largely the residuum of early experiences with pancreatic extracts of animal insulins, however, and have little connection to modern-day preparations of uncontaminated human insulin, which now has a time-tested safety record. Currently, severe immunological sequelae occur only rarely, and less severe events affect a small minority of patients. Still, the following questions persist for physicians and researchers:

- Why do IAs occur with the use of recombinant human insulins?
- What are the key factors that determine immunogenicity of insulin in humans?
- What are the mechanisms that lead to a break in immunological tolerance for insulin?
• What do IA detection and quantification techniques tell us about the nature of the antibodies?
• Do IAs lead to increased insulin dose requirements or hypoglycemic events?
• If the presence of autoantibodies to insulin (IAA) predicts the development of type 1 diabetes in patients not previously treated with exogenous insulin, what is the impact on residual β-cell function of antibody responses to exogenous insulin therapy in patients with established diabetes?
• Because antibodies cross the placenta, do IAs cause diabetes in a developing fetus or contribute to birth defects or postnatal complications?

This review will evaluate these questions by examining the development of IAs to exogenous insulin (Table 1) and by reviewing the literature on antibody responses to different types of insulin therapies with a focus on the clinical impact of IAs.

II. Human Experience with Exogenous Insulin Therapy

A. Animal insulin preparations

The first effective injectable insulins developed by Banting and Best (1) were crude acid-ethanol extracts of abattoir bovine or porcine pancreases. Potencies were highly variable, large volumes were required to reduce blood glucose in diabetic dogs, and injections often involved toxic reactions and abscess formation. The subsequent discovery of an insulin crystallization method by Collip in 1922 led to the first commercial insulin preparations for diabetes therapy (2).

As purification increased, complaints of local abscesses in trial patients decreased; however, reports emerged of anaphylactic, local, and systemic hypersensitivity reactions (2, 3). In one patient, Tuft (4) demonstrated immunological specificity for insulin protein with regard to urticaria and edema, and he was able to transfer dermal reactivity to nonsensitized individuals (Prausnitz-Kustner reaction).

Resistance to insulin therapy and the discordant appearance of cutaneous reactions to insulin were reported from the 1920s onward. The immunological nature of this resistance was shown by insulin neutralization in mice that received sera from insulin-resistant patients (5). Clinical hypersensitivity and insulin resistance were first linked to circulating antibodies to insulin in the work of Berson et al. (6).

Table 1. Factors affecting IA development

<table>
<thead>
<tr>
<th>Insulin-related</th>
<th>Purity</th>
<th>Molecular structure</th>
<th>Storage condition</th>
<th>Formulation</th>
<th>Excipients</th>
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<tbody>
<tr>
<td>Dimer and oxidation products</td>
<td>Patient-related</td>
<td>Age</td>
<td>HLA type</td>
<td>Endogenous insulin</td>
<td>Delivery route</td>
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</table>

HLA, Histocompatibility leukocyte antigen.

The use of isoelectric precipitation by Eli Lilly and Company increased the purity of exogenous bovine and porcine insulin by 10- to 100-fold over previous preparations (2). However, improved analytical methods showed that crystallized insulin included proinsulin and intermediates, nonconvertible insulin dimers, arginyl and ethyl insulin, deamidated insulin, monodesamido insulin, and various noninsulin peptides (7–9). RIAs done in 1982 showed that twice-crystallized Novo monocomponent insulin contained proinsulin and its intermediates as well as trace amounts of other peptide hormones: proinsulin-like immunoreactivity up to 20,000 ppm, glucagon 45 ppm, pancreatic polypeptide 43 ppm, somatostatin 1.4 ppm, and vasoactive intestinal peptide 0.3 ppm (10). These findings raised the possibility that noninsulin peptides were responsible for the antigenicity of therapeutic insulins.

Local reactions to nonchromatographed insulins occurred in up to 55% of recipients, although systemic reactions were rarely seen (11). These dermal and systemic allergic reactions to insulin were demonstrated to be associated with circulating IgE and antibodies to insulin, which were directed toward extracted but not native insulin (12). Patients being treated with insulin were shown to have a broad array of IA binding classes (see Section IV.B) (13).

The incidence of insulin resistance of an immunological nature continued to be quite rare—less than 0.1% of recipients (14, 15). From the late 1970s onward, manufacturers took further steps in insulin purification, including column chromatography, separation of peptides by molecular sieving and charge (16), HPLC (17), and reversed phase HPLC (18). These highly purified monocomponent insulins, which have less than 3 ppm proinsulin-like immunoreactivity, decreased but did not eliminate the development of IAs (19), dermal reactions, systemic hypersensitivity, and insulin resistance (20–22).

B. Recombinant human insulins

The first human insulins for clinical use were derived from porcine insulin by semisynthetic conversion (23). The next advance in insulin therapy was the development of recombinant DNA (rDNA) human insulin made by fermentation of Escherichia coli or Saccharomyces cerevisiae yeast that contained DNA for insulin A and B chains, proinsulin, or modified proinsulin. Later steps after cell lysis include removal of bacterial and yeast components, recombination of insulin chains, or removal of connecting peptides (24, 25).

Clinical trials revealed that semisynthetic human insulin was less immunogenic than porcine monocomponent insulin in patients newly diagnosed with type 1 diabetes. Schernthaner et al. (26) found IAs of the IgG class in 14% of patients receiving human insulins and in 29% of the patients receiving nonhuman insulins. Heding et al. (27) reported that 44% of pediatric patients with type 1 diabetes receiving human monocomponent insulin were antibody-positive, compared with 59% of similar patients receiving porcine monocomponent insulins. (These differences may be attributable to antibody measurement methodologies, which will be discussed in Section IV.A) In a 1-yr trial, Fineberg et al. (28) compared development of antibodies to rDNA human insulin in 100
insulin-naive children and adults with 121 similar individuals treated with purified porcine insulin. At the end of the trial period, 44% of patients treated with rDNA human insulin and 60% of patients treated with porcine insulin had developed significant levels of IA.

In a retrospective review, injection site reactions with highly purified mixed bovine (70% bovine, 30% porcine), purified porcine, and rDNA human insulin were reported to be 12, 3.9, and 2.4%, respectively (29). Sporadic case reports have been published regarding individuals who developed insulin resistance and/or systemic hypersensitivity reactions while being treated with rDNA human insulins, including short-acting and long-acting human insulin analogs (30–34).

**C. Insulin analogs**

Insulin’s tendency to self-associate in concentrated solutions to form dimers and hexamers is largely the reason that sc regular rDNA human insulin and animal insulin extracts have nonphysiological action profiles (35). In response to this problem, researchers developed rDNA human insulin analogs that have decreased tendencies to self-associate. Currently, three rapid-acting human insulin analogs are in clinical use: lispro, aspart, and glulisine. These analogs consist of molecular modifications that result in fewer, if any, differences from unmodified human insulin pertaining to antigenicity and receptor binding of insulin (36, 37). Amino acids essential for insulin receptor binding are A1 (Gly), A2 (Ile), A3 (Val), A19 (Tyr), B6 (Leu), B12 (Val), B23 (Gly), B24 (Phe), and B25 (Asp). In addition, IGF-I binding is affected by B10 (His) and B26–30 (Tyr-Thr-Pro-Lys-Ala). Insulin lispro has a reversal of the sequence of amino acids B28 and B29 (Lys-Pro vs. Pro-Lys), lowering the potential for dimer formation. Insulin aspart substitutes Asp B28 for B28 (Pro). Both of these rapid-acting insulins have IGF-I receptor binding equivalent to unmodified human insulin. Insulin glulisine substitutes Glu for B29 (Lys) and Lis for B3 (Asp), resulting in decreased self-association but somewhat longer residence time on the IGF-I receptor (38). The rapid-acting insulin analogs, when given sc, result in development of humoral IA, with no receptor binding of insulin (36, 37). Amino acids essential to insulin receptor binding are A1 (Gly), A2 (Ile), A3 (Val), A19 (Tyr), B6 (Leu), B12 (Val), B23 (Gly), B24 (Phe), and B25 (Asp). In addition, IGF-I binding is affected by B10 (His) and B26–30 (Tyr-Thr-Pro-Lys-Ala). Insulin lispro has a reversal of the sequence of amino acids B28 and B29 (Lys-Pro vs. Pro-Lys), lowering the potential for dimer formation. Insulin aspart substitutes Asp B28 for B28 (Pro). Both of these rapid-acting insulins have IGF-I receptor binding equivalent to unmodified human insulin. Insulin glulisine substitutes Glu for B29 (Lys) and Lis for B3 (Asp), resulting in decreased self-association but somewhat longer residence time on the IGF-I receptor (38). The rapid-acting insulin analogs, when given sc, result in development of humoral IA, with no significant differences shown between the analogs and unmodified human insulin (39–42). In all the clinical trials cited, IAIs were cross-reactive, i.e., they react equally to unmodified and analog insulins with no demonstrable effects on insulin dose, hypoglycemic events, or glycemic control.

The rapid-acting human insulin analogs are available premixed with modifications having intermediate-duration characteristics (e.g., insulin lispro protamine suspension). Antibodies directed to or cross-reacting antibodies with protamine have been associated with anaphylaxis during reversal of anticoagulation in patients treated with neutral protamine Hagedorn (NPH) insulin (43, 44).

Two commercially available long-acting human insulin analogs are detemir and glargine. Insulin detemir is des B30 Thr with a B29 side chain (lys-myristic acid [tetradecanoyl-]). When concentrated, detemir forms hexamers in sc injection sites and, after absorption of insulin monomers, binds reversibly to albumin. IA data from clinical trials of this analog have not been presented; however, type 3 hypersensitivity reactions and severe dermal reactions have been reported (45, 46).

Insulin glargine is modified by the addition of two arginines to B30 (B31 and B32) and replacement of Asp21 Asp with Gly. Treatment with glargin in patients previously treated with insulin for at least 1 yr did not result in increased IA levels (47).

**D. Subcutaneous and implantable insulin pumps**

In a small, 12-month study, 45 patients with type 1 diabetes were randomized to conventional therapy, multiple dose, or continuous sc insulin infusion (CSII) with highly purified porcine insulins (48). Researchers found an increase in IAIs in the multiple dose and CSII groups but not in the conventional group, suggesting that differences in methods of sc insulin administration affect antibody development (the study has not been repeated with contemporary human insulins) (49, 50).

Implantable insulin pumps (IPPs) have been associated in various studies with increases in IA levels, leading researchers to suggest causative mechanisms such as the storage of insulin in the pump reservoir or to the presence of surfactant or polyethylene glycol (48–50). In a 2002 study, two groups of patients with type 1 diabetes were challenged for 6 months with either continuous peritoneal insulin infusion (CPII) or CSII (400 or 100 U/ml, respectively) using Hoe 21 PH, a semisynthetic human insulin using polyethylene glycol. Antibodies in the CSII group increased significantly vs. the CPII group. Although these data argue against the insulin preparation itself as the culprit, they do not eliminate the possibility of storage degradation or an effect of silicone oil contributing to the increased antibody development associated with CPII (see Section V.B.3.c) (51).

**E. Inhaled insulin**

Despite well-established, long-term benefits of good glycemic control (52), many patients and physicians are reluctant to initiate exogenous insulin therapy (53, 54). In response to this reluctance, researchers have explored pulmonary delivery of insulin as an alternative to sc injection (55). Inhaled human insulin [Exubera (insulin human [rDNA origin]) Inhalation Powder] is the first inhalable insulin approved by the United States and the European Union for the treatment of type 1 and type 2 diabetes. The Exubera inhaler delivers dry powder human insulin contained in blister packets (56).

Diabetes trials with inhaled insulins have been reviewed in a recent article (57), and IA data have been presented for Exubera (Pfizer Inc., New York, NY) in patients with type 1 and 2 diabetes (58, 59), and also for the AERx iDMS system (Novo Nordisk, Bagsvaerd, Denmark) (60). [All inhaled insulins currently are used as premixed insulin analogs but do not have enough duration of activity to meet basal insulin needs (58, 61, 62).]

In the Exubera trials, IA development in patients with type 1 diabetes exceeded that in comparator regular sc human insulin groups; median IA binding increased from 3 to 29% in the Exubera group, compared with no change in the sc
group (58). Among patients with type 2 diabetes who were previously treated with insulin, median IA binding increased to 6% in the Exubera group and was unchanged in the sc group. In patients with type 2 diabetes who had not previously used insulin, no change in median levels of IA binding was seen in either the Exubera or comparator group (treated with an oral antihyperglycemic agent). Differences between patients with type 1 and type 2 diabetes were maintained throughout a 24-month extension study. The IAs were of the IgG class (Fig. 1), and no effects on insulin efficacy or adverse effects could be attributed to IA development (58, 59). Furthermore, in another study IA levels were shown to decline after cessation of Exubera (63).

The AERx iDMS device delivers a liquid droplet of human insulin onto a dosage strip for inhalation (64). In a 12-wk intensive treatment trial among patients with type 2 diabetes being treated with insulin, there was a significant increase in IAs in the inhaled insulin group, but no change in the comparator sc group (60). No clinical effects were attributed to IAs.

Causes of the increased antibody development with inhaled insulin compared with sc insulin therapy have not been identified. The excipients used in the formulation of powdered insulin are not known to incite an immune response, nor can structural differences or alterations in bioactivity be demonstrated (58, 59). However, differences in site of delivery per se may be paramount, because both liquid and powdered formulations of pulmonary insulin are more immunogenic than sc comparators.

F. Modifying factors in the antibody response to exogenous insulin

1. Immunological mechanism of antibody responses. The generation of a primary antibody response to foreign proteins involves the uptake by antigen-presenting cells (APC) such as dendritic cells (65). Dendritic APC are found throughout the body, including all current insulin delivery sites, i.e., the sc space, the lungs, and lymph nodes that drain the peritoneum. The protein antigen is processed to smaller peptide fragments in endosomal vesicles. These peptides bind to major histocompatibility complex (MHC) class II molecules and are transported to the surface of the APC. There are different types of class II molecules, and each has multiple alleles. MHC binding of antigen fragments is determined by the peptide sequence and type of MHC molecule. Thus, not all peptides formed associate with MHC.

The MHC-peptide complex on the APC surface binds to helper T cells with a corresponding T cell receptor that recognizes the peptide. In addition to MHC-peptide-T cell receptor binding, a second signal from the binding of B7 (costimulatory molecule) on APCs to CD28 on T cells will lead to T cell activation and the production of various cytokines. Costimulatory molecules are up-regulated by proinflammatory cytokines produced with tissue damage or infection. Protein antigen binding to the IgG B-cell receptors along with cytokines from the activated T cells leads to the proliferation and differentiation of B cells to antibody-secreting plasma cells.

Immunological tolerance prevents the development of antibody response to self-proteins. This may involve clonal deletion of T cells that recognize self-proteins during development in the thymus. Without costimulation by APC, T cells may become anergic and will not respond to antigen. Tolerance may also occur by means of regulatory T cells that actively down modulate T-helper cell activity. Down-regulation of immune responses to self and nonself antigens involves a subset of regulatory T lymphocytes that express CD4, CD25, and the transcription factor Foxp3 (66). These regulatory T cells, called suppressor T lymphocytes in the past, may be critical in warding off or terminating autoimmunity (67–69). It is likely that these regulatory cells may also play a role in the development and levels of immune responses to exogenous insulin. These cells are present throughout life in humans and have a very rapid turnover rate. The peripheral T regulatory cells are prone to apoptosis and may require the presence of continuous concentrations of self-antigens and/or growth factors such as TGF-β (70). Such conditions may be less prevalent in autoimmune diseases, such as type 1 diabetes, resulting in deficient T-regulatory cell numbers or function (71, 72). T-regulatory cell deficiencies have been demonstrated in a mouse model of autoimmune diabetes (73, 74). Tiittanen et al. (75) recently reported that insulin treatment may activate insulin-specific regulatory T cells in children with newly diagnosed type 1 diabetes.

The H2b strain of mouse does not form antibodies to porcine insulin; however, both helper and suppressor T cells are activated in that strain, allowing the secondary development of antibodies to bovine insulin but suppressing the response to porcine insulin. When T suppressor cells are removed, in vitro development of antiporcine IAs can be shown (76). In addition, nonresponder strains of mice immunized with porcine insulin have been shown to have primed T helper cells and dominant T suppressor cells that cross-react with autologous insulin. Thus, the balance between the effects of T suppressor and T helper cells determined the effectiveness of immune tolerance to heterologous and autologous insulins (77).

2. Sequence differences and impurities. Differences between the amino acid sequence of a protein and the sequence of a self-protein will increase its inherent immunogenicity. As shown in Table 2, the sequences of bovine and porcine insulin differ from human insulin by three amino acids and one amino acid, respectively. Therefore, the administration of bovine insulin or bovine-porcine insulin mixtures resulted in greater IA responses than porcine or human insulin (21, 46, 58, 60, 62, 71, 72, 75, 77).
been against noninsulin proteins (see preparations, some of the antibodies generated may have insulin complexes) in early bovine and mixed bovine-porcine formation as regular human insulin (40). Insulin lispro is thought to have comparable rates of antibody response in a subpopulation of patients (81). However, immunogenicity is also true for other biological therapeutics and has resulted in strict limitation on aggregates in final drug products. It is thought that insulin aggregates may remain for longer periods at the injection site or be more readily taken up by APC. Insulin aggregates are present in the circulation of patients with diabetes being treated with insulin (92), and antibodies that bind to insulin dimers have been demonstrated (92). Lymphocyte transformation studies suggest that insulin aggregates, rather than monomers, are the cause of persistent cutaneous allergy (93). Insulin aggregation and degradation products in a glycerol-based formulation of insulin for use in IPPs have been shown to be associated with increased immunogenicity (94).

Studies in animal models also indicate that aggregates are more immunogenic. Insulin samples taken directly from an insulin vial (material that contained no aggregates) and insulin from an ip pump reservoir (material with a high level of aggregates) were administered to rats, and IA levels were subsequently measured (95). The percentage binding in sera from rats immunized with insulin from the pump reservoir was approximately 2-fold higher than that obtained with

3. Formulation and aggregates. Insulin formulation can also affect immunogenic potential. Soluble forms of insulin, such as regular and semilente, are less allergogenic than intermediate- or long-acting preparations, such as NPH, lente, protamine zinc, and ultralente (21,89). Acid preparations are more immunogenic than neutral ones (21). Allergic reactions to both protamine and zinc have been reported and require special testing to distinguish from insulin allergy (90,91).

Aggregation can clearly affect insulin immunogenicity. This is also true for other biological therapeutics and has resulted in strict limitation on aggregates in final drug products. It is thought that insulin aggregates may remain for longer periods at the injection site or be more readily taken up by APC. Insulin aggregates are present in the circulation of patients with diabetes being treated with insulin (92), and

## TABLE 2. Comparison of amino acid sequences in human and animal insulin

<table>
<thead>
<tr>
<th></th>
<th>A chain</th>
<th>B chain</th>
<th>No. of differences</th>
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<tr>
<td>Human</td>
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<td>FVQNHLCSHSLVEALYLCGERGFFYTPKT</td>
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<td>Chimpanzee</td>
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<td>AfrGr</td>
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<td>Dog</td>
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<tr>
<td>Rat</td>
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<td>FVQNHLCSHSLVEALYLCGERGFFYTPKT</td>
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<tr>
<td>Sheep</td>
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<tr>
<td>Goat</td>
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</table>


78–80). Studies of insulin aspart, which differs from human insulin by one amino acid, have shown a robust antibody response in a subpopulation of patients (81). However, insulin lispro is thought to have comparable rates of antibody formation as regular human insulin (40).

Owing to the presence of contaminants (including glucagon, pancreatic polypeptide, somatostatin, proinsulin, and insulin complexes) in early bovine and mixed bovine-porcine preparations, some of the antibodies generated may have been against nonsulin proteins (see Section II.A) (30). Moreover, other contaminants may also have adjuvant effects and thereby enhance the generation of IAs (19). This finding is consistent with studies that have shown minimal difference in immunogenic potential between purified porcine and human insulin (28, 78, 82).

Most commonly, IAs that develop in response to exogenous bovine, porcine, or human insulins are cross-reactive with all three species (Fig. 2 and Table 3) (83–86). In rare cases, however, antibodies bind differentially to insulins from different species, correlating with clinical responses observed when switching insulin sources (87, 88).

### Fig. 2.

In 1988, Fineberg et al. (85) reported antibody-bound insulin in previously insulin-naive individuals with type 1 or type 2 diabetes treated with rDNA human insulin, purified porcine insulin, or mixed bovine-porcine insulin over 2 yr. On the left, patients with type 1 diabetes reach a peak response within 6 months, with the greatest response to mixed bovine-porcine insulin. On the right, antibody responses for patients with type 2 diabetes are diminished for all the insulins. The insets compare antibody response to human and porcine insulin.
insulin directly from the vial, suggesting that insulin aggregates formed in the reservoir were slightly more immunogenic. This may be relevant to elevated IA levels that have been observed in patients with implanted ip pumps (see Sections II.D and V.B.3.c).

4. Route of delivery. Animal and human studies suggest that the site of antigen delivery can affect antibody responses. Insulin-specific tolerance induction by iv injection in mice is antigen-specific and dose-dependent. The same dose of insulin given ip results in an augmented rather than an inhibited antibody response (96). Intraperitoneal injection of insulin in mice has been found to enhance the immune responses to multiple antigens, including insulin (76, 97, 98). Oral, parenteral, and aerosol insulin administration in diabetes-prone animal models can induce immune tolerance (see Section III.B).

Intraperitoneal insulin delivery via IPPs has been shown to increase levels of IAs in patients with type 1 diabetes (50, 99), but not in those with type 2 (see Sections II.D and V.B.3.b) (100). Intravenous insulin delivery via IPPs (101, 102), as well as oral insulin delivery, does not result in detectable IAs before therapy. After 3 months of therapy, the IAA-positive patients had higher IA levels than those with type 2 diabetes (41). The mechanism for this difference in response is not clear, but it may be due to defects in regulatory T cell function, as reported in nonobese diabetic (NOD) mice—a model of type 1 autoimmune diabetes (73). This defect may lead to enhanced immune responses to human insulin in patients with type 1 diabetes (see Sections II.F.1 and V). However, it is not clear whether exogenous antibody formation can be attributed to further expansion of autoreactive antiinsulin T cells and B cells that produce IAs. There are conflicting data on whether the presence of IAs predicts the IA response to exogenous insulin (106, 113, 114).

The findings from studies with inhaled insulin suggest that patients with type 1 diabetes develop greater levels of IAs than those with type 2 diabetes (58). Studies with insulin lispro also demonstrated that patients with type 1 diabetes had higher IA levels than those with type 2 diabetes (41). The mechanism for this difference in response is not clear, but it may be due to defects in regulatory T cell function, as reported in nonobese diabetic (NOD) mice—a model of type 1 autoimmune diabetes (73). This defect may lead to enhanced immune responses to human insulin in patients with type 1 diabetes (see Sections II.F.1 and V). However, it is not clear whether exogenous antibody formation can be attributed to further expansion of autoreactive antiinsulin T cells and B cells that produce IAs. There are conflicting data on whether the presence of IAs predicts the IA response to exogenous insulin (106, 113, 114).

Exogenous insulin administration may also result in antibody formation in individuals who do not have diabetes. Antibody formation induced by as few as six injections of human insulin is comparable to that seen in chronically treated patients with type 1 diabetes (115). IAs are also found more commonly in individuals who have endocrine

### Table 3. Cross-reactivity of IAs

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Median (µU/ml)</th>
<th>Lower quartile (µU/ml)</th>
<th>Upper quartile (µU/ml)</th>
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<td><strong>Patients with type 1 diabetes treated with EXU</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Human IA</td>
<td>353</td>
<td>29.0</td>
<td>13.0</td>
<td>44.0</td>
</tr>
<tr>
<td>Bovine IA</td>
<td>353</td>
<td>23.0</td>
<td>12.0</td>
<td>37.0</td>
</tr>
<tr>
<td>Porcine IA</td>
<td>353</td>
<td>25.0</td>
<td>12.0</td>
<td>42.0</td>
</tr>
<tr>
<td><strong>Insulin-naive patients with type 2 diabetes treated with EXU</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human IA</td>
<td>134</td>
<td>5.0</td>
<td>1.5</td>
<td>15.0</td>
</tr>
<tr>
<td>Bovine IA</td>
<td>134</td>
<td>4.0</td>
<td>1.5</td>
<td>14.0</td>
</tr>
<tr>
<td>Porcine IA</td>
<td>134</td>
<td>4.0</td>
<td>1.5</td>
<td>11.0</td>
</tr>
</tbody>
</table>

autoimmune syndromes, such as Graves’ disease, Hashimoto’s thyroiditis, and nonimmune thyroid disease (116).

Age may play an important role in the IA response with exogenous insulin administration. Immunological competence declines as an individual ages, causing decreased ability to form high-affinity antibodies, decreased ability to generate long-lasting memory cells, and delayed hypersensitivity responses (117–119). The age of the individual also affects the presence of IAAs before the development of type 1 diabetes and whether an individual is more or less likely to develop a significant antibody response to exogenous insulin (120).

In the studies of Fineberg et al. (41, 83, 121), the development of significant levels of antibodies to exogenous insulin has been shown to be inversely related to C-peptide levels and age. The two factors—age and C-peptide level—are independently correlated, although there is more evidence for a correlation to age than to C-peptide level. A logistic regression analysis of antibody development in 744 individuals who were insulin naive at the beginning of therapy revealed a 3% decrease in the chances of antibody development for every 1-yr increase in age (odds ratio, 0.97; 95% confidence interval, 0.96–0.99; P < 0.001) (122). Additional analysis showed a 46% decrease for every 1-nmol/liter increase in C-peptide levels (odds ratio, 0.54; 95% confidence interval, 0.38–0.78; P < 0.001).

III. Predicting Human Immunogenicity with Animal Models

Several animal species have been used for investigations into the immunogenicity of insulins. Antibody responses can be generated in rabbits, mice, rats, and guinea pigs with sc administration of human insulin and Freund’s adjuvant; however, given the differences in amino acid sequences of insulin between these species and humans (Table 2), these findings are not surprising. The immunogenic potential of insulin lispro, biosynthetic human insulin, and purified porcine insulin in Freund’s adjuvant were compared in rhesus monkeys over a 6-wk period (four per dose group) (123). Measurement of insulin-specific IgG antibody in serum by ELISA indicated that none of the test insulin preparations stimulated antibody formation; thus, these data indicated that human insulin is nonimmunogenic in monkeys. Like other species tested, however, the monkey is a poor predictor of immunogenicity of insulins, including inhaled insulin, in humans. In summary, it appears that animal models are poor predictors of the immunogenicity of exogenous insulin in humans. This is consistent with findings for other protein therapeutic agents. Many drugs are found to be strongly immunogenic in monkeys but weakly immunogenic in humans. Therefore, immunogenicity data from animal studies are usually not used to predict immunogenicity in humans but only to interpret findings in the animals studied (e.g., changes in toxicity, efficacy, and pharmacokinetics due to antidrug antibodies) (124).

However, data exist that suggest animal models may be helpful in predicting relative differences in immunogenicity when comparing modifications of insulin. As discussed in Section III.B, a study was conducted in rats to compare immunogenic responses with different levels of insulin aggregates. In another study, immunological tolerance for human insulin was induced by creating a transgenic mouse expressing the human insulin gene in the β-cells of the mouse pancreas. This model has been used to evaluate the immunogenicity of human insulin analogs (125). Twelve analogs having varying self-association properties were found to have differing immunogenic potential. Substitution of single amino acids in the A8–10 loop resulted in antibody responses in responder mice, whereas substitutions or deletions in B3, B9, B27, and B28 did not produce antibody responses. This model may help identify less immunogenic insulin analogs in the future. Comparing antibody responses to various insulin analogs in these mice to those in humans will be necessary to validate this approach.

A. Effects of inhalation of insulin

IA responses were evaluated in rats and monkeys exposed daily for either 1 or 6 months to the insulin inhalation powder used in Exubera (126). Exposures were up to maximally tolerated doses that were limited by hypoglycemia. IAs were measured by both ELISA and radioligand binding (RLB) assay in serum of rats and monkeys and bronchoalveolar lavage fluid in monkeys. A weak antibody response was observed in rat serum by RLB assay only, but IAs were not detectable in monkey serum or bronchoalveolar lavage fluid.
A comparable weak antibody response in rats was observed after sc injection. There were no histopathological effects of exposure in the respiratory tract or bronchial lymph nodes of either species. Specifically, no evidence was found for an exposure-related immunogenic response in the respiratory tract.

The toxicity of a different dry powder formulation of inhaled insulin was evaluated in beagle dogs exposed for 13 wk (127). Although the researchers did not describe any IA measurements, they found no effects of exposure on pulmonary pathological examination.

The IA response in rats, monkeys, and dogs to insulin inhalation is consistent with findings that inhalation of harmless antigens leads to immunological tolerance. Inhaled antigens are likely captured by intrapulmonary dendritic cells. Under noninflammatory conditions, dendritic cells then migrate to draining lymph nodes and stimulate generation of T regulatory cells that down-modulate the immune response to the inhaled antigen. In this manner, immune-mediated inflammatory responses to reexposure to harmless antigens are avoided (128).

The effects of a nebulized solution of rDNA human insulin were evaluated in the NOD mouse model by Harrison et al. (129). Mice were exposed to either nebulized insulin or ovalbumin, and IAs were measured using an immunoprecipitation assay. Serum IA levels from aerosol–insulin–ovalbumin, and IAs were measured using an immunoprecipitation/agglutination analytical methods (6). Therefore, the RLB assay was used, and it remains the most common format in the measurement of IAs and IAAs. Recently, filter plate separation of insulin/IA complexes from unbound insulin has been incorporated into RLB assay protocols allowing for smaller sample volume requirements and higher throughput (145). In general, RLB assays for total insulin-binding capacity involve coincubating aliquots of serum, buffer, and trace amounts of monoiiodinated 125I-insulin. Unbound and bound insulin in the serum is often removed before incubation by using an acidification process followed by treatment with dextran-activated charcoal. Nonspecific binding can be assessed by incubating a duplicate aliquot of the test serum with a high concentration of unlabeled insulin and trace levels of 125I-labeled insulin.

Immune complexes are precipitated by the addition of antihuman globulin, protein A/G (conjugated to sepharose beads), polyethylene glycol, or other precipitants. The pellets of immune complexes are separated by filtration or centrifugation, washed to remove unbound radiolabeled insulin, and measured on a gamma counter. The mean nonspecific binding disintegrations per minute (dpm) are subtracted from total binding dpm to determine specific binding. The specific binding dpm are converted to microunits or nanograms of IA-bound insulin, based on the specific activity of the labeled insulin. Data may be expressed as insulin-binding capacity (nanograms or microunits of insulin bound per milliliter of serum). Other laboratories express the data as percentage binding (counts in the pellet relative to the total counts of 125I-insulin added).

The ELISA for IAs involves adsorbing insulin to plastic wells of 96-well plates with subsequent addition of test sera. Insulin-specific antibodies in the test sera bind to the insulin on the plate, and the remaining antibody is washed away. Anti-Ig labeled with horseradish peroxidase or alkaline phosphatase is added and binds to the IA. Substrate for horseradish peroxidase or alkaline phosphatase is added to each well, and a color product is formed and monitored by measuring light absorbance. The amount of color product formed increases with the amount of antibody bound to the plate. Data for ELISA are often expressed as end point titers (minimally detectable) or titers that produce 50% maximal signal.

In a few studies of serum from patients with diabetes being treated with insulin, the ELISA was found to correlate with the RLB assay (99, 146–148). However, other studies have demonstrated a poor correlation between the two assays (149–151). Serum samples were found to have very high antibody levels/titers by the ELISA but very low titers when using the RLB assay and vice versa. The RLB has been shown to be more sensitive than the ELISA for detection of IAA. Furthermore, a positive RLB assay for IAA has been found to be a better predictor for the development of diabetes than a similar ELISA result (152).

The differences between the RLB and ELISA findings have been attributed to inherent differences between assay formats. The RLB is a solution-based assay in which low levels of 125I-labeled insulin are used. Thus, high affinity IAs are primarily measured in this assay (150, 151). In contrast, as serum is progressively diluted, the ELISA reaches the stage of excess antigen and thus has the capacity for measuring IAs.
of varying affinities (150, 151, 153). In addition, it is believed that the binding of insulin to solid phase (plastic wells) in the ELISA results in antibody-binding epitopes that are different from those available with 125I-labeled insulin in solution phase.

Although ELISA assays can be processed rapidly and in larger batch sizes and do not require radiolabeled insulin, most laboratories that measure IAs use the RLB assay for the reasons described above. Insulin autoantibody assays are designed with high sensitivity in mind because autoantibodies are found at significantly lower concentrations than are IAs that develop in response to exogenous insulin therapy; therefore, autoantibody assays may need further modification and validation to accurately measure higher IA concentrations.

Progress has been made toward standardizing assays for the measurement of IAs (154, 155), but there is no standardization for assays designed to quantify insulin-therapy-induced IAs, particularly when IAs are present at high concentrations. Because of the large volume of samples generated in the Diabetes Prevention Trial Type 1 study, micro methods were developed to analyze combined IAA glutamic acid decarboxylase 65 and ICA512 (splice variant of islet antigen-2 autoantigen, also referred to as IA-2A) autoantibodies in less than 3 h (156). Even slight differences in the concentration of labeled insulin used, washing conditions, precipitating agent (polyethylene glycol or protein A/G beads), or reagents can yield significantly different results, so the absolute concentration of antibodies determined by one assay cannot be assumed to have a quantitative correspondence with the results of another. Comparison of assays used by different laboratories would require bridging studies between the two methods using a set of laboratory standards of varying antibody levels. This has been accomplished with regard to IAA, using multiple laboratory quality controls, and expressing insulin binding as Juvenile Diabetes Foundation units (154). However, the lack of standardization for the IA assay in patients treated with insulin hampers the ability to correlate titers with clinical findings when IAs are measured by different laboratories using different assay conditions and sometimes different assay formats.

B. Immunoglobulin classes

IA responses consisting of virtually all Ig classes and IgG subclasses have been reported. Insulin-specific antibodies are primarily composed of IgG1–4 antibodies (157), but IgM, IgA, and IgE have been reported. Antiinsulin IgM has been detected during early insulin treatment (158–160), and both Andersen (157) and Reisman et al. (160) reported the presence of that class in patients with immunological insulin resistance. However, Patterson et al. (161) failed to detect IgM in patients with diabetes who were treated with insulin. Faulk et al. (159) reported detectable IgA in patients, and Kniker et al. (162) associated IgA with allergic reactions in patients with diabetes.

Most reports regarding allergic reactions implicate IgE alone or in combination with IgG (see Section V.A) (163–168). However, correlations have not always been found between patients exhibiting allergic reactions and assays for insulin-specific IgE (169), and patients with detectable antiinsulin IgE have not always exhibited allergic reactions (166, 169–171). IgG antibodies have been associated with cases of severe insulin resistance (164, 167); however, most studies show no correlation between IAs and increasing insulin dose requirements (see Section V.B.2.b). Antibodies to insulin—both sc and inhaled—are predominantly of the IgG class (58).

In a study of patients receiving either inhaled or sc insulin, distributions of IgG subclasses were similar for both therapies (172). In general, IgG1 levels were greater than those of IgG4, which were slightly greater than IgG2 and IgG3 levels in both study groups. Similarly, Füchtenbusch et al. (173) demonstrated that after 12 months of sc insulin treatment, patients with type 1 diabetes had predominantly IgG1 and IgG4 antibodies to insulin and that IgG2 and IgG3 were lower in concentration. These authors also showed that IgG1 levels tend to decline, whereas IgG4 levels rise with increased duration of sc insulin treatment.

Ig antibody class distributions resulting from inhaled insulin therapy have been reported in two articles describing immunogenicity data for Exubera and AERX iDMs (58, 60). Although a wide range of antibody levels were found in the Exubera studies, only IgG antibodies could be identified; IgA, IgE, and IgM antibodies were undetectable (58). In 54 patients treated with AERX-iDMS, percentage binding levels rose substantially from a baseline of 6 to 39% after 12 wk (60). This rise in antibodies was accounted for primarily by an increase in IgG antibodies, with small increases in IgE antibodies in four patients. These data are consistent with antibody development reported in studies of sc insulins and suggest that pulmonary antibody responses result from similar immunological mechanisms.

C. Antibody affinity

The RLB assay format has been used to assess relative affinity of IAs. In the classic equilibrium-binding assay, multiple radioligand binding incubations are set up for a single test serum. In this way, a fixed concentration of antibody in the test serum is incubated with increasing concentrations of unlabeled insulin and a fixed amount of 125I-insulin. Using higher concentrations of unlabeled insulin allows IAs with lower binding affinities to bind the labeled insulin. The amount of 125I-insulin bound in each tube with different concentrations of insulin is determined, and a Scatchard analysis of the data has been performed in several studies by plotting the bound/free vs. bound insulin concentration.

Using this method, Goldman et al. (84) and Brooks-Worrel et al. (174) found curvilinear Scatchard plots when serum samples from patients with diabetes treated with insulin were analyzed. Two populations of IAs were identified: high-affinity antibodies with low binding capacity and low-affinity antibodies with high binding capacity. These two subpopulations were typically described by reporting the binding affinity and binding capacity determined from the apparent linear regions of curvilinear Scatchard plots. The binding capacities, association constant (Ka), and calculated dissociation constant (Kd) of each of these two antibody subpopulations were determined. A mean Kd for the high-affinity and low-affinity antibody populations were 1.9 × 10⁻¹⁰ M and 1.6 × 10⁻⁶ M, respectively.
Similar findings were also observed in an earlier study in which a gel-filtration method was used to measure bound and unbound insulin (175). Scatchard analysis has also been extensively used to evaluate the relationship between antibody affinity and hypoglycemia (see Section V.B.3) (49, 176–180).

Concerns have been raised with regard to the use of Scatchard analysis to determine the binding affinities and binding capacities of polyclonal antibody responses (181, 182). Antibody responses to most antigens, including insulin, are polyclonal and comprise antibodies with a wide range of affinities that recognize different epitopes on the insulin molecule (86). Determining antibody affinity and binding capacity of two selected subpopulations in Scatchard analyses excludes information about many other potentially relevant subpopulations of antibodies. Scatchard analyses are best suited for investigations of monoclonal antibodies to determine binding affinity characteristics. When evaluating polyclonal antibody responses, Scatchard analyses are best suited to determine the variance of affinities (181).

A simpler way to describe data from the equilibrium binding assay was reported by Heise et al. (59). Insulin-binding capacities determined from the RLB assay and expressed as micromolars of insulin bound per milliliter of serum at specific insulin concentrations were reported. Binding capacities at corresponding insulin concentrations can be compared and analyzed directly. Reporting binding data in this way is not dependent upon applying a two-site binding model to determine the binding/dissociation constants of arbitrarily selected antibody subpopulations.

Heise et al. (59) used this reporting method to evaluate the clinical significance of low-affinity and high-affinity antibodies in patients with type 1 diabetes treated with inhaled insulin (Exubera). The researchers employed a parallel group design in which 23 patients were treated with sc insulin and 24 patients were treated with inhaled insulin for 24 wk. Mean ($\pm$SD) IA levels for the sc and inhaled insulin groups were $4.3 \pm 9.4$ and $101.4 \pm 140.4 \mu$U/ml, respectively. The binding capacities of samples measured with $10^{-10}$ M (high-affinity) and $10^{-8}$ M (low-affinity) insulin were determined. For the Exubera group, the binding capacities of lower affinity antibodies ranged from 667 to 3360 $\mu$U/ml and from 41 to 387 $\mu$U/ml for the higher-affinity antibodies. The greater binding capacities of the low-affinity antibodies are similar to those reported with Scatchard analysis in previous studies with sc insulin. The binding capacities of the low-affinity and high-affinity antibodies were compared with postprandial glucose area under the curve (AUC), duration of insulin action, hypoglycemic events, and fasting plasma glucose. No correlation was observed between these pharmacodynamic markers and binding capacities of high or low affinities.

**D. Insulin epitope and insulin antibody idotype analyses**

Numerous mouse monoclonal antibodies against human insulin have been generated to identify potential binding sites within the insulin molecule (51, 56, 59, 183, 184). These studies have shown that insulin administration could result in antibodies capable of recognizing many different epitopes. Monoclonal antibodies have been used in competition assays to determine the major sites recognized by naturally occurring polyclonal IAs. This approach has been used to evaluate epitopes recognized by IAs (185), but not by IAs.

Phage display libraries have also been used to investigate insulin epitopes. The displayed random hexapeptide phagotopes recognized by IAA and IA from different sources have been identified and sequenced (186–188), and consensus sequences have been determined and compared against the amino acid sequence of human insulin. These comparisons may lead to the identification of new immune markers of diabetes disease states based on insulin epitopes recognized by IAA and may be used to identify patients for type 1 diabetes prevention therapy. Phagotype analysis may allow IAs to be distinguished from IAs (186).

**E. Characterization of insulin antibody immune complexes**

It was discovered early on that IAs do not form precipitable immune complexes as observed with immune complexes against larger proteins (6). Using ultracentrifugation methods to analyze the nature of the IA immune complexes, it was determined that insulin may bind IAs and form monomers and dimers. The dimers consisted of two IgG molecules bound to one or two molecules of insulin (189). Two different epitopes on insulin are recognized (bivalent) and act as a bridge between two IgG molecules. Larger immune complexes are not formed. It is known that small immune complexes do not activate complement (C1q binding) and are not rapidly cleared (190). This is consistent with the finding that IA immune complexes remain in circulation and are not readily cleared. As discussed in Section V.B.1, IA immune complexes circulate and thereby act as a “sink” for insulin in the circulation. It was also determined that C1q binding to immune complexes of serum samples taken from patients with type 1 diabetes with IAs was not greater than normal controls (191). Immune complex size may also explain the rarity of immune complex hypersensitivity reactions or immune complex diseases in patients with high levels of IAs who receive daily exogenous insulin (see Sections V.A and V.F).

**V. Clinical Significance of Insulin Antibodies**

The incidence and severity of immunological complications of insulin therapy have dramatically decreased with the use of highly purified porcine or rDNA human insulin. Although the new preparations still produce IAs, the titers are lower, and they are rarely associated with clinical events. This section will review previous investigations into the relationships of IA and the following clinical issues: hypersensitivity reactions, hyper- or hypoglycemia, pregnancy, autoimmune markers, pulmonary function, immune complexes, glycemic variability, and diabetes risk. It should be noted that interpretation of these investigations is often limited by uncontrolled observations, small sample sizes usually consisting of individual case reports, and nonstandardized antibody measurement methods.

**A. Hypersensitivity reactions**

Before the 1980s, local hypersensitivity reactions were common complications of insulin therapy. Since the advent
of highly purified insulin in clinical practice, the incidence of allergic complications has markedly decreased (78). Local and systemic reactions with insulin administration may be mediated by insulin-specific IgE (type 1, immediate hypersensitivity reactions) or IgG (type 3, intermediate immune-complex-mediated reactions) antibodies. In addition, non-antibody-mediated type 4, delayed hypersensitivity reactions have been reported. Surveys of these published cases and reviews reveal that the exact type of local reaction is difficult to identify, and in-depth evaluation of such patients using skin tests and skin biopsies have not been commonly reported; thus, the frequency of insulin reactions ascribed in the literature may not be entirely accurate. Furthermore, local allergic reactions to insulin may be of mixed types.

1. Type 1 hypersensitivity. Local, immediate reactions are the least common cutaneous insulin reaction, occurring in less than 1% of patients (192). These reactions begin within minutes of an insulin injection, peak from 12 to 24 h later, and can be followed by generalized anaphylaxis (170). Type 1 reactions can occur at any time with respect to the initiation of insulin, particularly when there is a history of interrupted insulin therapy (78).

Anaphylactic reactions are often preceded by a series of local, immediate reactions (193). They are initiated by the binding of antiinsulin IgE to insulin. These complexes bind to mast cells, releasing into the circulation a variety of vasoactive substances that mediate the syndrome.

Patients with generalized reactions have higher insulin-specific IgE levels than do patients with only local immediate reactions (194). Antinsulin IgE levels can be 10-fold to 20-fold higher in patients with allergic disease than in patients without clinical allergies (167, 195). However, the demonstration of circulating antiinsulin IgE does not establish the diagnosis of insulin allergy because IgE can be found in patients with no apparent allergy (see Section V.B) (171, 196). Patients with antiinsulin IgEs can have concomitant insulin-specific IgG antibodies (88, 167).

2. Type 3 hypersensitivity. Local, intermediate reactions appear 4 to 8 h after insulin injections, peak at 12 h, and generally subside within a day (197). These phenomena are believed to be a vasculitic response to soluble antigen-antibody complexes (Arthus reactions) (192, 194, 198). Lipoatrophy—or loss of fat at insulin injection sites—may represent a persistent, localized Arthus reaction. In biopsies from affected sites, IgM, IgG, and C3 have been demonstrated in dermal vessel walls (199). Lipoatrophy was found to occur in up to 20% of patients before the wide availability of highly purified forms of insulin (197), but it has become virtually nonexistent in patients treated with purified porcine or human insulin. It is possible that lipoatrophy is mediated by contaminant-specific immune responses rather than by IAs. In recent years, the more commonly observed lipohypertrophy at insulin injection sites has been linked to immune responses to insulin as well (200).

3. Type 4 hypersensitivity. Local, delayed reactions are the most common hypersensitivity reaction and usually occur at the start of insulin therapy (192, 198, 201, 202). They generally begin 8 to 12 h after an insulin injection and peak at 24 to 48 h. Most local delayed reactions are mild and self-limited. Although histological analyses suggest that some of these reactions may be mediated by sensitized T lymphocytes (192, 194), it is not known how many of these delayed reactions are truly type 4 reactions. Occasionally, local reactions follow a biphasic course, with temporary improvement occurring between an immediate and a delayed reaction. Type 4 hypersensitivity reactions may also be attributed to contaminants in the older insulin preparations.

4. Allergic response to inhaled insulin. In a pooled analysis of phase 2 and phase 3 trials of Exubera, no evidence was found for excess adverse events of an allergic nature despite higher IA responses in patients who switched to Exubera-based regimens (Table 4) (58).

B. Glycemic effects of insulin antibodies

A number of cross-sectional studies involving small numbers of patients have suggested that IAs can be associated with alterations in a variety of insulin pharmacokinetic parameters. Some of these observed alterations might predict a clinical tendency to hyperglycemia or insulin resistance by neutralizing the biological effect of circulating bioactive insulin, whereas other observed alterations might predict a predisposition to clinical hypoglycemia by prolonging duration of insulin action. Most clinical trial data do not show pharmacodynamic consequences on glucose control corresponding to the pharmacokinetic observations, but case reports of immunological insulin resistance or hypoglycemia syndromes attributed to IAs continue to be published. This section will first review the published pharmacokinetic studies, then clinical data on relationships between IAs and the following glycemic parameters: insulin resistance, metabolic control, insulin dose requirements, and hypoglycemia.

1. Antibodies and insulin pharmacokinetics and pharmacodynamics. Insulin-IA interactions like insulin-insulin receptor interactions are reversible and follow the principles of equilibrium binding. Thus, the amount of insulin bound is dependent on antibody affinity, insulin concentration, and antibody concentration (insulin-binding capacity). Hypothetically, the amount and time course of bioavailable insulin in plasma (insulin pharmacokinetics) could be influenced by IAs and may be mediated by antibody affinity and binding.

Table 4. All-causality adverse events of an allergic nature in controlled phase 2 and phase 3 Exubera studies

<table>
<thead>
<tr>
<th>Event</th>
<th>Inhaled insulin (n = 920)</th>
<th>Subcutaneous insulin (n = 602)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects’ months of exposure</td>
<td>3791</td>
<td>2939</td>
</tr>
<tr>
<td>Body as a whole (includes potential injection site reactions and allergic reactions)</td>
<td>40 (4.3)</td>
<td>33 (5.5)</td>
</tr>
<tr>
<td>Lipodystrophy</td>
<td>4 (0.4)</td>
<td>4 (0.7)</td>
</tr>
<tr>
<td>Respiratory (includes asthma)</td>
<td>107 (11.6)</td>
<td>81 (13.5)</td>
</tr>
<tr>
<td>Skin and appendages (includes nonspecific rashes and dermatitis)</td>
<td>64 (7.0)</td>
<td>28 (4.7)</td>
</tr>
</tbody>
</table>

Data represent number of adverse events (%). Reprinted with permission of the Journal of Clinical Endocrinology & Metabolism (58).
capacity. However, evaluating the pharmacokinetic effects of IAs presents several challenges, not the least of which is validation of the accurate and reproducible measurement of insulin concentrations in the presence of IAs (203, 204). Free insulin results may not always be true reflections of bioactive insulin in the presence of IAs (205).

With an increase in IA binding, there is an apparent increase in the volume of distribution of insulin (206, 207). By acting as a “sink” for exogenously administered insulin, an increase in the apparent volume of distribution of free insulin related to IAs could in theory result in alterations in the disposition kinetics of free insulin.

It has been reported that patients with elevated levels of IAs or antibodies with higher binding capacity experience reduced initial rates of increase and delayed time to peak (120 to 180 min vs. 90 min), and prolonged return to baseline of plasma-free insulin levels after sc insulin injection in comparison with antibody-negative patients or patients with lower antibody-binding capacity (207–210). Although time to peak insulin level after administration of insulin appears to be delayed in most reports, the absolute magnitude of the peak does not appear to be markedly different between high and low levels of IAs (178, 208, 210). The presence of IAs has also been associated with lower maximal (Cmax) free insulin concentrations and AUCs after sc injection of NPH insulin and iv infusion of porcine insulin (178, 209). Prolonged half-life (up to 8-fold greater), increased distribution space (up to 10-fold greater), and faster metabolic clearance rates have been documented after iv infusion of recombinant human and/or bovine insulin in patients with type 1 diabetes (206, 207, 211). Alterations in pharmacokinetic parameters appeared to be correlated with various measures of antibody characteristics, such as insulin-binding affinity, insulin-binding capacity, or percentage of insulin binding.

Waldhauser et al. (211) found heterogeneous total insulin responses to iv insulin administration in subjects with more than 25 μg/liter insulin-binding capacity. Their data suggest a shift in the time course of the plasma free insulin profile but not in the overall extent of the plasma free insulin exposure.

Measurements of the pharmacodynamic responses to insulin are more clinically relevant than pharmacokinetic responses and not subject to questions regarding the validity of insulin measurements in the presence of IAs. Higher postprandial plasma glucose concentrations in subjects with IAs have been described in two pharmacodynamic studies (208, 210). In the first report (208), the effects of IAs appeared to vary with the insulin species under study. Postprandial plasma glucose concentrations after injection with rDNA human insulin did not differ among patients with low (<10 U/liter) or moderate (>10 U/liter) insulin-binding capacity, but postprandial plasma glucose concentrations were significantly greater in the moderate binding group when bovine insulin was administered.

In the second report, Van Haeften et al. (210) found no differences in postprandial insulin or glucose when comparing porcine to human insulin injections, although postprandial glucose excursions in response to a standard meal did appear correlated with the IA binding. Correspondingly, increases in plasma free insulin levels after injections of both insulins were negatively correlated with IA binding. The effect of IAs on postprandial insulin and glucose was largely accounted for by the association constant of the high-affinity IA binding sites (K1). Peak postprandial glucose was 237 ± 10 mg/dl in patients in the upper quartile, compared with 166 ± 12 mg/dl in patients in the lowest association constant quartile.

In contrast, a prospective, open-label, parallel-group trial of 47 patients with type 1 diabetes randomized to receive inhaled insulin (Exubera) or sc regular human insulin, was designed to evaluate whether IA development with inhaled insulin is associated with the loss of postprandial glucose control (59). Mean IA levels increased from baseline after inhaled insulin treatment but not after sc regular insulin treatment. The researchers found no significant differences in postprandial plasma glucose profiles between treatment arms, and no correlation between postprandial blood glucose exposure and antibody-binding affinity was apparent.

A body of literature suggests antibody-mediated prolongation of elevated insulin levels after sc injection (206–208, 211–213); however, pharmacodynamic consequences of this finding have not been consistently demonstrated. Importantly, increased rates of hypoglycemia have not been confirmed in clinical trials (see Section V.B.3.d). Although IAs may not cause hypoglycemia, Bolli et al. (212, 213) published two studies that demonstrated prolonged recovery times from experimentally induced hypoglycemia in patients with IAs. The researchers concluded that patients with insulin-dependent diabetes can have impaired glucose counterregulatory hormone reserve, which can be compounded by a prolongation of the half-life of insulin by IAs. To explain such an IA-mediated phenomenon mechanistically, one must postulate dissociation of free insulin from antibody bound insulin complexes (see Section V.B.3.d).

IAs have not consistently been shown to have the property of prolonging the duration of insulin action. Euglycemic clamp studies have been conducted to explore the effect of IAs on the pharmacodynamic response to exogenously administered insulin. In one study, glucose infusion rates did not vary between subjects with high and low insulin-binding capacity but were lower than in subjects without diabetes (lower glucose infusion rates indicate decreased insulin action) (211).

Peters et al. (209) noted that exposure (AUC) to free insulin and glucose infusion rates were lower in patients with greater than 10% antibody binding compared with subjects with less than 1.5% antibody binding. Gardner et al. (214) found no correlation between antibody status and the onset of action of insulin administered sc. Furthermore, the peak effects of insulin action as well as the duration of insulin action were similar both in patients who were antibody positive and in those who were antibody negative. Similarly, Heise et al. (59) were not able to detect a significant difference in duration of inhaled insulin action in subjects with or without IAs. They reported no correlation between IA-binding affinity and the duration of insulin action.

It seems possible that pharmacokinetic differences attributable to IAs may be observed, but the effects are small in the context of the myriad of other factors that influence glycemic response. The inconsistency between pharmacokinetic and pharmacodynamic observations may lie in the incomplete
understanding of the in vivo conditions that control insulin bioactivity in the presence of IAs. This is further complicated by the imprecise relationship between insulin pharmacokinetics and pharmacodynamics that exists in the absence of IAs (215, 216).

2. IAs and hyperglycemia/interference with insulin action

a. Immunological insulin resistance. IAs are determined to be present when binding of labeled insulin is demonstrated in vitro. Whether in vitro binding quantitatively reflects the binding of circulating insulin in vivo is difficult to directly determine (see Section V.B.1). If antibody binding did occur in vivo to significant levels, one might expect to observe increasing insulin dose requirements and, possibly, worsening glycemic control in patients with diabetes. Although a rare syndrome of severe insulin resistance has been described in patients with high IA levels, a mechanism for a causal relationship between the antibodies and the syndrome has not been clearly established. Furthermore, IAs do not correlate with measures of glycemic control or insulin dose requirements in most large population studies.

Insulin resistance can be defined as a daily insulin dose requirement that exceeds the normal daily pancreatic output in the nondiabetic state, i.e., approximately 40 ± 20 U/d (217). Severe insulin resistance is usually defined as insulin requirement of more than 200 U/d for at least 2 d (14, 217, 218). The incidence of insulin resistance in patients with diabetes was estimated to be 0.1% in a study at the Joslin Clinic between 1940 and 1960 (14). However, this incidence was acknowledged as likely to represent an overestimate because of referral bias, and IA measurements were not reported.

In a subsequent case series, Davidson and DeBra (218) characterized 35 patients with severe insulin resistance associated with the presence of high levels of circulating IAs. Patients studied in this series had mean daily insulin requirement of 550 U/d (range, 200 to 2000 U/d) and maximum insulin-binding capacity greater than 10,000 μU/mL. In addition, the following underlying explanations for large insulin requirements were ruled out: diabetic ketoacidosis, significant infection, significant dietary indiscretion, lipotrophic diabetes, significant complicating endocrine disease, insulin receptor defects, antibodies to insulin receptors, or factitious claim of insulin dose greater than 200 U/d.

At least 60% of patients diagnosed with immunological insulin resistance had a history of diabetes with onset after age 30 yr, suggesting that the syndrome is more commonly observed in patients with type 2 diabetes (20, 218). The onset and subsequent course of insulin resistance is often associated with symptomatic hyperglycemia, including episodes of ketoacidosis and hyperosmolar coma (217, 218). The duration of insulin therapy before the onset of severe insulin resistance has been reported to range from 1 month to 15 yr (217, 218), although 50–85% of patients with insulin resistance received insulin for less than 1 yr, and 10–25% received insulin for less than 1 month before the onset of severe insulin resistance (217, 218). Insulin allergy may coexist with immune insulin resistance in 10–35% of cases (20, 217). Some cases of insulin resistance that occurred as insulin allergy spontaneously subsided, prompting speculation that increasing concentrations of IgG can inhibit IgE-mediated clinical events (20, 167, 219). Although the dominant feature of this syndrome is decreased insulin action, episodes of hypoglycemia can occur. Furthermore, the frequency of hypoglycemia may diminish after resolution of the insulin-resistant state (218).

Immunological insulin resistance is thought to occur less commonly now than it did before the 1980s, but cases of at least partial resistance attributed to IAs continue to be reported—even in patients treated only with human insulin or insulin analogs (30, 164, 220–227). Severe insulin resistance has also rarely been described in the setting of high levels of serum insulin-binding activity associated with underlying chronic lymphocytic leukemia, lymphoma, multiple myeloma, and macroglobulinemia (228). Possibly, the severe resistance in these cases was due at least in part to the production of monoclonal paraproteins with significant insulin-binding activity, although monoclonal insulin binding was demonstrated in only two cases. Insulin resistance occurring in patients with hematological malignancy is not always associated with an insulin-binding monoclonal protein (87).

b. Treatment of immunological insulin resistance. Because immune insulin resistance tends to be self-limited (50% of patients are insulin resistant for <6 months and 75% for <1 yr), therapy is aimed at speeding a remission while avoiding metabolic complications (14). The initial treatment for severe insulin resistance has generally been to switch therapy to alternative insulin formulations. Before the advent of human insulin in clinical practice, such patients were successfully treated by switching to purified porcine insulin (20, 229), fish insulin (219, 230), and modified insulins, including sulfated insulin (218) and desalane-porcine insulin (231). The beneficial effect on immunological insulin resistance of sulfated insulin may have been mediated by CD8+ T cells (232).

In patients with type 2 diabetes, insulin cessation may be a successful treatment for immunological insulin resistance (221, 233). Switching insulin-resistant patients to human insulin (87) or insulin lispro (220, 224) or from U-100 to U-500 insulin has also been successful (234). When switching insulin formulations in patients with immune-mediated insulin resistance, substantially lower doses may be necessary to avoid hypoglycemia (217). In a case report, endogenous insulin stimulated by tolbutamide was found to be effective in a patient with type 2 diabetes who was felt to be unresponsive to exogenous insulin (235).

Glucocorticoids are a second-line therapy for patients with nonremitting immunological insulin resistance who do not respond adequately to switching insulin formulations (14, 15, 20, 236), or in whom ketoacidosis ensues (79). Prednisone at doses of 60 to 80 mg/d can result in lower insulin requirements in up to 75% of patients, often within a few days and even before declines in antibody levels (20). Responses in some cases can be dramatic, and severe hypoglycemia can ensue (14, 217, 236), leading some to recommend that glucocorticoid therapy be administered in a hospital or other monitored setting (217). Prednisone doses should be tapered when there is a clinical response. Often, 2 to 3 wk of glucocorticoid therapy is adequate to achieve a sustained response (20, 217). In rare cases, plasmapheresis (223, 237) or
cytotoxic immunosuppressive agents, such as 6-mercaptopurine (238), have been used for immune insulin resistance not related to an underlying paraproteinemia.

Although high levels of IAs have been demonstrated in patients with syndromes of severe insulin resistance, it is difficult to arrive at a causal relationship between the antibodies and the syndrome with available data. Insulin dose requirements are not predicted by insulin-binding capacity measurements, even in patients with severe insulin resistance (20, 218). Furthermore, there is substantial overlap in antibody levels between resistant and nonresistant patients (218). An approximate maximum antibody-binding threshold of 30,000 µU/ml, above which patients may be at risk for this syndrome, has been suggested; however, 15% of patients with the syndrome described in the literature have values below this range (217, 218).

If an antibody threshold exists, one still needs to postulate that there is an undiscovered attribute of the antibodies or related cofactors that determines why some patients develop insulin resistance, whereas other patients with comparable antibody levels do not. Subpopulations of antibodies identified by insulin affinity have been correlated with clinical resistance and response to therapy in case reports (223, 239, 240). Both high-affinity (221) and low-affinity (240) antibodies have been identified as correlating best with clinical findings.

IA levels clearly do not by themselves explain why some patients with this syndrome require 200 U/d and others require 2000 U/d (241). Furthermore, the mechanism by which IAs would cause insulin resistance is not clear. IAs could function as a circulating binding reservoir for insulin; however, in theory, once this reservoir is saturated, normal free insulin levels should be achievable with typical insulin doses (20, 217, 241, 242).

Cyclical expression of important idiotypic determinants—despite unchanged levels of total insulin binding—could explain clinical syndromes that occur and remit spontaneously. Although such cyclic idiotypic expression has been described in one report (243), it is not clear that this phenomenon involves the insulin-receptor-binding epitope, which would be necessary to explain the occurrence and remission of immunological insulin resistance. Rarely, insulin resistance is associated with antiinsulin receptor antibodies (244). Most patients with insulin resistance due to insulin receptor antibodies have evidence for systemic autoimmunity to noninsulin autoantigens (244), unlike the immune response to exogenous insulin in patients with diabetes (245).

Modern definitions of immunological insulin resistance require excluding the presence of insulin receptor antibodies (218).

c. Overall metabolic control and insulin dose requirements. Some pharmacodynamic studies that examined the relationship between IAs and postprandial glycemia have suggested that IAs can be associated with relative hyperglycemia after meals (49, 208, 210) (see Section V.B.1). A prospective study evaluating postprandial glucose tolerance during the development of IAs with inhaled insulin therapy showed no loss of postprandial glucose control (59).

Some investigators have reported IA levels correlated with higher average glucose in populations of patients (246–248), but most showed no correlation between IA and glucose control—usually measured as glycated hemoglobin (19, 58, 99, 114, 241, 249–265). Most studies published since 1980 have shown no evidence for a significant relationship between IA and average glycemia (58, 99, 114, 241, 250–256, 259–263, 265).

Researchers have postulated that IAs can be associated with mild degrees of insulin resistance, which would be detected clinically as mild to moderate increases in insulin dose requirements. Additionally, some studies have suggested that increasing insulin dose requirements correlate with IA levels (249, 264, 266–268); however, most studies have showed no relationship or correlations between IAs and decreasing dose requirements (58, 99, 114, 241, 247, 250, 252, 255, 259, 260, 262, 265, 266, 269–273). Evidence suggests that IA-positive patients who switch insulins to a less immunogenic preparation can experience reduced dose requirements in concert with declining IA levels (27, 264, 274, 275), or that switching insulin preparations can result in declining IA levels without decreased insulin dose requirements (19, 251, 263, 276–280).

3. IAs and hypoglycemia. Rare syndromes in which recurrent or prolonged hypoglycemia is the dominant feature have been attributed causally to IAs. Most frequently, this situation is encountered in insulin autoimmune syndrome (IAS, also called Hirata’s disease), in which nondiabetic patients with no history of insulin exposure spontaneously develop IAs and hypoglycemia. Evidence that IAs induced by exogenous insulin therapy can also cause hypoglycemia is limited to case reports. Although hypoglycemia is the most notable feature of these syndromes, some affected patients have also been reported to have clinical evidence of attenuated insulin action (including severe insulin resistance). It remains to be determined whether antibodies associated with pathological hypoglycemia can be distinguished in vitro from the far more commonly occurring IAs that are not linked to clinical hypoglycemia.

a. Hirata’s disease. IAS consists of high levels of IAs with or without concomitant Graves’ disease associated with fasting hypoglycemia in insulin-naive patients. The disease was first described in 1970 by Hirata et al. (281). This disorder is HLA-linked (282) and is the third most common cause of hypoglycemia in Japan, but has been sporadically reported outside Japan (283, 284).

In most patients, remission occurs within 6 months; however, life-threatening hypoglycemia may necessitate measures such as plasmapheresis (285, 286). Impairment in glucose tolerance is reported for some of these patients (283). Patients with IAS often have late postprandial hypoglycemia, possibly due to late release of endogenous insulin from the autoantibody pool (286). It is also possible that some instances of hypoglycemia associated with IAS can be attributed to the development of antidiotypic antibodies that have insulin agonistic properties. Among white patients in the United States, IAS manifests primarily as postprandial hypoglycemia; as in Japanese patients, the syndrome can be associated with polyclonal or monoclonal IgG insulin-binding antibodies (287).
b. Hypoglycemia and antibodies to exogenous insulin. Published case reports have attributed unusually prolonged episodes of hypoglycemia to high levels of antibodies to exogenous animal insulin (176, 178, 288, 289). The first case, reported in 1960 by Harwood (288), described a 44-yr-old woman with type 1 diabetes who for 9 yr experienced periods in which she would need to discontinue insulin therapy for up to 23 d because of prolonged hypoglycemia. She was found to have 106,000 μU/ml insulin-binding capacity with an unusually slow rate of dissociation of the antibody-antigen complex.

Seven case reports have been published of hypoglycemia occurring in patients with antibodies found in the setting of human insulin therapy (223, 239, 290–293). Interestingly, these cases were all reported from Japan, where IAS is thought to be more prevalent than in the rest of the world. These cases also occurred predominantly in patients over 70 yr of age, as is the case with IAS.

Hypoglycemia has also been reported in up to 54% of patients receiving a combined pancreas and kidney transplant (294). Tran et al. (295) compared patients who had repeated episodes of hypoglycemia or hypoglycemic symptoms after a pancreatic transplant matched with patients who did not have hypoglycemia after pancreatic transplant. They found a decrease in the ratio of fasting free insulin to total insulin in the patients who had a hyperglycemic response to a liquid meal challenge. This subgroup had a substantial increase in total, but not free, insulin concentrations. Although IAs were not directly measured, these data are consistent with the hypothesis that hypoglycemia was associated with high levels of circulating IAs (295).

Although anecdotal information from these case reports suggests that very high levels of IAs can be associated with unusual hypoglycemia syndromes, studies of large populations have failed to establish a relationship between IAs and hypoglycemia event rates (58, 59, 251, 259, 263, 265, 296, 297). In a contrasting study, Jeandidier et al. (294) tracked sections II.D, II.F.3, and II.F.4) (50). Lassmann-Vague et al. (99) found no correlations between IAs and hypoglycemic event rates experienced by 24 patients who had been treated for 2 yr with ip insulin therapy. IA levels also did not correlate with glycated hemoglobin, insulin requirements, or free insulin levels in these patients. In a contrasting study, Jeandidier et al. (49) tracked IA response in 62 patients with type 1 diabetes on ip pump therapy for 2 yr. Although an association between IA status and the number of low blood glucose values per month was reported, severe hypoglycemic event rates dropped dramatically in patients implanted with ip pumps regardless of IA responses.

The possibility that nocturnal hypoglycemia could be related to the antibody response—despite downward titrations of overnight basal insulin infusion—was first raised by Olsen et al. (50), who described four of 25 patients using IIPs with unexplained significant decreases in overnight basal insulin dose requirements. All four of these patients had peak IA levels above 200 μU/ml (range, 205 to 1021 μU/ml), although one patient remained symptomatic despite a decline in antibody level to 84 μU/ml while still receiving ip insulin therapy.

d. Clinical trials and limitations of literature. Although information from case reports suggests that high levels of IAs can be associated with prolonged periods of hypoglycemia, studies of large populations have failed to establish a relationship between IAs and hypoglycemia event rates (58, 59, 251, 259, 263, 265, 296, 297). In a pooled analysis of phase 2 and phase 3 trials involving more than 350 patients with type 1 diabetes and more than 400 patients with type 2 diabetes treated with inhaled insulin (Exubera), no relationships between hypoglycemic event rates and IA levels were observed (58).

Published case reports describe patients with unusual hypoglycemia syndromes who are found to have very high levels of IAs. Because of an inherent reporting bias, however, it is difficult to determine from the literature how many patients with high antibody levels do not have hypoglycemia. Likewise, it is difficult to know the frequency of similar syndromes in patients who have low IA levels.

Although both hypoglycemia and IAs are common in patients receiving insulin, a causal relationship between the two has been difficult to demonstrate. For the presence of antibodies to explain hypoglycemia in such patients, it must be presumed that antibodies first bind the insulin in circulation and then later dissociate from the insulin, allowing activation of cellular insulin receptors. Despite multiple attempts to characterize antibodies associated with hypoglycemia syndromes, it is not clear that consistent affinity/binding capacity profiles can distinguish antibodies in patients with and without hypoglycemia syndromes (49, 179).

However, recent studies have reported potential progress toward identifying in vitro characteristics of IAs associated with clinical hypoglycemia syndromes in small numbers of patients (177, 180, 222, 223, 298). One of those studies has suggested that insulin-receiving individuals with high antibody levels and recurrent hypoglycemia have a higher dissociation constant for insulin measured by surface plasmon resonance than monoclonal antibodies to human insulin (298). It remains to be seen whether these recently proposed assays correlate better with symptoms than with standard IA measurements.

It is also not clear what factors may precipitate the hypothesized unregulated dissociation of antibody-insulin complexes. Because some patients are reported to have nocturnal hypoglycemia, researchers often presume that binding equilibrium is shifted toward antibody-insulin dissociation when free insulin concentrations decline as time elapses overnight from evening insulin administration. However, were this the case, a correlation between IA and lower fasting glucose measured in the morning would be expected, and no such relationships have been demonstrated (58, 59, 251, 256).

Clinical trials comparing inhalation with sc delivery of insulin in patients with type 1 or type 2 diabetes have demonstrated reduced fasting plasma glucose levels by as much as 40 mg/dl associated with inhaled insulin, the mechanism for which is unknown (299). However, IAs were found not
to correlate with these fasting glucose levels (58) (Fig. 4, from Pfizer data on file). This is consistent with the observation that treatment group differences in fasting plasma glucose are fairly consistent in patients with type 1 or type 2 diabetes, whereas IA responses to inhaled insulin are significantly higher in patients with type 1 compared with patients with type 2 diabetes (58).

To be clinically significant, prolonged dissociation of IA complexes should result in a measurable prolongation of the duration of insulin action. This hypothesis was examined in a prospective 6-month pharmacodynamic study in which duration of insulin action was measured by 12-h euglycemic clamps (59). This study showed that in patients with IA responses to inhaled insulin (Exubera), duration of insulin action was unchanged, despite a rise in IA levels, and no differences were found between these patients and those treated with sc insulin. Furthermore, there were no correlations between high-affinity or low-affinity insulin binding on duration of insulin action, fasting glucose, or clinical hypoglycemic events (see Section V.B.1).

Clinical trial data suggest that hypoglycemia does not correlate with IAs in large populations of patients. Nevertheless, rare patients with high antibody levels may develop hypoglycemia syndromes owing in part to prolonged or unregulated appearance of free insulin in the circulation (178). Because the levels of IAs do not in themselves account for recurrent hypoglycemia, it seems likely that clinical hypoglycemia in this setting would have a multifactorial etiology, perhaps involving deficient glucose counterregulation coupled with a prolonged half-life of free insulin (213, 300).

C. Pregnancy

Maternofetal IgG transfer begins early in the second trimester, with most antibodies transferring to the fetus during the third trimester (301). Because organogenesis occurs during the first trimester, a lack of correlation between congenital malformations and maternal IAs is not surprising (302). Recently, it has been shown that transmission of maternal antibodies to exogenous insulin does not affect diabetes risk in offspring (303).

Pregnancy-related risks from IAs have not been clearly demonstrated. Some early papers reported associations between IAs and neonatal hypoglycemia (304–306). However, these papers did not adequately describe maternal glycemic control in the patients studied. Maternal glycemic control is important because it is known to influence many fetal and neonatal risks, including neonatal hypoglycemia and macroamnios (307). Most notably, despite the strong association between neonatal hypoglycemia and increased birth weight in the infant of a diabetic mother (307), no consistent linkage was found between IAs and birth weight, even in the early studies that linked IAs to neonatal hypoglycemia. Furthermore, hypoglycemia remains a common complication in neonates born to mothers with diabetes, despite the fact that insulin preparations of low immunogenicity are now in routine use (22, 308).

The potential for increased incidences of neonatal hypoglycemia, respiratory distress syndrome, and hypocalcemia has also been suggested in small studies published from 1980 to 1990 (304, 305, 309, 310); however, these reports also lacked adequate documentation of maternal glycemic control. Subsequent studies, performed in the modern era of prenatal care for women with diabetes, argue against a connection between IAs and fetal morbidities (254, 311). Additionally, multiple studies have failed to show a relationship between maternal IAs and birth weight. A randomized trial comparing human insulin with animal insulin during pregnancy found that improved glycemic control, not IAs, influenced infant birth weight (312). Wellik et al. (313) showed no correlation between IAs and neonatal glucose level or birth weight. Three recent studies with substantially larger sample sizes reported no relationships between IAs and birth weight (314–316). The most recent report found similar birth weights in offspring of 138 mothers with type 1 diabetes across a range of maternal IA levels and cord blood insulin levels (314). Islet autoantibody concentrations also were found to have no influence on birth weight.

Two hypotheses have been put forward to explain a possible relationship between maternal IAs and neonatal hypoglycemia (79), both of which would predict clear correlations between IA levels, birth weight, and neonatal hypoglycemia risk. In the first, maternally derived antibody interference with insulin action in the fetal circulation could result in compensatory fetal hyperinsulinemia. In this scenario, fetal hyperinsulinemia results in increased birth weight and neonatal hypoglycemia. Although neonatal cord blood C-peptide levels (reflecting endogenous insulin secretion) were found to correlate with IAs in one study (305), the finding was not reproducible (302, 315). Recently, no relationship

![Fig. 4](https://academic.oup.com/edrv/article-abstract/28/6/625/2355076/26)
was found between cord blood insulin levels and birth weight (314). Furthermore, multiple studies, including the only randomized prospective study, have found no relationship between IAs and fetal birth weight (312).

A second hypothesis suggests that insulin is transferred to the fetus via IA complexes. These complexes could then dissociate in the fetal circulation, releasing bioactive insulin. Were this to happen during fetal life, increased birth weight would be expected. As discussed, multiple studies have failed to show relationships between maternal IAs and macrosomia. If IA complex dissociation occurred after birth, unusually prolonged neonatal hypoglycemia syndromes might be expected, given that the biological half-life of circulating IgG is approximately 23 d (317). No distinguishing clinical characteristics of neonatal hypoglycemia associated with IAs, such as unusual prolongation, have been described in babies born to mothers with IAs.

D. Autoimmune diseases

Antibody responses to exogenous insulin have not been shown to cause generalized immune activation resulting in autoimmune disease states. Lassmann-Vague et al. (245) measured a panel of autoantibodies before implantation with pumps for ip insulin delivery and then subsequently every year in 28 patients with type 1 diabetes. At baseline, 19 of 28 patients were negative for all tested autoantibodies (antithyroglobulin, antithyroidperoxidase, gastric parietal cell, smooth muscle, mitochondrial, liver-kidney microsome, antinuclear, antiendomysium, and antigliadin antibodies). During 2 yr of ip insulin treatment, the sera of the patients negative at baseline remained negative throughout the study, despite the expected IA response (see Sections II.D and V.B.2.c). Nine patients with preexisting autoantibodies had no change in most autoantibody titers. Two of these patients had increases in antithyroidperoxidase titers, whereas three patients had decreases in these titers. No difference was seen in IA responses to ip insulin delivery in the nine patients with preexisting autoantibodies relative to those who did not have preexisting antibodies.

E. Inhaled insulin antibodies and pulmonary function

Inhaled human insulin is associated with higher levels of IAs compared with sc human insulin (see Section II.E). Inhaled insulin (Exubera) is also associated with small treatment group differences in pulmonary function tests, which develop early after treatment initiation, are not associated with clinical sequelae, are nonprogressive with up to 2 yr of therapy, and are reversible with treatment discontinuation (63, 318). This pulmonary function test change does not, however, appear to be mediated by the immunological response to inhaled insulin. Teeter and Riese (63) showed that the treatment group difference in forced expiratory volume in 1 sec (FEV₁) is manifest as early as 2 wk after inhaled insulin initiation, at which time the mean IA response has not yet evolved. Furthermore, Fineberg et al. (58) showed no correlation between IA levels and FEV₁ in pooled analyses of inhaled insulin (Exubera) clinical trials.

F. Immune complexes

A series of reports published from the 1960s through the 1980s yielded conflicting results regarding insulin-antinsulin immune complexes in the development of long-term diabetic complications in animal models (319). Studies in humans have not shown consistent links between IAs and diabetic complications. Although some studies have shown increased levels of immune complex formation in patients with diabetic microangiopathic complications compared with patients without complications (320–322), these immune complexes often do not contain insulin or IA (321–324). Furthermore, insulin administration does not contribute to this immune complex formation (325). The nonspecific immune complexes observed in patients with diabetes may reflect general inflammatory reactions associated with angiopathies (321).

Overall, no direct evidence has shown that immune complexes, insulin immune complexes, or IAs are capable of mediating vascular or glomerular damage. Associations have been reported, but they have been largely based on the prevalence of immune complex detectability rather than on quantitative measurements of the amount of immune complex present (326). Andersen (327) showed that high porcine insulin-binding levels were more frequent in patients with complications from diabetes than in patients without complications but found no significant differences in mean antibody levels between groups. Virella et al. (328) found correlations between the presence or absence of detectable insulin-antiinsulin immune complexes and the presence or absence of some diabetic complications.

Although immune complexes in insulin-treated patients have been associated with procoagulant markers in some studies (323, 329, 330), the majority of studies have shown no relationship between IA and diabetic angiopathic complications. Specifically, no relation has been found between IAs and histological findings of nephropathy (331), muscle basement membrane thickness (332), clinical nephropathy (114, 333, 334), clinical retinopathy (114, 273, 321–323, 332–336), clinical autonomic neuropathy (337), clinical peripheral neuropathy (338), or complement activation in patients with diabetes (339).

The absence of a pathogenic effect may be related to the properties of the immune complex. It has been observed that insulin and IA immune complexes do not precipitate (at antigen or antibody excess) and cannot be detected by standard immunodiffusion methods (340). In addition, insulin–antibody immune complexes are monomers or dimers, and large immune complexes are not formed. Small immune complexes do not readily bind to Clq complement and are, therefore, not readily cleared by the reticuloendothelial system of the liver or spleen (190). Small immune complexes have a similar half-life to circulating Ig molecules, which explains the high levels of circulating total insulin (antibody bound) in patients with high levels of IAs (see Section IV.E).

G. Buffering effect of insulin antibodies

Both glycemic stability and instability have been attributed to the presence of IAs. Instability has been described
only in case reports and is characterized either by both hyperglycemia and hypoglycemia occurring within a single 24-h interval or by periods lasting days to weeks of hyperglycemia alternating with periods of hypoglycemia of similar duration (49, 176, 178, 223, 229, 239, 290, 291, 341, 342). Some of these case reports describe patients with relative overnight hypoglycemia and daytime hyperglycemia, although there is no clear mechanistic explanation for such diurnal patterns (see Section V.B.3.c). Because these glycemic instability syndromes are rare and variable in nature and because in vitro parameters are not predictive of clinical findings (see Section V.B), it is difficult to establish cause and effect relationships between the antibodies and such glucose variability syndromes.

Conversely, because studies in patients being treated with insulin—as well as those in individuals with insulin autoimmune-hypoglycemia syndrome—have suggested that the presence of high levels of IAAs is associated with a retarded disappearance rate of insulin, some authors have suggested that antibodies may serve as a “buffer” to glucose variability (see Section V.B.1) (6, 178, 223, 286, 343, 344). Limited studies demonstrated that this slowed rate of insulin disappearance appeared to decrease the likelihood of diabetic ketoacidosis, contributing to stability of glycemic control (269, 345–347). However, for the majority of patients, the levels of antibodies seen with insulin therapy are unlikely to result in significant effects on glycemic variability (348).

H. Insulin antibodies and risk for diabetes

1. IAs and β-cell loss. Although the spontaneous appearance of IAA in nondiabetic patients is known to be predictive of type 1 diabetes development (349, 350), there is no evidence that IAAs or IAs themselves causally mediate β-cell destruction. Support for this comes from studies in which nondiabetic patients with circulating antibodies to insulin were followed for the onset of diabetes (351–354). For example, Bock et al. (351) investigated whether insulin treatment of patients without diabetes who were undergoing insulin shock therapy for psychiatric disorders would be at increased risk for the development of diabetes. In their retrospective analysis of 481 patients observed for an average of 22 yr, one patient developed type 1 diabetes, and 12 developed type 2 diabetes. These instances did not differ from the background population. Only two of 27 patient samples examined were positive for IAs, and none was positive for islet cell antibodies. The researchers concluded that exogenous insulin used in diabetes prevention trials was safe and would not increase the risk for diabetes.

2. Cellular immune response to exogenous insulin. Most of the reports on insulin-specific T cell responses have focused on the autoreactive T cells involved in the pathogenesis of type 1 diabetes. Studies using NOD mice have demonstrated the presence of CD4+ and CD8+ cells that recognize insulin and lead to the destruction of β-cells in this model of spontaneous autoimmune diabetes (355). However, multiple interventions, including parenteral, oral, or aerosolized insulin treatment (see Sections III.A and III.B) have been shown to delay the onset of diabetes in these mice.

T cell clones have been generated from the draining pancreatic lymph nodes of patients with type 1 diabetes that were found to be responsive to insulin (356). These data indicate that insulin may be one of the initial antigens recognized by autoreactive T cells before the onset of clinical type 1 diabetes and exogenous insulin treatment.

A few studies have evaluated insulin-specific T cell responses in patients with diabetes being treated with insulin. The most commonly used assay to measure insulin-responsive T cells involves coinoculating peripheral blood mononuclear cells with or without insulin for 6 to 10 d. The proliferative response is measured by the incorporation of radio-labeled nucleotide (357). T cell responses in patients with type 1 diabetes treated with porcine and bovine insulin or porcine-bovine mixtures were first reported in 1975 (358, 359). Patients with recent-onset diabetes (32%) and those with long-standing illness (47%) were found to have positive T cell responses to human insulin (360). Since T cell help is required for B cell development and antibody production, IA levels might be expected to correlate with T cell responsiveness. However, poor correlations between IA and/or IAA levels and T cell responsiveness have been observed (360–362). Patients with high IAs or IAAs had very low T cell responses, and patients with low IA or IAA levels had high T cell responses. The relatively infrequent finding of a strong cellular immune response to insulin in patients with type 1 diabetes treated with exogenous insulin may be partly explained by the activation of regulatory T cells (75).

Additional investigations suggest that T cells respond to different regions (epitopes) of the insulin molecule. T cell responses are greater with the B chain than the A chain of insulin (363, 364). Theoretically, insulin-specific T cells measured in these studies may be autoreactive T cells generated by endogenous insulin, autoreactive T cells expanded by exogenous insulin, and/or T cells initially generated by exogenous insulin; however, based on the assays used, it is not possible to differentiate among these possibilities. T cell responses with exogenous insulin treatment likely will be further investigated if insulin treatment is found to prevent the onset of type 1 diabetes in humans, as observed in NOD mice.

3. Exogenous insulin and prevention of type 1 diabetes in clinical studies. Results from the NOD mouse studies led to the Diabetes Prevention Trial Type 1, which was designed to test the ability of sc and orally administered insulin to prevent type 1 diabetes in subjects known to be at risk based on the presence of autoimmune markers (e.g., IAAs and islet cell antibodies) (365). The sc insulin administration arm of the trial showed no acceleration or delay of type 1 diabetes onset. The same was true for the oral insulin treatment arm when all patients were included (366). A subset analysis of the oral insulin treatment group, however, demonstrated a significantly lower annualized rate of diabetes onset in patients with baseline IAA levels of at least 80 nU/ml treated with oral insulin compared with patients treated with placebo.

Additional studies showed no effect of oral insulin on residual β-cell function in patients with new onset type 1 diabetes (103, 104). More recently, 38 children at risk for type 1 diabetes were treated with intranasal insulin and showed no evidence for accelerated loss of β-cell function (367). Fur-
thermore, IA responses to intranasal insulin were demonstrated, as were immune changes consistent with mucosal tolerance to insulin. A small trial suggested that low-dose sc insulin may have favorable immunomodulatory effects in adult patients with latent autoimmune diabetes (368).

VI. Conclusion

Regardless of purity and origin, therapeutic insulins continue to be immunogenic in humans; however, severe immunological complications occur rarely, and less severe events affect just a small minority of patients. Today’s human insulins are free of noninsulin peptides and variations in insulin structure that contributed to the antigenicity seen with pancreatic insulins in the past; however, insulins must be manufactured, stored, and delivered by nonphysiological means, possibly contributing to the humoral and cellular immune responses seen in patients treated with insulin.

IAs may be detectable in insulin-naive individuals who have a high likelihood of developing type 1 diabetes, have had viral disorders, have been treated with various drugs, or have autoimmune disorders or paraneoplastic syndromes. This suggests that under certain circumstances immune tolerance to insulin can be overcome. Factors that can lead to more or less susceptibility to humoral responses to exogenous insulin include the recipient’s immune response genes, age, the presence of sufficient circulating autologous insulin, and the site of insulin delivery. Down-regulation of immune responses to self- and nonself-antigens involves a subset of regulatory T lymphocytes that may be critical in warding off or terminating autoimmunity (67–69). These regulatory cells may also play a role in the development and levels of immune responses to exogenous insulin.

Researchers have postulated that IAs can be associated with insulin resistance, which would be detected clinically as increases in insulin dose requirement. However, most studies have shown no relationship between dose and IAs (58, 99, 114, 241, 247, 250, 252, 255, 259, 260, 262, 265, 266, 269–273). In long-term, follow-up studies of children with type 1 diabetes, neither the presence of IAs nor the development of IAs affected insulin dose requirements (114).

Case reports indicate that high levels of IAs can be associated with prolonged or recurrent hypoglycemia, but studies of large populations have failed to establish a relationship between IAs and hypoglycemia event rates. Reports describe patients with hypoglycemia syndromes who are found to have markedly high levels of IAs, but it is difficult to determine from the literature how many patients with high antibody levels do not have hypoglycemia.

It is not clear what factors may precipitate the hypothesized unregulated dissociation of antibody-insulin complexes. Because some patients are reported to have nocturnal hypoglycemia, researchers often presume that binding equilibrium is shifted toward antibody-insulin dissociation when free insulin concentrations decline. Clinical trial data suggest that hypoglycemia does not correlate with IAs in large populations of patients; however, it cannot be excluded that a small number of patients with high antibody levels develop hypoglycemia syndromes owing in part to prolonged or unregulated appearance of free insulin in the circulation (178). Clinical hypoglycemia in this setting may be multifactorial in etiology, involving deficient glucose counterregulation coupled with a prolonged half-life of free insulin (213, 300). Most studies published since 1980 have shown no evidence for a significant relationship between IA and average glycemia.

Some early studies suggested that IAs were associated with increases in neonatal morbidity, including hypoglycemia, respiratory distress syndrome, and hypocalcemia (304, 310). However, studies in which pregnant women with diabetes were monitored for glycemic control argue against a connection between IAs and fetal risk. No distinguishing clinical characteristics of neonatal hypoglycemia associated with IAs, such as unusual prolongation, have been described in babies born to mothers with IAs.

Human studies have not shown consistent links between IAs and diabetic complications. Although studies have shown increased levels of immune complexes in patients with diabetic microangiopathic complications (320–322), these immune complexes often do not contain insulin or IAs (321–324). Neither does insulin administration contribute to immune complex formation (325). The majority of studies have shown no relationship between IAs and diabetic angiopathic complications, including nephropathy, retinopathy, and neuropathy.

The absence of a pathogenic effect may be related to the properties of the immune complex. Researchers have observed that insulin and IA-immune complexes do not precipitate (at antigen or antibody excess) and cannot be detected by standard immunodiffusion methods (340). In addition, binding data suggested that insulin binding is unique, which limits the formation of large immune complexes. Small immune complexes do not readily bind to C1q complement and are not, therefore, readily cleared by the reticuloendothelial system of the liver or spleen (190). Small immune complexes have a similar half-life to circulating Ig molecules. This explains the high levels of circulating total insulin (antibody bound) in patients with high levels of IAs.

In conclusion, humoral antibody responses to exogenous insulin continue to be largely unavoidable. Little proof exists that the development of antibodies to exogenous insulin therapy affects glycemic control, insulin dose requirements, and hypoglycemia, or contributes to β-cell failure or to the long-term complications of diabetes. Current human insulin and insulin analog therapies have resulted in decreased IA levels when contrasted with animal insulins. Local reactions to the most recent formulations of insulin continue to be observed but are infrequent, and systemic reactions are rare. Until therapeutic insulin can be delivered in a physiological manner, we are likely to continue to observe infrequent immunological sequelae. The development of IAs to exogenous insulin reflects the exquisite sensitivity of the immune system to even minor perturbations.

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