Development of a Transgenic Mouse Model to Study the Immunogenicity of Recombinant Human Insulin

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ABSTRACT: Mouse models are commonly used to assess the immunogenicity of therapeutic proteins and to investigate the immunological processes leading to antidrug antibodies. The aim of this work was to develop a transgenic (TG) Balb/c mouse model for evaluating the immunogenicity of recombinant human insulin (insulin) formulations. Validation of the model was performed by measuring the antibody response against plain and particulate insulin in TG and nontransgenic (NTG) mice. Intraperitoneal administration of insulin (20 μg/dose, 12 doses over a period of 4 weeks) did not break the immune tolerance of the TG mice, whereas it did elicit antibodies in NTG mice. The immune tolerance of TG mice could be circumvented, albeit at low titers, by administering insulin covalently bound to 50-nm polystyrene nanoparticles. The TG mouse model was employed to compare the immunogenicity of oxidized aggregated insulin, oxidized nonaggregated insulin, and three commercially available formulations of insulin variants (i.e., Levemir®, Insulatard®, and Actrapid®). Oxidized insulin, aggregated or nonaggregated, was moderately immunogenic in TG mice (50% and 33% responders, respectively), whereas the immunogenicity of the commercial formulations was low. This model can be used to compare the immunogenicity of insulin formulations and to study immune mechanisms of antibody formation against insulin. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:1367–1374, 2014

Keywords: immune response; protein aggregation; protein formulation; oxidation; nanoparticles; physicochemical properties

INTRODUCTION

With the development of recombinant DNA technology, it has become possible to produce well-defined recombinant human (rh) therapeutic proteins.[1,2] Despite the fact that these proteins are structurally very similar to their endogenous counterparts, almost all rh proteins for therapeutic use are immunogenic.[3,4] Many factors influence the immunogenicity of a protein drug, which can be categorized into patient-dependent (e.g., type of disease, genetic background), treatment-dependent (e.g., dose, dosing schedule, route of administration, comedication), and product-dependent factors.[5,6] An increasing number of publications support aggregation of therapeutic proteins, which can be induced upon protein oxidation[7] and can involve covalent cross-links,[8] as one of the major product-related factors influencing immunogenicity.[9,10] For rh interferon-alpha (IFNα) and rh interferon-beta (IFNβ), transgenic (TG) immune tolerant mouse models have been shown to be a valuable tool to study product-related factors contributing to the immunogenicity and the underlying mechanisms.[11–13] For human insulin, the first TG mouse model was described by Ottesen et al.[14] to study the impact of the modifications of the insulin sequence on the formation of new epitopes. However, whereas they used complete Freund's adjuvant (CFA) to trigger the immune system, for studying formulation-related factors an adjuvant-free animal model is preferred, because the addition of CFA alters the formulation, may affect protein's structure (unpublished observation from our laboratory), and likely affects the biodistribution of the protein. The primary aim of this work was to create a CFA-free TG mouse model to study the immunogenicity of rh insulin (insulin) formulations, based on the TG mouse strain previously used by Ottesen et al.[14] To compensate for loss of sensitivity in the absence of adjuvant, the administration scheme and the antibody assay were optimized. After evaluation of the model by administration of untreated insulin and insulin covalently bound to nanoparticles (NP), which were used as negative and positive control, respectively, we studied the applicability of the mouse model by comparing the immunogenicity of oxidized and aggregated insulin, oxidized nonaggregated insulin, and three different commercially available formulations of insulin variants (i.e., Levemir®, Insulatard®, and Actrapid®).

MATERIALS AND METHODS

Materials

Insulin containing 0.4% (w/w) zinc ions was provided by Merck (Oss, The Netherlands). Three commercially available insulin formulations, that is, Insulatard® (long-acting insulin suspension obtained with zinc and protamine), Levemir® (long-acting insulin modified on Lys B 29 with a fatty acid), and Actrapid® (neutral unmodified insulin solution) were a gift from the Leiden University Medical Center. Copper(II) chloride, ascorbic acid, ethylenediaminetetraacetic acid (EDTA), ammonium bicarbonate (ABI), sodium citrate, citric acid, arginine, disodium hydrogen phosphate,

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n-hydroxysulfosuccinimide sodium salt (NHS-sulfo), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide HCl (EDAC), and 2-*N*-morpholino-ethanesulfonic acid (MES) were bought from Sigma–Aldrich (Schnelldorf, Germany). Glacial acetic acid and acetonitrile were purchased from Boom (Meppel, The Netherlands). Slide-A-Lyzer dialysis cassettes (3.5 and 10 kDa) were purchased from Thermo Fisher Scientific (Etten-Leur, The Netherlands). Polystyrene NP (diameter 50 nm) that contain surface carboxyl groups were purchased from Polysciences GmbH (Eppelheim, Germany). All chemicals were of analytical grade and used without further purification. Deionized water was purified through a Purelab Ultra System (ELGA LabWater Global Operations, Marlow, UK) prior to use.

Preparation of Unmodified Insulin Solutions

Insulin solutions were prepared by dissolving 1.1 mg insulin in 100 μL 0.1 M HCl. Subsequently, 50 mM sodium phosphate buffer (PB), pH 7.4, was added up to 1 mL and the pH adjusted to 7.4. The concentration was calculated by UV spectroscopy, using a molecular weight of 5.8 kDa and an extinction coefficient of 6200 M^{-1}/cm at 276 nm,[15] and eventually adjusted to 1.0 mg/mL with PB. Insulatard®, Levemir®, and Actrapid® were diluted to 0.2 mg/mL with PB, prior to injection.

Preparation of Insulin Covalently Bound to 50-nm Polystyrene NP

Insulin was conjugated with 50-nm polystyrene NP following the procedure described by Kalkanidis et al.[16] Briefly, to prepare 9.00 mL of 1 mg/mL covalently bound insulin, 3.6 mL of NP [2.5% (w/v), aqueous suspension] were suspended in 2.25 mL of 0.2 M MES. Then, 1.125 mL of 28.80 mg/mL EDAC in water were added dropwise over a period of 10 min. After that, 1.125 mL of 360 mM NHS-sulfo in water were added, and the reaction mixture was left to equilibrate for 2 h at room temperature on a rotating plate (final pH was 6.0). After 2 h, the pH was brought to 6.9–7.0 with 1 M NaOH, and 900 μL of 10 mg/mL insulin in PB pH 7.0 were dissolved in the mixture (final insulin concentration was 1 mg/mL, pH 7.0). Conjugation was carried out overnight at room temperature before 24 h dialysis against PB, pH 7.4, using a 10-kDa dialysis cassette. (Further we will refer to this suspension as NP-ins). In order to verify whether insulin was covalently bound to the NP, a mixture of insulin and NP [hereafter called insulin adsorbed to NP (ADS-ins)] was prepared following a similar procedure but without NHS-sulfo and without EDAC.

Preparation of Oxidized Aggregated and Oxidized Nonaggregated Insulin

Oxidized and aggregated insulin (OA-ins) was prepared as previously reported.[17] Briefly, the hydroxyl radicals generated through the Cu^{2+}/ascorbate oxidative system yield tyrosine oxidation products, including 2-amino-3-(3,4-dioxocyclohexa-1,5-dien-1-yl) propanoic acid, which is a Michael acceptor for nucleophilic addition with insulin's amino groups, resulting in covalent insulin aggregates. To obtain oxidized nonaggregated insulin (O-ins), metal-catalyzed oxidation (MCO; via the oxidative system Cu^{2+}/ascorbate) was performed in 50 mM sodium citrate buffer (CB), pH 3.0 for 3 h at room temperature. To this end, insulin solution of 1 mg/mL was prepared by dissolving insulin directly in CB. MCO was performed by adding to 1 mL of 1 mg/mL insulin, 100 μL of 0.4 mM $CuCl_2$ in CB to a final concentration of 40 μM. The reaction was performed in 2-mL Eppendorf tubes covered with aluminum foil to protect the reaction mixture from light. After 10 min of incubation of insulin with Cu^{2+}, to allow copper to bind to insulin, the oxidation reaction was started by the addition of 11 μL of 400 mM ascorbic acid in CB to a final concentration of 4 mM. The reaction was quenched after 3 h of incubation at room temperature by adding 11.2 μL of a 100-mM EDTA in CB, pH 3 to a final concentration of 1 mM. The oxidized sample was extensively dialyzed at +4°C against CB. Next, a second dialysis against 250 mM ABI buffer, pH 8.0, for 24 h, was performed. These procedures, as previously reported,[17] prevented insulin aggregation, because of protonation of free amino groups in the insulin molecule under acidic conditions.

SDS-PAGE

Acrylamide gradient gels (10%–20% tris-tricine; Biorad, Veenendaal, The Netherlands) were run as described before.[18] The cathode electrophoresis buffer was 0.1 M tris(hydroxymethyl) aminomethane, 0.1 M tricine, and 3 mM SDS, pH 8.3. The anode electrophoresis buffer was 0.1 M Tris pH 8.9. Gel electrophoresis was performed with a Biorad Protean III system (Biorad). Samples were boiled for 2 min before application to the gel. A polypeptide marker solution (Biorad) was included for determination of the molecular weight.

Centrifugation

Centrifugation in the presence of SDS was performed prior to size-exclusion chromatography (SEC) analysis for NP-ins and ADS-ins, to investigate the amount of insulin adsorbed and covalently bound, calculated by comparison with native insulin. Two milliliter of NP-ins and ADS-ins (final insulin concentration 1 mg/mL) were loaded into Ultra–Clear centrifuge tubes (1/2 × 2 in., 13 × 51 mm^2; Beckman Coulter, Inc, Brea, California), in the presence of 20 mg SDS [i.e., 1% (w/v) SDS]. Native insulin, 2 mL of 1 mg/mL, was loaded in another tube without SDS. Next, the tubes were centrifuged with a Beckman Titanium Centrifuge Type 70 Ti (Ultra Fixed Angle Rotor) (Beckman Coulter) for 30 min at 250,000*g*. Supernatant was diluted twofold in PB, pH 7.4, prior to SEC analysis.

Size-Exclusion Chromatography

Size-exclusion chromatography experiments were performed using an insulin HMWP column, 7.8 × 300 mm^2 (Waters, Milford, Massachusetts), an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, California), consisting of a 1200 series HPLC pump, degasser, autosampler G1329A, and a variable wavelength detector G1316A. The SEC eluent (0.5 mL/min) was composed of a mixture of 1 g/L arginine in water–acetonitrile–glacial acetic acid 65:20:15 (v/v/v), as described in the European and United States Pharmacopoeias,[19,20] and chromatograms were acquired using UV absorption at 276 nm.

Dynamic Light Scattering

Dynamic light scattering (DLS) measurements were performed with a Malvern Zetasizer Nano ZS (Malvern, Herrenberg, Germany) equipped with a 633-nm He–Ne laser and operating at an angle of 173°. The software used to collect and analyze the data was the Dispersion Technology Software version 6.01 from Malvern. Each sample, containing 5 μL of NP, NP-ins, or ADS-ins, diluted with 495 μL water, was measured in single-use polystyrene half-micro cuvettes (Fisher Emergo, Landsmeer,