Oxidation, aggregation and immunogenicity of therapeutic proteins

Torosantucci, R.

Chapter 8

Development of a transgenic mouse model to study the immunogenicity of recombinant human insulin

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Abstract

Mouse models are increasingly used to assess the immunogenicity of recombinant human (rh) therapeutic proteins and to investigate the immunological processes leading to anti-drug antibodies (ADAs). In 1994 a transgenic (TG) mouse model for studying the immunogenicity of insulin variants was described (Ottesen et al., Diabetologia 37:1178-1185). However, this model requires the use of complete Freund's adjuvant (CFA), which changes the formulation and thus limits its applicability for studying the effect of formulation-related factors on immunogenicity. The aim of this work was to develop an adjuvant-free TG mouse model for evaluating the immunogenicity of rh insulin (insulin) formulations. 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 non transgenic (NTG) mice. This tolerance in TG mice could be circumvented by insulin covalently bound to 50 nm polystyrene nanoparticles as well as by oxidized and aggregated insulin and to a lesser extent by commercially available insulin products.

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, co-medication) and product-dependent factors [5-6]. An increasing number of publications support aggregation of therapeutic proteins as one of the major product-related factors influencing immunogenicity [7-8].

For rh interferon alpha (IFNα) and rh interferon beta (IFNβ), transgenic immune tolerant mouse models have been shown to be a valuable tool to study the product-related factors contributing to the immunogenicity and the underlying mechanisms [9-11]. For human insulin, the first transgenic (TG) mouse model was described in 1994 by Ottensen et al. [12] to study the impact of the modifications of the insulin sequence on the formation of new epitopes.

However, while they used complete Freund's adjuvant (CFA) to trigger the immune system, we prefer CFA free models to study formulation-related factors because the addition of CFA alters the formulation, CFA may affect protein's structure (unpublished observation from our lab) 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. Next, to study the applicability of the mouse model, we compared the immunogenicity of oxidized and aggregated insulin, oxidized non-aggregated insulin, and three different commercially available formulations of insulin variants (i.e. Levemir®, Insulatard®, 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, i.e.

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, ethylenediamine tetraacetic acid (EDTA), ammonium bicarbonate (ABI), sodium citrate, citric acid, arginine, disodium hydrogen phosphate, n-hydroxysulfosuccinimide sodium salt (NHS-sulfo), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide HCl (EDAC), 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). 3.5 kDa Slide-A-Lyzer dialysis cassettes were purchased from Thermo Fisher Scientific (Etten-Leur, the Netherlands). Polystyrene nanoparticles (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 of 1 mg/mL were prepared by dissolving insulin in 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^{-1} at 276 nm [13]. Insulatard®, Levemir® and Actrapid® were diluted to 0.2 mg/mL with PB, prior to injection.

Preparation of oxidized aggregated and oxidized non-aggregated insulin

Oxidized and aggregated insulin (PB-MCO) was prepared as previously reported [14]. To obtain oxidized non-aggregated insulin, metal catalyzed oxidation (MCO, via the oxidative system Cu^{2+}/ascorbate) was performed in 50 mM sodium citrate buffer, pH 3.0 (CB) for 3 hours at room temperature. To this end, insulin solution of 1 mg/mL was prepared by dissolving insulin directly in CB. MCO was performed by addition 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 1mM. The oxidized sample was extensively dialyzed at 4 °C against CB. Next, a second dialysis against 250 mM ammonium bicarbonate (ABI) buffer, pH 8.0, for 24 h, was performed. These procedures, as previously reported [14], prevented insulin aggregation. Further we will refer to this oxidized non-aggregated insulin as ABI-MCO.

Preparation of insulin covalently bound to 50 nm polystyrene nanoparticles

Insulin was covalently bound onto 50-nm polystyrene nanoparticles (NP) following the procedure described by Kalkanidis et al [15]. 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 minutes. After that, 1.125 mL of 360 mM NHS-sulfo in water, were added and the reaction mixture was left to equilibrate for 2 hours at room temperature on a rotating plate (final pH was 6.0). After 2 hours, 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). Coupling was carried out overnight at room temperature before 24 hours dialysis against 50 mM PB, pH 7.4 (further we will refer to this suspension as NP-ins). In order to verify whether insulin was covalently bound to the NP or only adsorbed, an insulin control (hereafter called insulin adsorbed on NP (ADS-ins)) was prepared following a similar procedure but without NHS-sulfo and without EDAC. Because of the nature of the reagents (NHS-sulfo and EDAC), carboxylic groups in insulin can be potentially activated during coupling with NP. Hence, a second control (hereafter called cross-linked insulin (CR-ins)) was prepared in the same way as described above for the preparation of NP-ins, but without the addition of NP.

SDS-PAGE

Acrylamide gradient gels (10-20% tris-tricine, Bio-Rad, Veenendaal, the Netherlands) were run under reducing and non-reducing condition as described before [16]. 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 presence of SDS was performed prior to size exclusion chromatography (SEC) analysis for NP-ins and ADS-ins (not CR-ins, which was centrifuged as described below), to investigate the amount of insulin adsorbed and covalently bound, calculated by comparison with native insulin. 2 mL of NP-ins and ADS-ins (final insulin concentration 1 mg/mL), were loaded into Ultra–Clear centrifuge tubes (1/2 x 2 in., 13 x 51 mm, Beckman Coulter, Inc, Brea, CA), in 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) for 30 minutes at 50000 rpm, (250000 g). **(**Beckman Coulter, Fullerton, CA). Supernatant was diluted two-fold in 50 mM PB, pH 7.4, prior to SEC analysis. CR-ins was centrifuged for 10 minutes, 4 °C at 16162 g with a bench centrifuge (Sigma 1-15 bench centrifuge, Shropshire, UK), without SDS, to remove insoluble aggregates that might block the SEC column.

Size exclusion chromatography (SEC)

SEC experiments were performed using an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA), consisting of a 1200 series HPLC pump, degasser, autosampler G1329A, and a variable wavelength detector G1316A. The SEC eluent 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 United States and European Pharmacopoeias [17-18], and chromatograms were acquired using UV absorption at 276 nm.

Dynamic light scattering (DLS)

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, The Netherlands). Water as a dispersant (viscosity 0.8872 cP and RI 1.330) was used to measure different formulations of nanoparticles, mentioned above. To measure plain insulin, 500 µl of 10 mg/mL human insulin in 50 mM PB, pH 7.4, and PB as a solvent (viscosity 1.0200 cP and RI 1.335) was used. The measurements were made at a position of 4.65 mm from the cuvette wall with an automatic attenuator and at a controlled temperature of 25°C. For each sample, 15 runs of 10 s were performed, with three repetitions. The intensity size distribution, the Z-average diameter (Z-ave) and the polydispersity index (PdI) were obtained from the autocorrelation function using the "general purpose mode". The default filter factor of 50% and the default lower threshold of 0.05 and upper threshold of 0.01 were used.

Circular dichroism spectroscopy (CD)

Far-UV CD measurements were performed as previously described [16].

Mouse studies

Breeding

Balb/c mice, transgenic for the human insulin gene, were obtained from Dr. J. Kapp. TG animals were bred with non-transgenic (NTG) Balb/c mice to obtain heterozygous TG offspring and NTG littermates, used in the *in vivo* studies presented here. Animals were bred at the Central Laboratory Animal Institute (Utrecht University, the Netherlands). NTG Balb/c mice were purchased (since breeding did not result in sufficient NTG littermates) at the Centre d'Elevage Janvier (Le Genest-Saint-Isle, France). Food (Hope Farms, Woerden, the Netherlands) and water (acidified) were available *ad libitum*. The presence or absence of the human insulin gene was determined by real

time PCR (rtPCR) in chromosomal DNA isolated from ear tissue. All animal experiments described were approved by the Animal Ethical Committee of the Utrecht University.

Functional immune system: response to foreign protein

To test whether the transgene interfered with the general capacity to produce antibodies we immunized both TG (n=4) and NTG (n=4) animals with 5 µg of human serum albumin (HSA, i.p.) on five consecutive days for three weeks, according to Hermeling et al. Blood was taken before the start of the immunization and on days 9 and 18 by cheek puncture. On day 28 mice blood was collected via heart puncture under isoflurane anesthesia followed by cervical dislocation. The obtained plasma was stored at -80˚C until further analysis.

Validation of the insulin mouse model: Immunogenicity of insulin and NP-ins

TG and NTG mice were treated for 4 weeks with 3 i.p. injections per week (Monday/Wednesday/Friday) of 20 µg of unmodified insulin (19 mice per group). The same schedule and number of animals was used to study if insulin bound to nanospheres (NP-ins) would be able to induce antibody formation Blood was drawn from all mice per group by cheek puncture before the injection schedule started and additionally on day 14. On day 28, blood was collected via heart puncture under isoflurane anesthesia followed by cervical dislocation for all mice. Plasma was stored at -80˚C until further analysis.

Application of the insulin mouse model: Immunogenicity of insulin formulations

98 animals, 49 TG and 49 NTG, were used to compare the potential immunogenicity of PB-MCO, ABI-MCO, Levemir®, Insulatard® and Actrapid®, with insulin and NP-ins. To this end, 7 mice per group were used and the same injection schedule was used as described above. Blood was drawn before the injection schedule started and on day 28 via heart puncture under isoflurane anesthesia followed by cervical dislocation for all mice. Storage was performed at -80 ˚C until further analysis.

Binding antibody assay

Plasma was analyzed for binding antibodies against insulin or HSA by direct ELISA, as described in detail by Hermeling et al [10]. Microtiter plates were coated with 100 µl of 5 µg/ml native insulin or 5 µg/ml HSA, depending on the assay, overnight at 4˚C. Samples were considered positive when the OD at 450 nm of the 20 or 10 fold diluted plasma was higher than the cut-off value, calculated according to Mire-Sluis et al [19]. Positive and negative control samples were included in each ELISA plate. The plots of OD 450 versus dilution were fitted to a sigmoidal curve and the reciprocal of the dilution at the midpoint was considered the titer of the plasma. Sigmoidal curves were calculated using a fourth order polynomial degree regression curve, elaborated with GraphPad version 4.03. Each plate contained negative plasma from NTG animals (i.e., before injection of any formulation) and positive plasma from NP-ins treated NTG and TG animals. To confirm the specificity of the antibodies for insulin, and their cross-reactivity, selected antibody-positive plasma samples of TG and NTG mice (2 samples per group) were spiked for 1 hr with increasing amounts of insulin (100 µg max), bovine serum albumin (BSA, 100µg max), bovine insulin, bovine insulin's chain A or Chain B, before adding the samples to the plate and performing the ELISA as described before. Samples from NTG mice treated with NP-ins were diluted 200-fold while samples from TG mice treated with NP-ins and from NTG and TG treated with insulin, were 20-fold diluted.

Statistical analysis

Antibody titers were first checked for normal distribution. After determining they were not normally distributed a non-parametric Mann-Whitney U test was used to compare antibody titers between groups. Significant differences between groups in the number of responders were determined with a Fisher's test. A calculated probability (p) value equal than or below 0.05 was considered to be statistically significant.

Results and Discussion

Physicochemical characterization of NP-INS, PB-MCO and ABI-MCO

Conjugation of human insulin to carboxylated polystyrene nanoparticles: evaluation of covalent binding, conjugation efficiency and characterization of particle size

Chemical covalent linkage between insulin and polystyrene nanoparticles (NP) was achieved following a previously described procedure [15]. To investigate the nature of the coupling, the amount of insulin covalently coupled versus the adsorbed one, and to exclude the presence of protein aggregates, we used SDS-PAGE, SEC and DLS analysis. Results suggest that mainly particles coated with covalently bound human insulin, with a Z-average of 55.21 ± 0.11 were present . No substantial increase in particle size was observed when insulin was mixed with non-activated particles (50.37 ± 0.21) indicating little or noncovalent binding of insulin to the NP.

Furthermore, the presence of free insulin in the mixture of ADS-ins likely explains the slightly lower average diameter. The low PdI measured with DLS (<0.1 for all NP formulations studied) points to the absence of NP aggregates.

The absence of aggregates in NP-ins and the successful conjugation of insulin to NP was further confirmed with SDS-PAGE (Figure 1, panel A, B and C). In particular panel A indicates the covalent nature of the bond between insulin and NP, since free insulin was barely detected. Panel B shows that the broad band at the top of Figure 1, panel A is solely due to NP and provides evidence that the coupling procedure with NHS-sulfo and EDAC does not induce aggregates formation as for these reagents used with insulin but without NP (Figure 1, panel C).

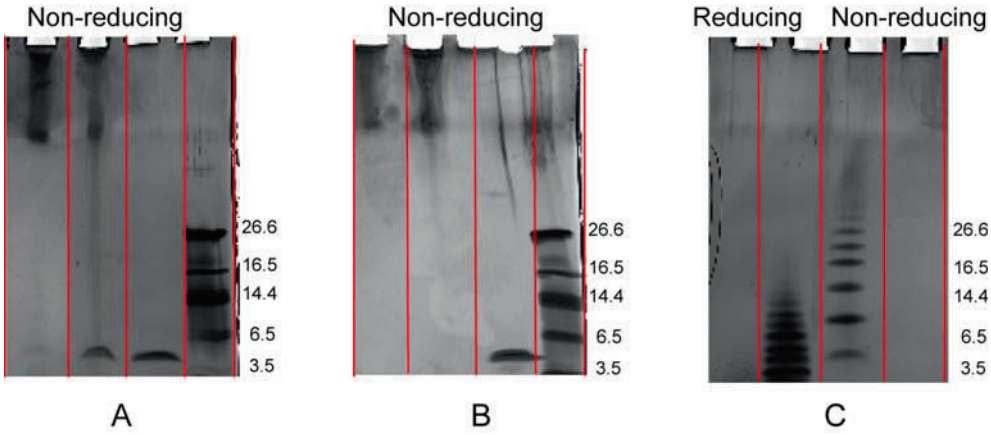


Figure 1. SDS-PAGE results: A, from left to right: nanoparticles-insulin (NP-ins), insulin adsorbed on nanoparticles (ADS-ins), rh insulin (insulin), polypeptide standard (M.W. indicated in kDa); B, from left to right: NP-ins, polystyrene nanoparticles (NP), insulin, polypeptide standard; C, from left to right: buffer, reducing SDS-PAGE of cross-linked insulin (CR-ins), non-reducing SDS PAGE of CR-ins, buffer.

Aggregate content and structural changes in PB-MCO and ABI-MCO

PB-MCO has been extensively characterized before [14, 16]. SEC and CD analysis were used to confirm our previous results: PB-MCO insulin contained 23.1 ± 3.6% aggregates and its secondary structure was substantially perturbed, as demonstrated by the reduction of the intensity of the entire spectrum and an increased 208/223 nm ratio, relative to insulin (PB-MCO 1.30 ± 0.03; insulin 1.19 ± 0.01), consistent with our previous data. ABI-MCO showed an amount of aggregates comparable to that of insulin, based on SEC analysis (ABI-MCO 0.7 ± 0.2%; insulin 0.4 ± 0.2%). CD analysis instead suggested that the content of alpha helix in ABI-MCO had decreased, parallel to an increase in random coil structure [20], based on the higher 208/223 ratio, which was 1.40 ± 0.02. This is likely due to oxidation of amino acid residues on insulin chain A and B which are normally involved in forming an alpha-helix structure, namely Tyr A14 and A19 and, His B10 and Tyr B16 [21].

Furthermore, the introduction of new oxygen atoms on His, Phe and Tyr residues, may alter the native hydrogen bond network of insulin, inducing insulin to assume a non-native like secondary structure [22].

Validation of the insulin mouse model and immunogenicity of insulin formulations

Immunization of transgenic and non-transgenic mice with human serum albumin

Mice were injected with 5 µg HSA daily for five consecutive days for three consecutive weeks. Anti-HSA IgG titers at days 11 and 18 were determined. TG animals showed an antibody response against HSA which was comparable to that in NTG animals, showing that the transgene did not interfere with the general capacity of the TG animals to produce antibodies. This is in line with previous TG mouse models for IFNα-2b [9, 23], IFNβ-1a [10-11] and insulin [12], which were also shown to be similarly responsive to the administration of a foreign protein as their NTG counterpart.

Immunogenicity of insulin and NP-ins

NTG and TG mice were injected with 20 µg of native insulin for 4 weeks (3 i.p. inj./week). Figure 2 shows the total IgG titers at day 28.

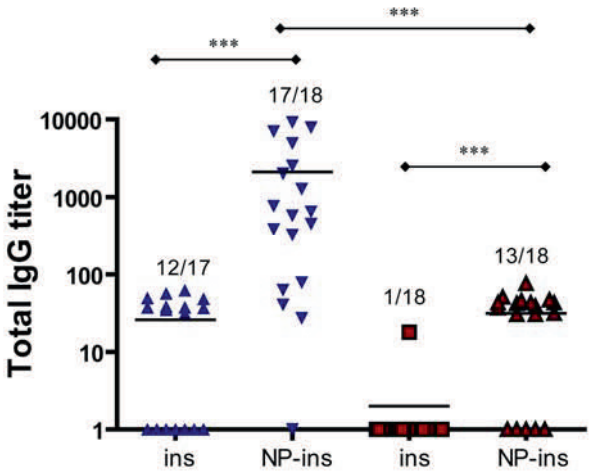


Figure 2. Total anti-insulin IgG titers after 28 days in NTG (blue) and TG (red) animals treated with rh insulin (ins) and nanoparticles-ins (NP-ins). Non-responders were assigned an arbitrary titer of 1 and they have been included in the calculation of the average titers depicted in this figure. Values above the bars represent the number of positive out of total mice. *** indicates $p < 0.001$. Five animals died during the studies for unknown reasons.

Of the TG mice injected with insulin only 1 out of 18 develop antibodies against human insulin. In contrast, nearly 50% of the NTG mice treated with insulin developed measurable antibody titers after two weeks of treatment (data not shown) which increased to about 70% after 4 weeks of treatment.

This shows that the TG mice are tolerant for human insulin, while NTG mice are not. To validate that TG mice (and the NTG controls) can form anti-human insulin antibodies they were treated with NP-ins. This formulation contains a particulate form of insulin which is likely to expose repetitive epitopes.

Several studies have demonstrated that presentation of such structures to the immune system, can result in antibody formation [7, 24].

Indeed, both NTG and TG animals do develop antibody against human insulin by the administration of NP-ins. For the TG mice 50% and 72% showed antibodies after 14 (data not shown) and 28 days, respectively. 94.4 % of NTG mice were antibody positive after 14 (data not shown) and 28 days. Based on Fisher's test, the number of responders (both TG and NTG) was higher for NP-ins treated mice compared to mice treated with insulin ($p=0.022$).

In addition to the differences in number of responders, we also found differences in antibody titers. When combining all treatment data, NTG mice displayed significantly higher antibody titers than TG mice. In more detail, NTG mice treated with NP-ins had higher antibody titers compared to (i) NTG mice treated with insulin ($p<0.001$, at days 14 and 28) and to (ii) NP-ins treatment in TG mice ($p<0.001$) (Figure 2). Similarly, TG mice treated with insulin show significantly lower antibody titers as with NP-ins ($p<0.001$). The immunogenicity of NP-ins, where insulin is covalently bound on the surface of the particles is likely due to the particulate character [25] and to the exposure of repetitive native-like epitopes [7, 24].

As observed before in other mouse models [10, 26], the same formulation induced a higher antibody titer in NTG mice than in TG mice, likely due to the absence of the human insulin gene in NTG mice. Furthermore, human and murine insulin feature high homology (i.e., > 92%, because of 4 different amino acid residues). Hence, there might be cross reactivity between insulin (either mouse or human, which are in theory both produced in TG animals) and ADAs. This could compromise detection of anti-insulin antibodies by ELISA due to binding between the circulating insulin and anti-human insulin antibodies, as observed by Thomas et al [27].

In addition, murine and human insulin contain an identical A-chain loop, which is a well known antigenic determinant on insulin [28-29]. Furthermore, due to the high sequence homology between human and murine insulin and a comparable rigid structure of the A-chain loop, human insulin might not be as immunogenic for NTG animals as we see for other foreign proteins with lower degree of homology. Perhaps this is why not all NTG mice immunized with human insulin were seropositive and the antibody titers in the seropositive ones were low. Concluding, differences in titers among TG mice may arise from differences in their transgene number (for human insulin).

Specificity of the antibodies for human insulin

Spiking plasma with increasing amounts of insulin and BSA confirmed the specificity of the antibodies measured: whereas spiking with insulin significantly diminished the OD at 450 nm for all positive mice ($p<0.05$), BSA did not inhibit the OD 450 signal ($p>0.05$), confirming the specificity of the antibodies for native insulin (Figure 3).

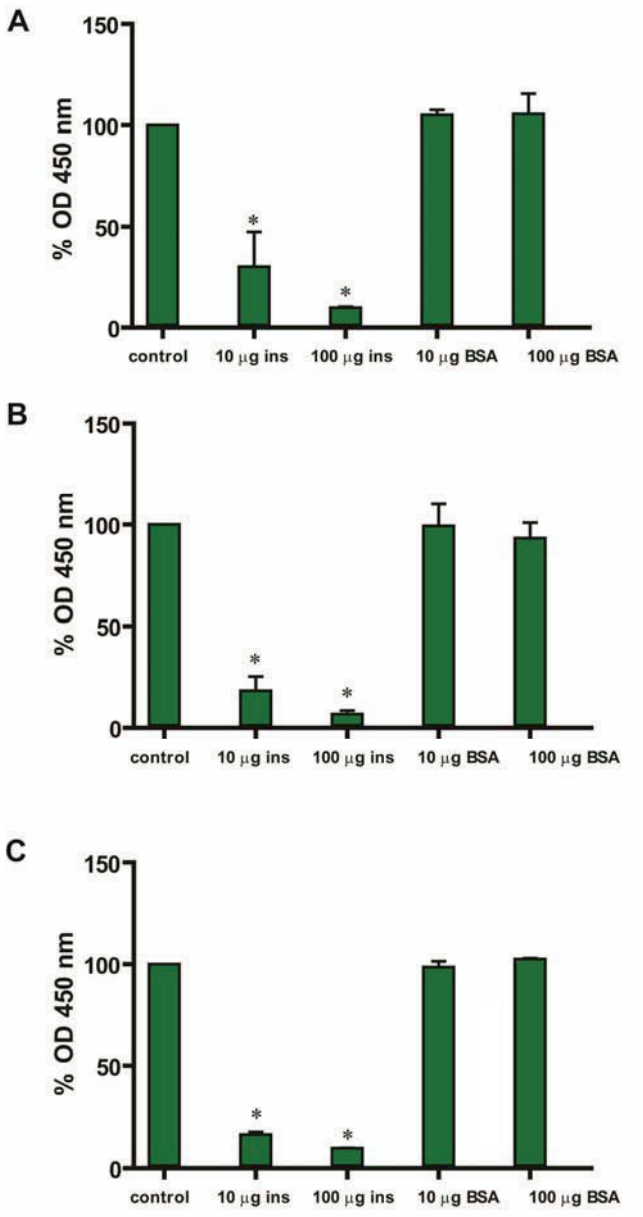
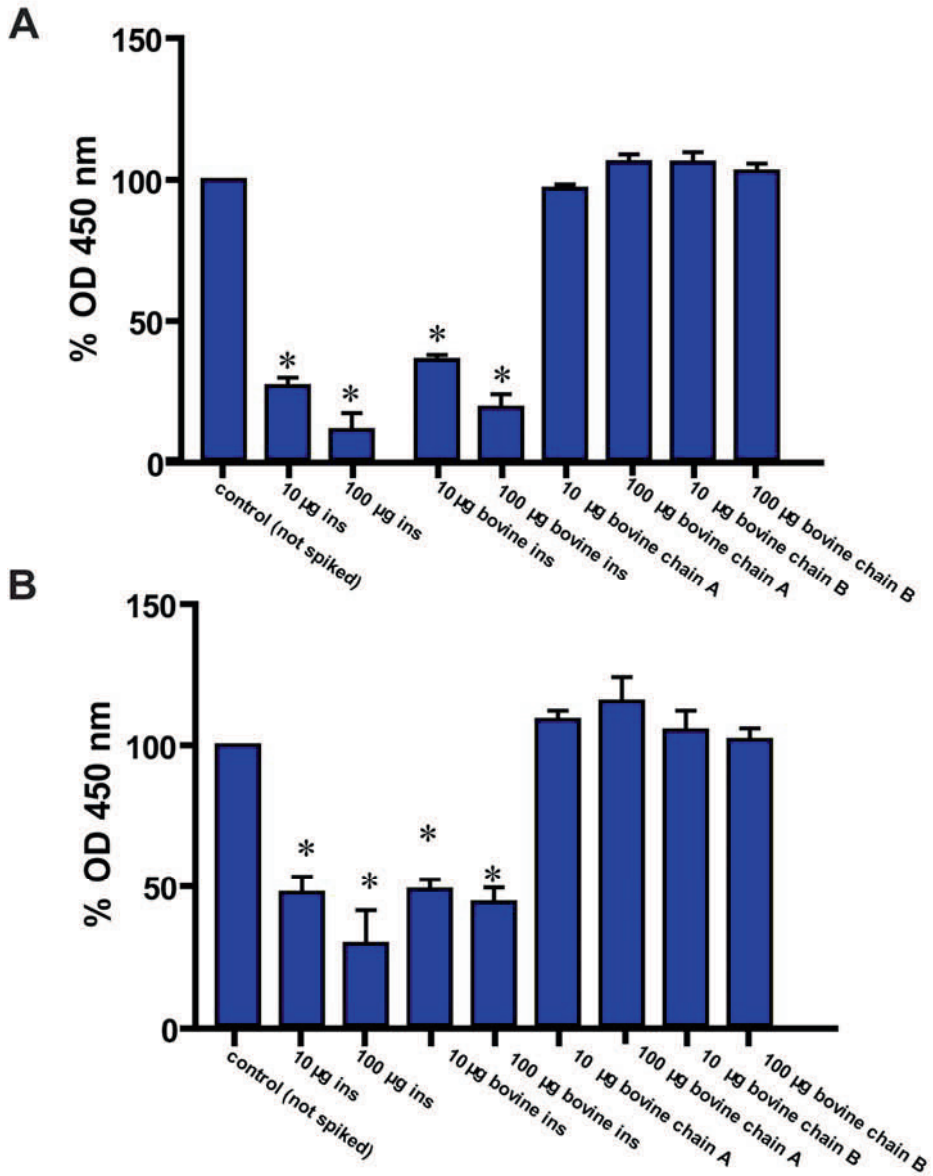


Figure 3. Spiking of plasma of NTG mice treated with nanoparticles-ins (NP-ins) (panel A), TG mice treated with NP-ins (panel B), NTG mice treated with rh insulin (ins) (panel C). Plasma was incubated with 10 or 100 μg ins or bovine serum albumin (BSA), as indicated at the x-axis. In the control sample no protein was added. Two antibody positive mice were used for each group. Data were normalized against the control and represented as mean + upper value. * indicates that the OD % was significantly lower than the control ($p<0.05$).

Cross-reactivity of the antibodies

In order to check whether the antibodies raised against human insulin cross-react with bovine insulin or its separate chains A and B, positive plasma samples were spiked with increasing amounts of human insulin, bovine insulin, bovine insulin's chain A and bovine insulin's chain B (Figure 4).



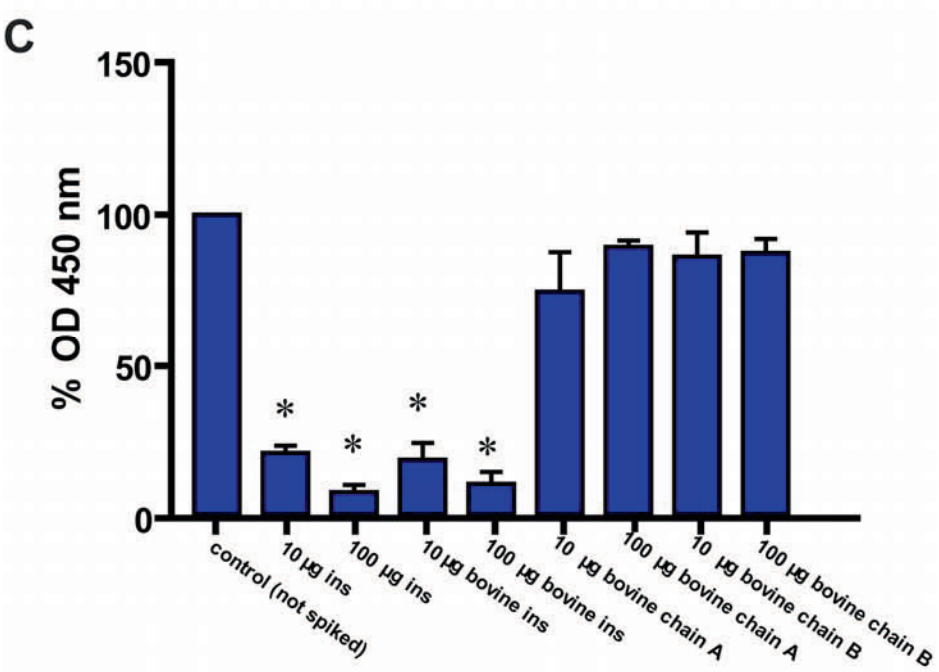


Figure 4. Spiking of plasma of NTG mice treated with nanoparticles-ins (NP-ins) (panel A), TG mice treated with NP-ins (panel B), NTG mice treated with rh insulin (ins) (panel C). Plasma was incubated, separately with ins, bovine insulin, bovine insulin's chain A and bovine insulin's chain B (i.e. 10 and 100 µg). In the control sample no protein was added (not spiked). Two antibody positive mice were used for each group. Data were normalized against the control and represented as mean + upper value. * indicates that the OD % was significantly lower than the control ($p<0.05$).

While bovine insulin, which shares with human insulin ~ 94% of the primary structure (i.e. 48 over 51 amino acids are identical) (Figure 5) as well as higher order structural features (i.e. secondary and tertiary structure), was recognized by the anti-human insulin antibodies, separated insulin's chains from bovine source were not. These findings suggest that the antibodies cross-react with bovine insulin and recognize conformational epitopes rather than linear epitopes.

A-CHAIN

	1	11	21
Human	GIVEQCCTSI	CSLYQLENYC	N
Bovine	GIVEQCCASV	CSLYQLENYC	N
Mouse/Rat I+II	GIVDQCCTSI	CSLYQLENYC	N

B-CHAIN

	1	11	21	30
Human	FVNQHLCGSH	LVEALYLVCG	ERGFFYTPKT	
Bovine	FVNQHLCGSH	LVEALYLVCG	ERGFFYTPKA	
Mouse/Rat I	FVKQHLCGPH	LVEALYLVCG	ERGFFYTPKS	
Mouse/Rat II	FVKQHLCGSH	LVEALYLVCG	ERGFFYTPMS	

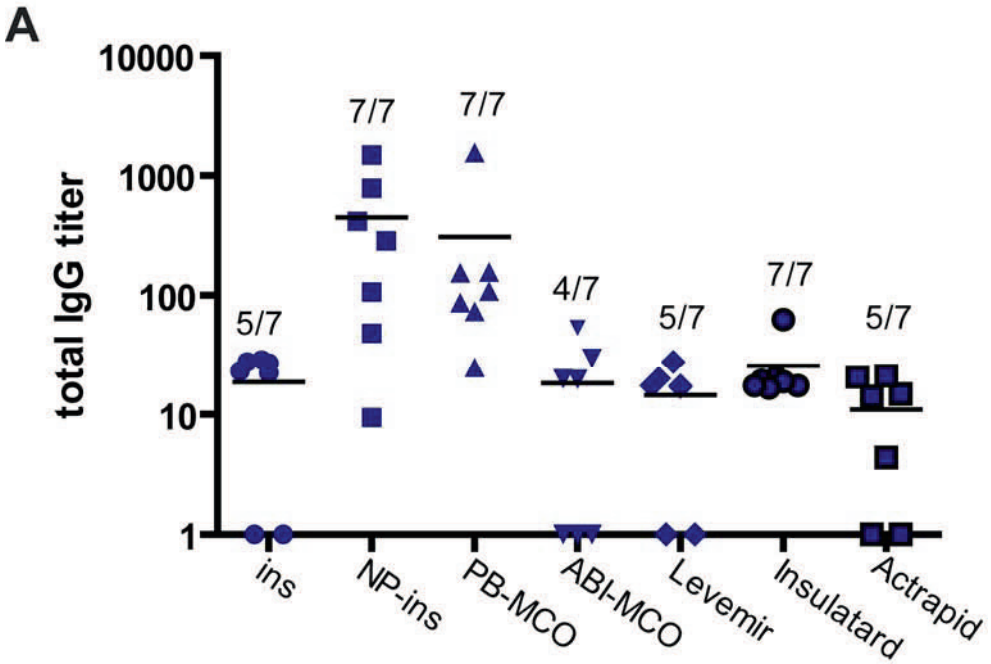
Figure 5. The amino acid sequences of human, bovine, mouse and rat insulin. Differences with human insulin are highlighted in red.

Immunogenicity of insulin formulations

In panel A and B of Figure 6 are shown the total anti insulin IgG titer in NTG and TG animals respectively. As expected, TG mice did not develop antibodies against native insulin, while 50% of the mice treated with NP-ins were antibody positive, in agreement with what was observed during our validation experiment. Similarly, half of the TG animals treated with PB-MCO were antibody positive and had comparable titers to those treated with NP-ins. This is in agreement with earlier results where IFNα-2a (unpublished results from our group), IFNα-2b [23], IFNβ-1a [26] and IgG1 [30], oxidized and aggregated with the oxidative system Cu^{2+}/ascorbate, induced ADAs. Although ABI-MCO formulations lacked aggregates, 33% of the animals were found to be positive.

This suggests that the chemical modifications induced during oxidation and/or the perturbation of the secondary structure contributed to the slightly enhanced immunogenicity of this formulation. Levemir® and Insulatard® were poorly immunogenic in TG animals (1 mouse over 7 was positive for both formulations), while Actrapid® did not elicit any immune response.

Significantly lower titers ($p<0.05$) were detected in TG animals treated with NP-ins, PB-MCO and Insulatard® compared to NTG (Figure 6, panel B) mice treated with these formulations. In ABI-MCO and Levemir® treated animals, the titers of TG and NTG mice were comparable.



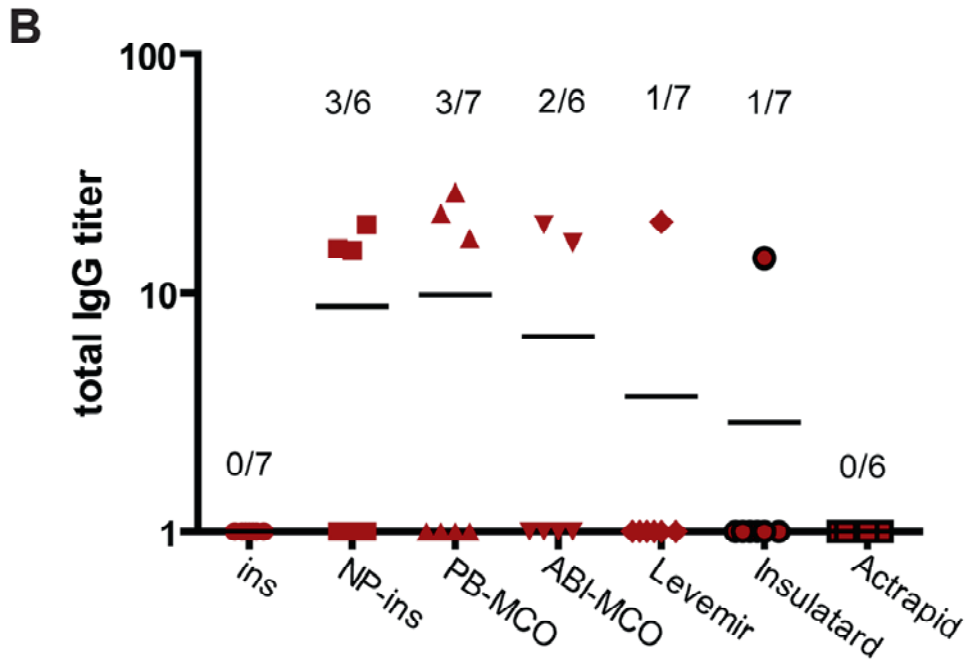


Figure 6. Total anti-insulin IgG titer after 28 days in NTG animals (panel A). Non-responders were assigned an arbitrary titer of 1 and they have been included in the calculation of the titers depicted in this figure. Values above the bars represent the number of positive out of total mice. Samples: rh insulin (ins), nanoparticles-insulin (NP-ins), oxidized and aggregated insulin (PB-MCO), oxidized non aggregated insulin (ABI-MCO), long acting insulin modified on Lys B 29 with fatty acid (Levemir®), long acting insulin obtained as a zinc suspension (Insulatard®), fast acting insulin (Actrapid®). Total anti-insulin IgG titer after 28 days in TG animals (panel B). Animal treatment and graph layout are the same of panel A. Please note that since TG mice treated with ins and Actrapid® had no detectable antibody and no responders (variance of the group=0), statistical testing involving this two groups was not allowed. Titers among groups with detectable antibody and responders were comparable. Three animals died during the studies for unknown reasons.

Conclusion

Here we reported the validation and application of a TG mouse model, immune tolerant for human insulin, for the study of product-related factors influencing the immunogenicity of insulin. In these studies it was confirmed that the combination of covalent aggregation and chemical modifications, induced via the oxidative system Cu^{2+}/ascorbate, likely contributes to the immunogenicity of therapeutic proteins. This mouse model represents a

promising tool to study in more detail the immune mechanisms of antibody formation against therapeutic proteins, using insulin as a model protein.

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