

Effects of PEG conjugation on insulin properties

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Received 5 November 2001; accepted 22 January 2002

Abstract

The goal of this research was to determine whether the site-specific attachment of poly(ethylene glycol) to insulin could enhance the physical and pharmacological properties of insulin without negatively affecting its biological activity or immunological properties. Electrophilically activated derivatives of low-molecular-weight monomethoxypoly(ethylene glycol) (mPEG) were chemically coupled to insulin via its amino groups at positions phenylalanine-B1 or lysine-B29, with an amide bond being formed between the polymer and protein. The site-specific attachment of mPEG to insulin did not substantially alter insulin's secondary/tertiary structure, self-association behavior, or potency *in vivo*. However, mPEG attachment did significantly enhance insulin's resistance to aggregation. In addition, the pegylation of insulin almost completely eliminates the resultant conjugate's immunogenicity, allergenicity, and antigenicity. Finally, the conjugates were observed to remain in the systemic circulation for longer periods of time than unmodified insulin after subcutaneous administration. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Diabetes; Poly(ethylene glycol); PEG–protein conjugates; Protein delivery; Pharmacokinetics; Immunogenicity; Protein stabilization; Parenteral delivery

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1. Introduction

Parenteral administration of insulin formulations remains the primary therapy utilized for the treatment of insulin-dependent diabetes mellitus (IDDM) since insulin's discovery over 75 years ago [1]. Many of the factors that make the current therapies inadequate are inherent shortcomings intrinsic to the insulin molecule. Contemporary researchers are applying genetic and protein engineering techniques to further probe the structure–function relationships of insulin in the continuing quest to produce pharmaceutical insulin preparations that closely mimic the natural secretion of insulin and, therefore, maximize the likelihood of achieving a euglycemic state in those afflicted with diabetes. Specifically, insulin faces many problems typical to protein pharmaceuticals, including poor physical and chemical stability, increased susceptibility to proteolysis, increased immunogenicity and antigenicity, and a relatively short plasma half-life [2].

Poly(ethylene glycol) (PEG) is a linear or branched neutral polyether with the chemical formula $\text{HO}-(\text{CH}_2\text{CH}_2\text{O})_n-\text{H}$. PEG is highly soluble in water and many organic solvents (e.g., methylene chloride, ethanol, toluene, acetone, and chloroform), and is readily available in various sizes (molecular weights) and functionalized architectures (e.g., amino-, carboxyl-, and sulfhydryl-terminated). Low-molecular-weight (< 1000 Da) PEGs are viscous and colorless liquids, whereas higher-molecular-weight PEGs are waxy solids whose melting points are proportional to their molecular weights with an upper limit near 67°C [3,4]. PEG has been found to be nontoxic and is approved by the FDA for use in drugs (parenterals, topicals, suppositories, nasal sprays), foods, and cosmetics [5]. Molecular weights of PEG used in biomedical applications usually ranges between a few hundred to 20 000 Da [6]. The molecular weight distribution (polydispersity) of these PEG polymers is generally low, but the commonly used monomethyl ethers of PEG exhibit broader molecular weight distributions [7]. In solution PEG is also a

highly hydrated polymer, where each monomer (ethylene oxide unit) can bind three molecules of water [3]. Lim and Herron executed molecular simulations of the behavior of single surface-bound PEG ($M_r = 600$ Da) chains in water [8]. Their results show the polymer exhibiting a large degree of segmental flexibility in aqueous solution. In addition, it is thought that PEG has the ability to influence the structure of several molecular layers of more loosely associated hydrating water molecules [9]. These findings taken together might explain why PEG is remarkably effective in excluding other polymers (natural and synthetic) from its presence in aqueous environments. The exclusion of other polymers is the primary driving force behind PEG's ability to reject proteins, form two-phase systems with other synthetic polymers, and makes this polymer both nonimmunogenic and nonantigenic [6]. When PEG is covalently attached to a protein, it typically transfers many of the polymer's favorable characteristics to the resultant conjugate. Because of the many beneficial properties mentioned above, PEG is ideally suited for protein modification. A non-exhaustive list of examples of PEG–protein conjugates described in the literature includes the conjugates of proteins such as adenosine deamidase [10,11], bovine liver catalase [12], uricase [13], collagen [14], human growth hormone [15], nerve growth factor [16], trypsin [17], alkaline phosphatase [18], asparaginase [19], superoxide dismutase [20], hemoglobin [21], interleukin-2 [22], granulocyte colony-stimulating factor [23], ovalbumin [24], ribonuclease A [25], and insulin [26–28].

This conjugation technology has been used to improve the therapeutic efficacy of recombinant human proteins because it alleviates many problems associated with therapeutic protein formulations. First, most parenterally administered proteins are rapidly cleared from the body by the reticuloendothelial system (RES), kidney, spleen, or liver [29]. Moreover, clearance depends on molecular size, charge, and the presence of specific cellular receptors for the proteins of interest. The attachment of PEG to

a protein affects its molecular size, charge, and receptor-binding capabilities, which can serve to decrease the conjugate's overall rate of clearance. Second, the metabolism of proteins by enzymes leads to the rapid loss of biological activity of therapeutic proteins. By sterically shielding the protein domains susceptible to proteolytic attack, PEG decreases the protein degradation that renders it biologically inactive. Third, even recombinant human proteins elicit immune responses after repeated use. Again, by sterically masking the therapeutic protein's immunogenic/antigenic determinants, PEG-attachment commonly results in conjugates that are nonimmunogenic and nonantigenic. The result of changes in the parental protein's characteristics by pegylation almost invariably increases the plasma half-life and resistance to proteolytic degradation, and decreases immunogenicity and antigenicity of the resultant PEG–protein conjugate.

Insulin is one of the most highly characterized substances ever studied. Since its discovery in 1922 [1], literally thousands of research articles have described almost every aspect of insulin's structure, function, and properties in both crystalline and solution states. The human insulin molecule consists of two polypeptide chains (A- and B-chains), the A-chain is composed of 21 amino acids (denoted A1–A21) and the longer B-chain is comprised of 30 amino acids (B1–B30). Overall, there are 23 polar residues in the insulin monomer, and all lie on the molecule's surface [30]. The nonpolar surface residues form extensive regions on two sides of the insulin monomer. One side comprises entirely B-chain residues from the α -helix and the C-terminus, and the other side comprises almost entirely residues from the B-chain's α -helix and N-terminus. These two surfaces are mechanistically involved in the molecule's organization into higher-ordered states such as insulin dimers and hexamers.

Like many other globular proteins, insulin tends to adopt a three-dimensional conformation in which its hydrophobic surfaces are buried by either folding or self-association mechanisms. However, changes in insulin's folding or self-association can be brought about by a variety of physical and chemical factors. Insulin can also undergo conformational changes that result in the linear aggregation (fibrillation), forma-

tion of viscous gels, or formation of insoluble precipitates, especially under the influence of heat [31]. The physical stability of insulin has a tremendous impact on the ability to design suitable insulin dosing regimes for diabetic patients. Insoluble insulin fibrils have been shown to elicit increased immunogenic responses and are not biologically active. As a result of fibril formation, many undesirable properties may be introduced into an insulin preparation and the inhibition of fibrillation is necessary for a preparation to generate desirable and reproducible pharmacological effects.

As a result of the inherent immunogenicity of insulin [32,33] many patients produce anti-insulin antibodies, mostly of the IgG and IgE subclasses [34]. High levels of anti-insulin IgE antibodies have been found in diabetic patients with insulin allergy [35,36] and high levels of anti-insulin IgG antibodies are associated with insulin resistance syndrome [37,38], which is defined as a daily insulin requirement of more than 200 U. Consequently, the therapeutic effectiveness of insulin may be counteracted by its rapid elimination from the patient's circulation in the form of immune complexes, and repeated administration may lead to allergic and/or anaphylactic symptoms. Furthermore, 1 in 500 insulin-dependent diabetics treated with recombinant human insulin experience local insulin allergies and in rare cases (1 in 1000) systemic allergic reactions where anaphylaxis can occur [39]. Additional problems associated with anti-insulin antibodies include antibody-mediated insulin resistance [33,40], lipoatrophy at the site of injection, antibody-mediated suppression of endogenous insulin secretion, and microvascular and macrovascular disease [41]. Thus, the presence of high levels of insulin-specific antibodies may have a significantly damaging effect on any clinical attempts to improve glucose control utilizing current insulin therapies [42–44].

The goal of modern insulin replacement therapy is to normalize blood glucose levels by imitating normal physiological patterns of insulin secretion in a dosage form. To date, a few reports have arisen in the literature describing how the pegylation of insulin influences the properties of this very important therapeutic protein. However, no comprehensive study on the effects of PEG conjugation to human insulin on its physicochemical, immunologi-

cal, and pharmacological properties has been reported. The work described in this review was motivated by the need to develop insulin preparations with improved physicochemical, immunological, and pharmacological properties. The ultimate goal of these studies was to determine if the site-specific pegylation of human insulin can favorably alter any of the deleterious properties ascribed to it without negatively affecting the conjugate's efficacy. This review illustrates the use of site-specific PEG conjugation to increase insulin's physical stability, reduce its immunogenicity and antigenicity, and extend its circulation half-life. The following issues will be addressed in this review: (1) the preparation and physicochemical characterization of PEG–insulin conjugates with differing molecular weights and sites of substitution; (2) assessment of the physical stability and in vivo biological activity of the conjugates; (3) investigation of the immunological (immunogenicity, antigenicity, and allergenicity) properties of the conjugates; (4) determination of selected conjugates' pharmacodynamic and pharmacokinetic parameters in a dog model.

2. Preparation and characterization of PEG–insulin conjugates

2.1. Preparation

This section describes the synthesis, purification, physicochemical characterization, and biological activity of a variety of pegylated insulin conjugates made in our laboratory differing in molecular weight and/or site of substitution. Our goal was to improve human insulin's physical properties without significantly altering its biological activity via site-specific pegylation. Fig. 1 shows a schematic of this native human insulin molecule and specific modification sites selected for the work. All three amino groups of insulin have historically been used as sites of modification with macromolecules such as transferrin [45], poly-*N*-vinylpyrrolidone (PVP) [46], dextran [47], and PEG [26,27,48] or organic small molecules such as glucopyranoside [49] and palmitic acid [50] with limited success in producing insulin derivatives possessing beneficial properties of clinical relevance.

We do not expect the conjugation of methox-

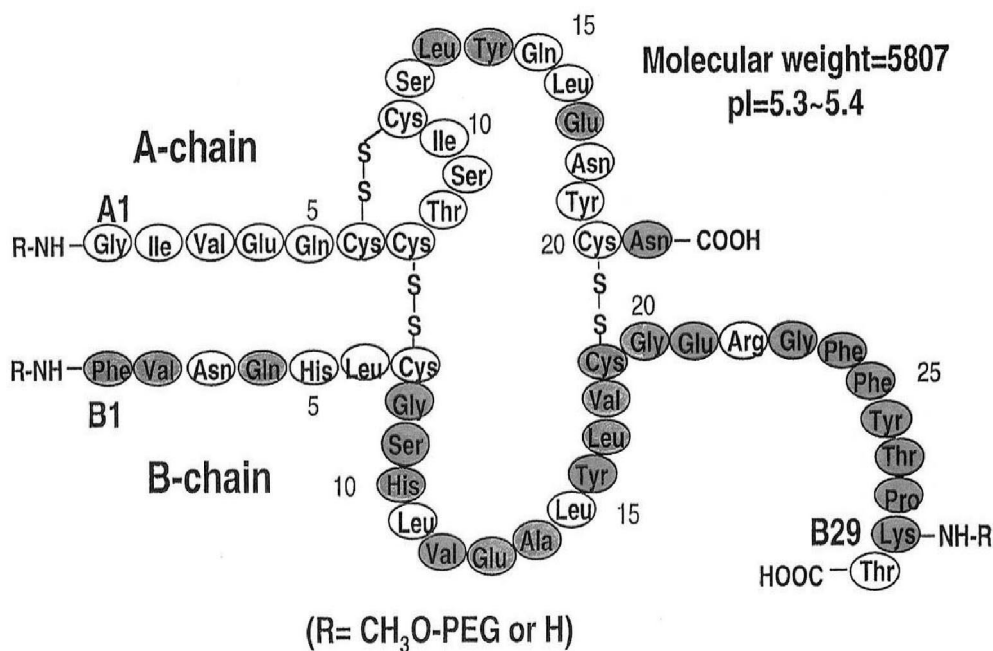


Fig. 1. Schematic representation of the primary structure of human insulin. Shaded residues are involved in self-association (or aggregation) events that result in insulin dimers (orange) and hexamers (blue). The three amino groups (A1, B1, B29) available for PEG conjugation are also labeled.

ypoly(ethylene glycol) (mPEG) to the PheB1 ($N^{\alpha B1}$) or LysB29 ($N^{\epsilon B29}$) amino groups of insulin to negatively impact its bioactivity since it is known that neither of these residues are essential to its bioactivity [50–52]. Conjugates substituted at positions B1 (mPEG–B1-insulins) and B29 (mPEG–B29-insulins) were prepared and characterized according to methods described in a previous report from our group [53]. For the sake of completeness, we have provided a brief overview of these methods followed to prepare and characterize the conjugates used in subsequent studies intended to determine the effect of pegylation on insulin's properties.

The preparation of $N^{\alpha B1}$ -methoxypoly(ethylene glycol)–insulins (mPEG–PheB1-insulins) began by synthesizing and purifying a di- $N^{\alpha A1}$, $N^{\epsilon B29}$ -*tert*-boc-insulin (diboc-insulin) intermediate according to procedures described earlier [54]. Briefly, *tert*-boc was reacted with insulin in a dimethyl sulfoxide–triethylamine (DMSO–TEA) mixture. The crude reaction mixture containing mono-, di-, and tri-substituted boc-insulins was extensively dialyzed and lyophilized before isolation of diboc-insulin. A fast protein liquid chromatography (FPLC) system fitted with a preparative cation-exchange column was equilibrated with a 7 M urea–1 M acetic acid mobile phase and a 0.1 to 0.3 M NaCl gradient was used elute the various boc-insulin species. The purity of diboc-insulin was assessed using a Mono S analytical cation-exchange column under the same conditions. Once the diboc-insulin was determined to be highly pure (single chromatographic peak), it was ready for pegylation.

The mPEG(750)–PheB1-insulin (F750) and mPEG(2000)–PheB1-insulin (F2000) conjugates were prepared by reacting 750 or 2000 Da mPEG–SPA (Shearwater, AL, USA) with diboc-insulin in DMSO–TEA. The mPEG(750 or 2000)–PheB1-diboc-insulin solutions were then extensively dialyzed and lyophilized. Quantitative deprotection of the lyophilized product was achieved by reaction with trifluoroacetic acid (TFA) containing 10% anisole at 0 °C. Finally, the conjugates were purified to homogeneity using the same chromatographic procedure described above for diboc-insulin.

Both $N^{\epsilon B29}$ -methoxypoly(ethylene glycol)–insulin (mPEG–LysB29-insulin) conjugates were synthesized according to a modified version of a method

used for the fatty acid acetylation of insulin [55]. Briefly, Zn^{2+} -insulin was dissolved in 60% dimethylformamide (DMF) (pH_{app} 9.5) at room temperature. At this pH the LysB29 amino group should be most able to react with the electrophilically activated PEG. An excess of mPEG–SPA (750 or 2000 Da) was added by immediate injection to the Zn^{2+} -insulin solution, keeping the apparent pH_{app} of the reaction solution constant by NaOH addition. The same methods were used to isolate both types (PheB1 and LysB29) of the mPEG–insulin and derivatives. The conjugates will be referred to in all subsequent studies as follows: F750 for mPEG(750)–PheB1-insulin; F2000 for mPEG(2000)–PheB1-insulin; K750 for mPEG(750)–LysB29-insulin; and K2000 for mPEG(2000)–LysB29-insulin.

2.2. Physicochemical characterization

Each conjugate's purity was assessed using a reversed-phase high-performance liquid chromatography (RP-HPLC) system fitted with an analytical C_{18} column. The presence of a single chromatographic peak confirmed the conjugates' purity was > 95% in all cases. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) analysis was used to determine the number of mPEG chains attached and the conjugate's molecular weight. Monomeric insulin has a calculated molecular weight of 5807.2 Da, and the number-average molecular weights of mPEG(750)–SPA and mPEG(2000)–SPA used in the conjugation reactions were 827 and 2006 Da, respectively. The mass spectra of both mPEG(750)–insulin (average molecular weight 6600 Da) and both mPEG(2000)–insulin (average molecular weight 7790 Da) conjugates clearly demonstrated that only one mPEG chain was attached to insulin. In addition, the individual ion peaks consistently differed by 44 Da (the molecular weight of an ethylene oxide monomer unit). These results confirm that only one mPEG chain was attached to insulin in all of the conjugates prepared and that their polydispersity is solely due to the polydispersity intrinsic to PEG.

N-Terminal protein sequence analysis was utilized to indirectly determine the site of PEG conjugation, with the knowledge that the Edman degradation reaction will not proceed at any N-terminal amino

group that is covalently bound to PEG. An N-terminal amino acid molar ratio of [GlyA1/PheB1] ≈ 1 is indicative of conjugation to residue LysB29 (or none at all) and [GlyA1/PheB1] $\approx \infty$ is indicative of conjugation to residue PheB1. We found that the mPEG–PheB1-insulin conjugates had a [GlyA1/PheB1] molar ratio at least three orders of magnitude larger than either Zn^{2+} -insulin or the mPEG–LysB29-insulin conjugates. Thus, the combined MALDI-TOF mass spectrometry and Edman degradation results provide conclusive evidence that only one mPEG chain was attached to each insulin molecule and the appropriate site-of-substitution was achieved with a high degree of purity ($> 95\%$ by RP-HPLC).

Circular dichroism studies were utilized to gain a semi-quantitative understanding of how the pegylation of insulin at two different sites affects its three-

dimensional structure. A large number of studies have used circular dichroism spectroscopy measured in the far-ultraviolet range to examine the conformation of insulin [56–62]. Fig. 2 shows the far ultraviolet CD spectra of the various conjugates and two control species. In general, the features of the spectra for insulin and its pegylated derivatives are similar. In some cases there are slight differences in the dichroic intensity of the spectra. The deconvolution program CDNN (version 2.1) was used to further analyze the data [63]. This program compared each samples' far-ultraviolet dichroic spectra to a set of 33 basis spectra representing the major secondary structure elements (e.g., α -helix, β -sheets, β -turn, random coil) commonly seen in peptides and proteins. Furthermore, the neural network chosen to analyze the data accounted for both aromatic and disulfide contributions to a sample's dichroic intensity in the far-ultraviolet region. The results given in Table 1 confirm that the secondary structure of native insulin is largely conserved after the covalent attachment of low-molecular-weight mPEG (750 or 2000 Da) to either site of substitution. All of the samples were composed of roughly the same proportions of the elements: 46% α -helix, 11% β -sheets, 14% β -turn, and 25% random coil. Any differences between the conjugates and insulin were within the error limits of the software ($\pm 6.45\%$). The values we calculated were very close to the values of Melberg and Johnson who determined that Zn-free-insulin was composed of 57% α -helix, little or no β -sheets, 18% β -turn, and 24% random coil elements [58]. In fact our results more closely match values estimated from the crystal structure of insulin (53% α -helix, 8% β -sheets, 18% β -turn, and 21% random coil) [64]. The spectral characteristics of the conjugates provide

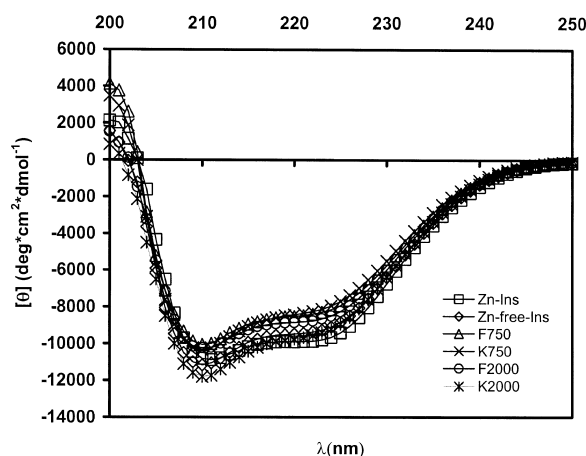


Fig. 2. Far UV CD spectra of insulin and its pegylated derivatives at 1×10^{-4} M in PBS.

Table 1

Secondary structure elements for various insulins calculated from far-ultraviolet CD spectra^a

	Zn-Ins	Zn-free	F750	F2000	K750	K2000
% Helix	48.4	47.4	46.6	43.3	45.6	48
% Antiparallel	6.1	5.9	7.3	7.1	7.3	5.4
% Parallel	5.5	5.3	5.8	5.4	5.7	5.2
% Beta-turn	13.3	14.3	13.0	14.9	13.6	14.7
% Random coil	24.8	25.2	25.4	26.6	25.7	25.0
Total	98.1	98.2	98.1	97.3	97.8	98.3

^a Expressed as the percent of total secondary structure elements. Average error of the neural network was 6.45%.

evidence that attachment of mPEG to insulin does not materially alter its secondary/tertiary structure.

It is commonly known that the average association-state of an insulin preparation substantially affects its pharmacological disposition [2,65–69]. Aqueous insulin solutions contain mixtures of monomeric, dimeric, and hexameric association-states that coexist under a dynamic equilibrium. The average association-state(s) in insulin preparations depend on protein concentration, temperature, ionic strength, and the presence of stabilizing molecules such as Zn^{2+} , phenol, chloride, or *m*-cresol [2,30,68,70,71]. Insulin monomers have two nonpolar surfaces that are directly involved in the reversible self-association process and dimerization proceeds through inter-monomer interactions involving residues ValB12, TyrB16, PheB24, TyrB26, and ProB28 [30]. The burial of hydrophobic side chains and creation of four inter-monomer hydrogen bonds from PheB24 to ProB28 result in an antiparallel β -sheet conformation between the monomers. Insulin hexamers are formed through both hydrogen bonding and hydrophobic interactions between surfaces formed by residues LeuA13, TyrA14, PheB1, ValB2, LeuB6, AlaB14, LeuB17, and ValB18. Addition of Zn^{2+} -ions further stabilizes the insulin hexamer through complexation of a single Zn^{2+} -ion with an imidazole group from each of three dimers that constitute the insulin hexamer [30,56]. It is important to note that the hexameric form of insulin is not bioactive and is only partly absorbed across the capillary endothelium into the systemic circulation [68]. In addition, the dissociation of insulin hexamers into dimers and monomers is seen as the rate-limiting barrier to subcutaneous absorption that directly affects the preparation's pharmacological response [66,72].

Investigations into the effects of insulin pegylation on its self-association were carried out using analytical ultracentrifugation. Sedimentation equilibrium experiments were used to gain insight into the association states of various insulin preparations including the four mPEG–insulin derivatives. Data analysis describing the concentration distribution was achieved by fitting the final absorbance versus radius data to various models of insulin self-association using nonlinear least-squares techniques and the analysis program NONLIN [73,74]. This software performs simultaneous nonlinear least-squares fits to

one or more sets of ultracentrifuge data according to the following equation:

$$M = \frac{2RT}{(1 - \nu\rho)\omega^2} \times \frac{dC/dr}{rC}$$

where dC/dr is the solute concentration as a function of radial position within the centrifuge cell, ω is the angular velocity, ν is the solute's partial specific volume (ml/g, volume of water displaced by 1 g of solute), ρ is the density of the solution, R is the gas constant, T is the thermodynamic temperature, M is the solute's molecular weight, and r is the radial position of the measurement within the centrifuge cell. In each case, the final fit resulted from the simultaneous fitting of three to six different concentration distributions. The partial specific volume (ν) was fixed at 0.72 ml/g, representing the measured value for insulin [57].

Table 2 gives the apparent molecular weights and average association-states for the unmodified insulin species and pegylated insulin derivatives. In every case a single-species (monomer, dimer, or hexamer) model best described the data, where the final fit comprised three separate data files with varying speeds and concentrations. In the presence of zinc, insulin appears to be primarily hexameric in solution. The data from Zn-free insulin indicate that its association-state is dimeric. It is well known that in

Table 2
Molecular weights of various insulins determined from sedimentation equilibrium experiments

Sample ^a	$M_{w,app} \pm \text{S.D. (kDa)}$	$(M_{w,app}/M_{w,m}) \pm \text{S.D.}^b$
Zn^{2+} -insulin	30.0 ± 9.0	5.17 ± 1.55
Zn-free insulin	10.4 ± 2.0	1.79 ± 0.34
Lispro ^c	5.70 ± 0.60	0.98 ± 0.10
F750-insulin	13.3 ± 3.0	2.0 ± 0.45
F2000-insulin	13.33 ± 3.0	1.71 ± 0.38
K750-insulin	14.41 ± 2.0	2.17 ± 0.30
K2000-insulin	15.98 ± 3.0	2.05 ± 0.38

^a All samples prepared in Tris–EDTA buffer (50 mM Tris, 5 mM EDTA, pH 7.4) except Zn^{2+} -insulin, which was prepared in Tris buffer only.

^b $M_{w,m}$ is the calculated molecular weight of the derivatives' monomeric species.

^c Monomeric Lispro[®] was prepared after extensive dialysis against distilled water to remove any stabilizing substances (phenol, glycerol, and Zn^{2+}) from the commercial formulation.

the absence of zinc that insulin exists as a dimer. However, in the presence of zinc insulin exists as a hexamer at the concentrations used in these experiments. Lispro[®] (Lilly, USA) is a mutant form of insulin where two amino acid residues (ProB28 and LysB29) are transposed, which results in an increased rate of subcutaneous absorption because the Lispro[®] hexamers more rapidly dissociate into monomers that are easily absorbed into systemic circulation. As expected, Lispro[®] was monomeric in solution. This is consistent with many reports indicating Lispro[®] is predominantly monomeric at high concentrations without the addition of stabilizers like phenol, Zn^{2+} , and glycerol. Finally, all of the mPEG-insulin derivatives appear to be dimeric in solution, regardless of their molecular weight or site-of-substitution.

The residuals from the curve fits were observed to be small and randomly distributed around zero, indicating the model chosen was able to accurately describe the data. The fits and residuals from three different concentration distributions were used in the final calculation of the values in Table 2. The presence of the PEG made it difficult to assign an accurate ν value to the pegylated insulin derivatives. Because the molecular weights obtained for the control samples were comparable to previously published reports and the weights of the conjugates were reasonable, the use of native insulin's ν value seems justified in the realm of calculating an average association-state. A likely explanation for this is that mPEG behaves as a random-coil attachment on the protein and does not change ν significantly.

2.3. Physical stability

An accelerated shake test was used to assess the physical stability of the four PEG–insulin conjugates in comparison to both Zn^{2+} - and Zn-free-insulin. This test is commonly described in the literature as providing an accurate measure of an insulin preparation's physical stability in an accelerated manner. The fibrillation kinetics of the four insulin conjugates, Zn^{2+} -insulin and Zn-free insulin (control samples) were studied by subjecting the solutions to horizontal shaking (100 strokes/min) at 37 °C. At prescribed time points, individual samples were

withdrawn, filtered (to remove the insoluble aggregates), and then analyzed for residual insulin (conjugate) concentration using RP-HPLC. The time elapsed until 50% of the initial protein concentration remained in solution ($T_{50\%}$) was taken as a relative measure of the samples' physical stability.

The results are summarized in Table 3 and corroborate previous results from our group for both glucosyl- and glucosyl-PEG–insulin conjugates [49,54]. The PheB1-insulin conjugates possess substantially higher (36–40 $\times$) physical stabilities and the LysB29-insulins were somewhat more-stable (4–8 $\times$) than control samples. We propose that the increased fibrillation-resistance of F750 and F2000 is due to two complementary effects. One is the specific steric blocking of the N-terminus in the B-chain by mPEG conjugation resulting in the specific prevention of this surface from participating in the hydrophobic interactions that drive insulin fibril growth [75,76]. The second contributing effect to PheB1-insulin's increased physical stability is nonspecific and steric in nature and increases with an increase in mPEG molecular weight. Both K750 and K2000 exhibited increased physical stability, showing a heightened resistance to fibrillation, but not to the same extent as the conjugates substituted at PheB1. This can be explained by the lack of LysB29's participation in fibrillation reactions, with any stabilizing effects caused by nonspecific steric-hindrance of the intermolecular interactions that are involved in fibrillation.

Table 3
Long-term physical stability of various insulins evaluated by shake testing^a

Sample ^b	$T_{50\%}$ (days) ^c
Zn^{2+} -insulin	0.5 ± 0.3
Zn-free insulin	0.4 ± 0.2
F750	18.4 ± 2.8
F2000	20.7 ± 4.1
K750	4.3 ± 1.1
K2000	8.6 ± 1.7

^a Accelerated shake-test done at 100 strokes/min and 37 °C.

^b Protein solutions prepared in PBS (38.0 mM, pH 7.4) containing 0.01% Na-azide.

^c Elapsed time to 50% of the initial protein concentration remaining (mean $\pm$ standard deviation).

2.4. In vivo biological activity

The in vivo biological activity of the conjugates' was measured using a blood glucose depression assay in a rat model. Male Spague–Dawley (SD) rats were kept under specific pathogen-free conditions in the animal facility. HumulinR[®] (Lilly) or the conjugate preparations were injected at a dose of 0.3 U/kg into the jugular vein. Blood samples were obtained at prescribed time points and assayed for glucose concentration using a commercially available glucometer. The blood glucose level (BGL) versus time area-under-curves (AUC_G), nadir BGL (C_{nadir} , % of basal value), and time to C_{nadir} (T_{nadir}) were determined from averaged experimental data. For assessment of the statistical significance of the differences, the Student *t*-test was employed and *P*-values for significance were set to 0.05.

Table 4 compares the bioactivities (AUC_G) of each of the four mPEG–insulin conjugates to that of HumulinR[®] (Lilly's regular insulin formulation) control samples. We found that F750 and K750 conjugates had equivalent bioactivities to HumulinR[®] after intravenous (i.v.) administration. However, the F2000 and K2000 conjugates possessed bioactivities that were slightly lower than HumulinR[®]. The attenuated glucose depression of the F2000 and K2000 conjugates is most likely due to increased steric hinderance of the larger polymer (relative to the smaller one) on the insulin–receptor interactions. As expected, no differences in bioactivity were observed between conjugates with different sites-of-substitution because neither PheB1 nor LysB29 are directly involved in receptor binding [51].

3. Immunological properties of PEG–insulin conjugates

Insulin is typically administered to diabetics via subcutaneous injection, and once this insulin has absorbed from the injection site into the systemic circulation it is cleared from target tissues primarily through receptor-mediated events. Clinical experience has shown that many insulin-dependent diabetes patients produce anti-insulin antibodies, mostly of the IgG and IgE subclasses [34]. High levels of anti-insulin IgE and IgG antibodies are commonly found in diabetic patients with insulin allergy [35,36] and insulin resistance syndrome [37,38], respectively. Consequently, the therapeutic effectiveness of pharmaceutical insulin preparations may be counteracted by their rapid elimination from the patient's circulation in the form of immune complexes and repeated administration can lead to allergic and/or anaphylactic symptoms.

We studied the immunological properties of mPEG–insulin conjugates in two types of mice: A/J (low insulin responders) and C57BL/10ScSn (high insulin responders). The humoral immunogenicity, cellular immunogenicity, antigenicity, and allergenicity of conjugates with different sites of substitution and molecular weights of mPEG attached were compared to those of unmodified recombinant human insulin. To avoid any confusion, in the context of these studies, immunogenicity is defined as the in vivo capacity of an antigen or allergen to induce the corresponding immune response. Similarly, in the context of this paper, antigenicity is defined as the ability of an antigen to bind to circulating antibodies. Finally, allergenicity is defined as the

Table 4
In vivo rat bioactivity study results for various insulins

Sample	T_{nadir} (min)	$C_{nadir} \pm S.D.$ (mg/dl)	AUC_G (mg $\times$ min/dl)	Bioactivity ^a (%)
HumulinR [®]	45	43 $\pm$ 1.38	11,700	100
F750	45	41 $\pm$ 3.52	11,250	104
F2000	30	60.5 $\pm$ 5.67	14,090	83
K750	45	39 $\pm$ 4.97	10,448	112
K2000	30	57 $\pm$ 4.96	13,770	85

^a Calculated relative to HumulinR[®].

capacity of an allergen to combine *in vivo* with homologous reaginic antibodies, thus triggering systemic anaphylaxis or local-skin reactions whether in direct skin tests or in passive cutaneous anaphylaxis (PCA) assays [77].

Here we compare the immunological properties of conjugates with distinct sites of substitution (N-terminus of the A-chain, N-terminus of the B-chain, and amino group of lysine in the C-terminal region of the B-chain) and varying sizes of low-molecular-weight mPEG (600, 750, and 2000 Da) to those of recombinant human insulin. Only derivatives with one mPEG chain/insulin monomer were used in this study because conjugates containing two or three mPEG chains/insulin monomer were previously found to have significantly reduced biological activities and, therefore, were not considered to be clinically relevant candidates for further study [78]. Likewise, only low molecular weight mPEG–insulin conjugates were used in these studies because the attachment of higher-molecular-weight mPEG (5000 Da) was found to considerably depress the conjugate's bioactivity [78].

Four experimental end-points were examined to determine the subjects' immunological response to insulin or PEG–insulin immunization. First, the effect of site-specific pegylation on the humoral immunogenicity of insulin was ascertained by measuring the amount of antigen-specific antibodies (IgG and IgE) present in the sera of immunized mice. Second, the effect of insulin pegylation on insulin's ability to interact with anti-insulin IgG antibodies (antigenicity) was assessed by enzyme-linked immunosorbent assay (ELISA) measurements using insulin or its pegylated derivatives as the detecting antigens. Third, the effect of insulin pegylation on delayed-type hypersensitivity reactions (cellular immunogenicity) was investigated by measuring hind footpad swelling in antigen-primed animals. Finally, a passive cutaneous anaphylaxis test was used to determine the effect of pegylation on immediate-type hypersensitivity reactions that include local allergy and systemic anaphylaxis.

Experiments described in this section were performed under the supervision of Professor Blanka Rihova in her laboratories at the Institute of Microbiology of the Czech Academy of Sciences in Prague. The authors wish to explicitly acknowledge

her contribution to these studies. Table 4 shows the insulin species used in these experiments. All of these conjugates were prepared as described in the previous section except G600 (mPEG–A1-insulin), which was prepared as previously described by Uchio et al. [78].

3.1. Humoral immunogenicity and antigenicity

The experimental groups of mice of inbred strain A/J and C57BL/10 were selected (10 mice/per one experimental group/per cage) and 100 μ l of blood was taken as a control before immunization. The preimmunization level of antibodies (so-called natural antibodies) was taken as a baseline and subtracted from all sera subsequently tested. The first immunization in a dose of 5 μ g of sample/mouse/immunization was given intraperitoneally as an alum precipitate (to increase the animals' response to immunization). A second dose was given after 3 weeks and 12 days later the mice were exsanguinated. Serum levels of insulin-specific IgG and IgE antibodies were measured by ELISA indirect double-layer method with antigen-adsorbed microplates, as previously reported [79,80].

It should be noted that in all of the mice immunized with any one of the conjugates, both unmodified insulin (Zn-Ins) and the respective PEG-insulin conjugates (G600, F750, K750, F2000) were used as detecting antigens in the ELISA tests. However, in all of the cases (except when the mice were twice immunized with Zn-Ins) where the conjugates were used as the detecting antigens, no measurable levels of antibodies were measured. Therefore, all of the data presented in this section (with the exception of the antigenicity data) refer to levels of insulin-specific IgG and IgE antibodies detected using unmodified insulin as the antigen.

In both types of mice we tested 12 days after the last immunization, the highest levels of insulin-specific IgG and IgE antibodies were found in the mice immunized solely with unmodified human insulin. Significantly, no insulin-specific IgG or IgE antibody responses were observed in mice immunized at least once F2000.

In C57 mice (high insulin responders), the experimental group immunized with only unmodified insulin produced from 14 to 315 times more insulin-

specific IgG antibodies than any other group studied (Fig. 3a–c). In C57 mice there were at least $10\times$ more insulin-specific IgG antibodies detected in groups immunized with insulin followed by a conjugate (G600, F750, K750, F2000) than in groups immunized in the opposite order. In addition, there were no significant differences in the immunogenicities of low-molecular-weight (600 or 750 Da mPEG) conjugates possessing totally distinct sites of substitution (N-terminus of the A-chain, N-terminus of the B-chain, and C-terminus of the B-chain). The only measurable levels of circulating insulin-specific IgE antibodies found in C57 mice were from those immunized exclusively with unmodified insulin.

In A/J mice (low insulin responders), the ex-

perimental group immunized with only unmodified insulin produced from 8 to $64\times$ more insulin-specific IgG antibodies than any other group studied (Fig. 4a–c). A/J mice immunized with insulin followed by a conjugate always produced higher titers than mice immunized in the opposite order, although the differences were not as distinct as those found in C57 mice. Differences did exist between the low molecular weight mPEG–insulin conjugates with different sites of PEG-substitution, although these differences were not significant.

In general, we found that pegylation of human insulin, irrespective of the site of substitution or molecular weight of mPEG attached, resulted in a 10 – 1000 times reduction in the amount of circulating

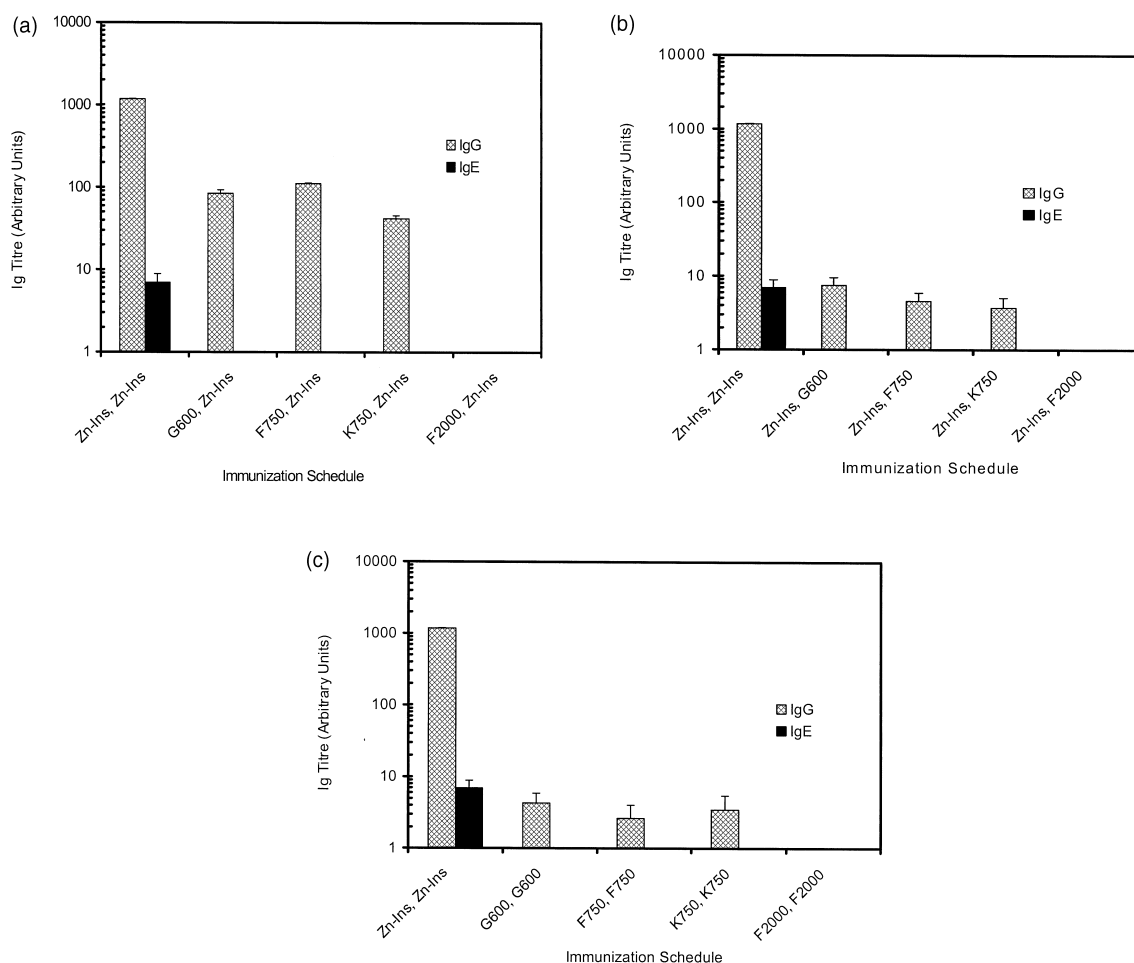


Fig. 3. (a)–(c) Levels of insulin-specific antibodies in C57BL/10 mice following immunization at 0 and 3 weeks.

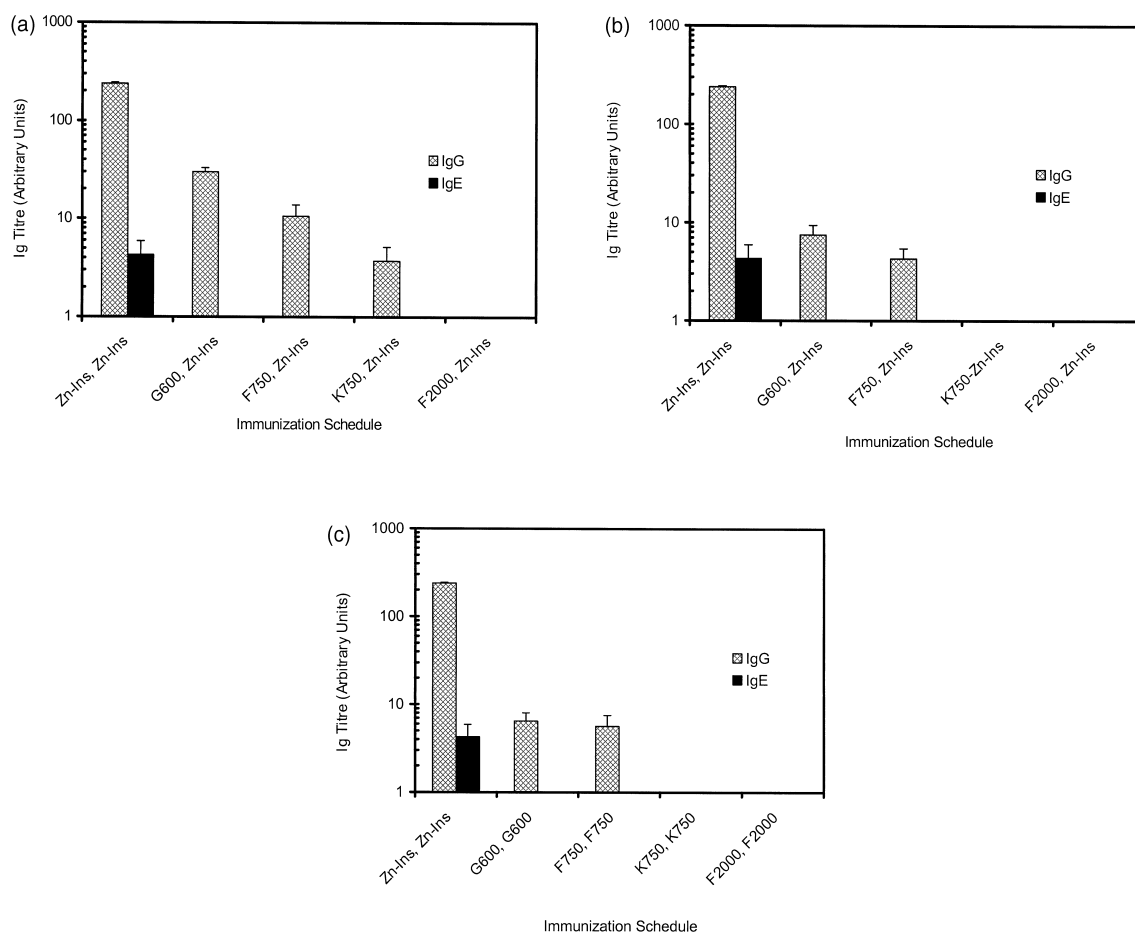


Fig. 4. (a)–(c) Levels of insulin-specific antibodies in A/J mice following immunization at 0 and 3 weeks.

insulin-specific IgG antibodies. These findings are in full agreement with those of other groups who assessed the immunogenicity of pegylated proteins like IL-2 [22], bovine liver catalase [12], bovine serum albumin [81], ovalbumin [77], and hen egg lysozyme [82]. Also, pegylation of recombinant human insulin resulted in the elimination of any detectable IgE antibody response in the test sera measured. Both of these conclusions endorse the concept that pegylation of human insulin results in a nonspecific reduction in the conjugate's immunogenicity. These conclusions are supported by the findings of other groups who have studied the effects of pegylation on the immunogenicity of protein antigens and found that conjugates containing increased amounts (size or number of mPEG chains)

exhibited lower immunogenicities than their less-modified or unmodified counterparts [10,12,22,77,81,83–85].

To assess the antigenicity of the PEG–insulin conjugates relative to unmodified insulin, all of the conjugates (G600, F750, K750, F2000) and Zn-Ins were used as the detecting antigens in ELISA tests used to quantify the amounts of anti-insulin IgG antibodies in those mice who were immunized solely with unmodified insulin. The results of these experiments are given in Table 5 and clearly demonstrate that the covalent attachment of PEG to insulin substantially (by two to three orders of magnitude) diminishes the conjugates' ability to bind to anti-insulin IgG antibodies.

B cell epitopes, or antigenic determinants, are the

Table 5
Antigenicity of insulin (PEG–insulins) in A/J and C57BL/10 mice

Antigen	IgG titer	Relative antigenicity ^a (%)
A/J mice		
Zn-Ins	1176.3±13.93	100
G600	5.66±2.64	0.48
F750	2.14±1.41	0.18
K750	4.0±1.52	0.34
F2000	0	0.0
C57BL/10 mice		
Zn-Ins	238.86±4.92	100
G600	3.73±1.87	1.56
F750	2.83±1.41	1.18
K750	2.30±1.52	0.96
F2000	0	0.0

^a Relative antigenicity of the conjugates determined by comparing IgG titers detected using these antigens relative to titers detected using Zn-Ins as the antigen.

regions on an antigen molecule to which antibodies bind and are usually found on the surface of protein antigens. B cell epitopes on protein antigens are of variable size comprising up to about 20 amino acids. Antibodies bind in a more or less exact three-dimensional fit with an epitope. Thus, the three-dimensional structure of a protein antigen may be essential for antibody binding. Because the production of specific antibodies against protein antigens is mediated through the recognition of conformationally-distinct regions of insulin by membrane-bound antigen receptors (IgM and IgD) on the B cell surface, it can be deduced that the decreased number of circulating antibodies in mice immunized with the PEG–insulin conjugates might be due to a nonspecific ‘masking’ of one or more of insulin’s B cell-recognition epitopes by the polymer. Thus, an increase in the molecular weight or number of PEG chains attached to insulin would be expected to result in a more pronounced steric ‘masking’ effect, as was seen in this study.

A qualitative ranking of the conjugates’ humoral immunogenicity, from highest to lowest, is as follows: G600>F750≈K750>>F2000. From our results there seems to be a direct correlation between a conjugate’s molecular weight of attached mPEG and reduction in its humoral immunogenicity. When the amount of mPEG attached approaches 2000 Da (F2000), the immunogenicity of the conjugate is

virtually nonexistent (e.g., undetectable). Taken together with the fact that a tri-substituted insulin conjugate with 600 Da mPEG attached to positions A1, B1, and B29 [mPEG(600)₃–insulin] did not elicit any measurable immune response in A/J and C57 mice [78], we assert that a threshold amount of mPEG attached/insulin monomer of roughly 2000 Da is necessary for a conjugate to neither elicit immune responses or be recognized by anti-insulin antibodies in the animal models tested here. This assertion is supported by the work of other analyzing the immunological properties of PEG–protein conjugates [12,86,87]. These groups also found that a minimum amount of PEG needs to be coupled to a protein for the conjugate to be rendered nonimmunogenic and nonantigenic. Conversely, we did not observe any relationship between a conjugate’s site of substitution and its immunogenicity/antigenicity.

3.2. Cellular immunogenicity

Cellular immunogenicity of insulin and its pegylated derivatives was examined by measuring hind footpad swelling in mice caused by delayed-type hypersensitivity (DTH) reactions [88]. The term delayed-type hypersensitivity includes the delayed skin reactions associated with type IV hypersensitivity and is defined as an immunologically specific inflammatory reaction involving the infiltration of mononuclear cells that is maximal at 24–48 h after sensitization [89]. On day 14 after the last immunization with insulin or its derivatives, the DTH response was assessed by a hind footpad-swelling test. This assay is widely used for detecting DTH in rodents and measures the gross manifestation of DTH reactions [88,89].

Table 6 gives a summary of the results of the DTH experiment. As seen in the antigen-specific IgG and IgE experiments, there was no DTH reaction in any of the mice immunized with F2000. The most significant DTH response was measured in animals immunized solely with Zn-Ins, but low responses were also observed in animals immunized with unmodified insulin prior to immunization with one of the mPEG–insulin derivatives (G600, F750, K750, F2000). When mice were immunized first with one of the conjugates no measurable DTH reactions occurred, regardless of the type of antigen used in

Table 6
DTH and PCA responses in inbred A/J and C57BL/10 mice immunized with insulin or one of its pegylated derivatives

Immunization schedule	Footpad swelling ^a		PCA ^b	
	C57	A/J	C57	A/J
Zn-Ins, Zn-Ins	84±13	89±29	1.65±0.6	1.12±0.3
Zn-Ins, G600	39±9	29±11	0	0
Zn-Ins, F750	31±14	24±16	0	0
Zn-Ins, K750	41±14	0	0	0
Zn-Ins, F2000	0	0	0	0
Zn-Ins, Zn-Ins	84±13	89±29	1.65±0.6	1.12±0.3
G600, Zn-Ins	0	0	0	0
F750, Zn-Ins	0	0	0	0
K750, Zn-Ins	0	0	0	0
F2000, Zn-Ins	0	0	0	0
Zn-Ins, Zn-Ins	84±13	89±29	1.65±0.6	1.12±0.3
G600, G600	0	0	0	0
F750, F750	0	0	0	0
K750, K750	0	0	0	0
F2000, F2000	0	0	0	0

^a DTH responses were tested on day 14 after the last immunization. Control animals were injected with 0.9% NaCl only. The data are presented as the mean±0.01 mm (differences in thickness between antigen and saline injected footpad)±S.D. of four mice per group.

^b The reaginic titre was read as the reciprocal of the furthest dilution giving a colored spot of at least 5 mm in diameter.

subsequent immunizations. Thus unmodified recombinant human insulin is the only antigen, when intradermally injected into previously sensitized individuals, capable of eliciting strong delayed-type hypersensitivity reactions in the mouse models used in this study.

Because DTH is a T cell-mediated event [88–90], the results of these experiments suggest that there must be differences in the way unmodified insulin and its pegylated derivatives are either recognized, processed intracellularly, or presented to antigen-specific T cells by antigen presenting cells (APCs). Although the mechanism by which the mPEG–insulin conjugates do not evoke DTH responses was not characterized here, we contend that pegylation might nonspecifically ‘mask’ one or more of insulin’s T cell epitopes and thus prevents it from being recognized normally by the cellular immune system. We believe the effects are nonspecific because there were no observable differences in the DTH responses between mice immunized with

conjugates having distinct sites of substitution and sizes of mPEG attached. We do acknowledge that the footpad test, although being widely used for detecting DTH reactions in rodents, can be justifiably criticized for its lack of sensitivity and objectivity.

3.3. Allergenicity

PCA is a skin test that involves the *in vivo* passive transfer of homocytotropic antibodies that mandate type I immediate hypersensitivity (e.g., IgE in man) from a sensitized animal to a previously nonsensitized individual by injecting the antibodies intradermally [80]. After injection, these antibodies become anchored to mast cells through their Fc receptors. This is followed by intravenous injection of antigen mixed with a dye such as Evan’s Blue. Crosslinking of the cell-fixed (e.g., IgE) antibody receptors by the injected antigen induces a type I hypersensitivity reaction in which histamine and other pharmacological mediators of immediate hypersensitivity are released. Vascular permeability factors act on the vessels to permit plasma and dye to leak into the extravascular space, forming a blue area that can be measured with calipers.

In order to confirm the presence of insulin or mPEG-modified insulin specific reaginic (IgE) antibodies, representative samples were also subjected to a PCA test, the results of which are depicted in Table 6. Sera that were positive in the insulin-specific IgE ELISA, e.g., sera from mice immunized with unmodified insulin only, also showed slightly positive PCA results. All other sera that had negative or very low positive responses in the IgE ELISA tests were also negative in the PCA test.

It has been demonstrated that IgE plays an important role in mediating immediate hypersensitivity reactions in response to antigen in humans [91,92] as well as in animal models [93,94]. Allergic reactions of the immediate type are triggered by multivalent allergens on account of their ability to cross-link IgE antibodies fixed to mast cells and basophils and the resultant release of vasoactive constituents from these cells [77]. For pegylated allergens (like insulin) to effectively be used in a clinical setting, it would be desirable that they not only suppress an ongoing IgE response but also be devoid of the capacity to

cross-link IgE antibodies and elicit unwanted allergic reactions.

The elimination of IgE responses to insulin was achieved in all of the cases evaluated here. The only mice, from both models investigated, that demonstrated PCA reactions were those animals that received sera from donor mice exclusively immunized with unmodified insulin (Zn-Ins). Thus it appears that the pegylation of recombinant human insulin, at any of the attachment sites studied, produces conjugates that are non-allergenic and do not elicit immediate-type hypersensitivity reactions. These findings are very much in line with those from another group who examined the allergenicity of pegylated ovalbumin and found that although the native protein stimulated strong PCA reactions the PEG-modified ovalbumin failed to elicit any significant PCA reaction [77,95,96]. The results of the present study are encouraging because they suggest that mPEG–insulin conjugates could be administered to patients who have insulin allergies or high levels of circulating IgE antibodies in large doses without the risk of unleashing systemic anaphylactic reactions.

The present study also attempted to measure the immunogenicity (humoral and cellular), antigenicity, and allergenicity of human insulin covalently-modified with mPEG. The initial hypothesis was that distinct differences would be observed between the immunological properties of conjugates with different sites of substitution (N-terminus of A-chain, N-terminus of B-chain, C-terminus of B-chain) and/or size of polymer attached (600, 750, or 2000 Da). However, the results of these studies indicate that the effects of mPEG-conjugation on insulin's immunological properties are nonspecific in nature. This conclusion was found to hold true in all of the experimental end-points we examined. In the experiments done to characterize the humoral (circulating IgG and IgE levels) and cellular (DTH reaction) immunogenicity of mPEG–insulin derivatives, no significant differences were observed between the conjugates. In addition, no significant differences were observed between the conjugates in their abilities to interact with anti-insulin IgG antibodies. Likewise, when the effects of mPEG-modification on insulin's allergenicity (PCA) were examined, identical results were obtained for all of the conjugates. Thus, our original hypothesis that the site-specific

pegylation of human insulin will result in conjugates with distinct immunological properties proved to be inaccurate.

Because protein antigens generally possess both B cell and T cell epitopes, it appears from the results of these studies that the attachment of low-molecular-weight mPEG (600–2000 Da) to insulin may serve to nonspecifically 'mask' both types of epitopes. Additionally, there did appear to be some molecular weight-dependence observed in the studies evaluating the humoral (B cell-mediated) immunogenicity and antigenicity of the conjugates, but no such dependence was observed in the experiments characterizing either the cellular (T cell mediated) immunogenicity or allergenicity of the conjugates. This suggests that insulin could contain two or more B cell epitopes that are spatially separated and the efficient masking of these epitopes by mPEG requires the polymer to be approximately 2000 Da in size.

Two clinical manifestations of circulating anti-insulin antibodies are postprandial hyperglycemia and extended periods of hypoglycemia, both of which are extremely undesirable. In addition, it is clear that diminishing the immunogenicity and allergenicity of an insulin preparation is an important factor in increasing the therapeutic efficacy of that preparation. These results, although not mechanistically characterized, imply that the pegylation of recombinant human insulin effectively suppresses the immunological properties of this important therapeutic protein in a way that is safe, nontoxic, and previously US Food and Drug Administration (FDA) approved (in other PEG–protein conjugates). Finally, the PEG–insulin conjugates studied here could be safely used in diabetes patients that have pre-existing anti-insulin antibodies due to their extremely low antigenicities.

4. Pharmacological properties of PEG–insulin conjugates

Two PEG–insulin conjugates (F750 and F2000) were injected via both intravenous and subcutaneous routes into male beagle dogs and their biological activities, and pharmacokinetic parameters were compared to Lilly's regular human insulin formula-

tion HumulinR[®] (Hum). Model-independent non-compartmental methods are commonly used to calculate the pharmacokinetic parameters of insulin preparations [97], and in this study we have also utilized this approach to compare the pharmacological properties of insulin and two of its pegylated derivatives. A chronic dog model was used to evaluate the changes in some pharmacokinetic (PK) and pharmacodynamic (PD) parameters due to modification of insulin with 750 or 2000 Da mPEG at residue PheB1. Healthy beagle dogs (13–18 kg were maintained at the Animal Resource Center and given at least 2 weeks between treatments.

Prior to insulin (conjugate) injection, samples of blood were taken to establish the basal levels of insulin (conjugate), canine c-peptide, and glucose. Hum, F750, and F2000 preparations (0.3 U/kg) were administered via i.v. injection into the jugular vein in order to assess total bioavailability of the insulin preparations. The three insulin preparations (0.3 U/kg) were also injected in the nape of the neck of dogs to examine their absorption characteristics from a subcutaneous depot. Serum samples were analyzed for insulin, glucose, and c-peptide content according to the methods described below.

4.1. Biological activity and pharmacodynamic parameters

The serum samples obtained from treated dogs were assayed prior to freezing for glucose concentration. The blood glucose depression abilities of the insulin derivatives after intravenous administration are depicted in Fig. 5. This plot shows that there are some minor differences between the glucose-lowering abilities of native insulin and its pegylated derivatives after intravenous injection into dogs. The onset of action (lowering of blood glucose) and duration of action for HumulinR[®] (Hum), F750, and F2000 are similar which suggests that the pegylation of human insulin does not result in any unfavorable effects on its biological activity in vivo if directly injected into systemic circulation. After intravenous administration of these insulin derivatives the dogs' glucose levels rebounded to their initial levels within about 3 h.

The blood glucose lowering effects of Hum, F750, and F2000 after subcutaneous injection of 0.3 U/kg

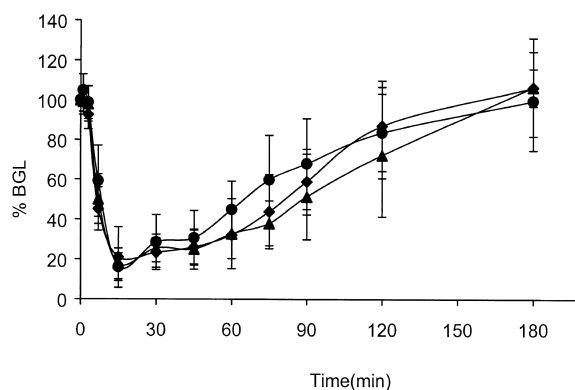


Fig. 5. Blood glucose levels in male beagle dogs following intravenous administration (0.3 U/kg) of HumulinR[®] (♦), or conjugates F750 (▲), or F2000 (●). Data are means $\pm$ S.D., $n = 6$.

are depicted in Fig. 6. The main conclusions that may be drawn from these data are the pegylation of insulin at residue B1 results in a slower onset of glucose depression in the animal model studied. A relationship between time to onset of activity and molecular weight was observed between the two conjugates and insulin. The higher-molecular-weight conjugate (F2000) required the longest amount of time to decrease blood glucose levels, while the lower-molecular-weight conjugate (F750) exhibited an intermediate (between Hum and F2000) amount of time to decrease blood glucose levels. After the initial lag time to full action, the duration of biological activity (ability to lower blood glucose) is comparable between the three insulin derivatives.

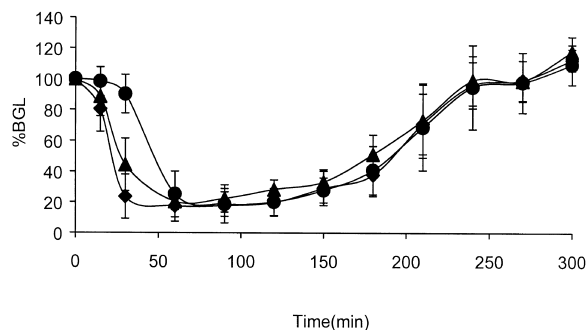


Fig. 6. Blood glucose levels in male beagle dogs following subcutaneous administration (0.3 U/kg) of HumulinR[®] (♦), or conjugates F750 (▲), or F2000 (●). Data are means $\pm$ S.D., $n = 6$.

Thus, the conjugates and unmodified insulin behave similarly once they reach systemic circulation.

Values of the BGL versus time AUC_G were calculated by the trapezoidal method. The nadir BGL (BGL_{nadir} , % of basal value) and the time to BGL_{nadir} (T_{nadir}) were selected to be the pharmacodynamic parameters compared between preparations, and these values were determined from the experimental data. For assessment of the statistical significance, a two-tailed Student's *t*-test (paired two samples means) was used, and mean values were considered to be statistically different when $P < 0.05$.

Table 7 summarizes the pharmacodynamic parameters calculated for the different insulin treatments after intravenous and subcutaneous administration. When the area under the blood glucose depression curves (AUC_G) were calculated for the three derivatives after intravenous or subcutaneous administration, we found that F750, F2000, and Hum elicited the same ($\pm 10\%$) biological effect. Notably, the differences in the biological activities of Hum, F750, and F2000 are statistically indistinguishable from each other; therefore, all intravenous and subcutaneous treatments are considered to be biologically equivalent within the errors of measurement. Pegylation of human insulin did alter the time to onset of biological action (glucose depression) after subcutaneous administration with an increase in the time to onset with increasing molecular weight of mPEG attached.

The equivalent biological activities found between both of the mPEG–insulin conjugates and unmodified human insulin, as measured their ability to lower blood glucose concentrations, are consistent with the findings of another group who examined

similar PEG–insulin conjugates in male Wistar rats and domestic-bred male rabbits [48]. These authors also found that conjugates substituted at position PheB1 with PEG ($M_r = 1500$ Da) were equally capable of lowering the animals' blood glucose levels after intravenous administration. The mentioned authors also determined that their PEG(1500)–PheB1-insulin conjugate was fully bioactive after administration to both male Wistar rats and male domestic-bred rabbits via the subcutaneous route [48]. The fact that two independent research groups came up with the same results in very different animal models suggests that the conjugation of low-molecular-weight PEG ($M_r < 2000$ Da) to insulin at residue PheB1 does not adversely affect its biological activity in vivo.

4.2. Pharmacokinetic parameters

Commercially available human insulin and canine C-peptide radioimmunoassay (RIA) kits were used to measure serum concentrations of insulin (mPEG–insulin conjugates) and endogenous insulin secretion, respectively. Each of the insulin kits used in these studies was carefully validated for the analysis of unmodified human insulin, F750 and F2000 using appropriate reference solutions prepared in insulin-free matrix. Both types of assays were considered valid for the individual treatments if the calculated values of two levels of control samples were found to fall within 2 standard deviations of their known value.

Serum insulin (mPEG–insulin) concentrations were adjusted for endogenous insulin using canine C-peptide concentrations as an indicator of endogen-

Table 7
Summary of dog model in vivo biological activity data for various insulins^a

Parameter	Treatment		
	HumulinR [®]	F750	F2000
Intravenous ^b AUC_G (mg $\times$ min/dl)	9494 $\pm$ 1064 (100%)	9209 $\pm$ 1521 (103%)	10 320 $\pm$ 1642 (92%)
Subcutaneous ^b AUC_G (mg $\times$ min/dl)	8984 $\pm$ 890 (100%)	9998 $\pm$ 591 (90%)	10 053 $\pm$ 1244 (90%)

^a Area under glucose depression curves (AUC_G) calculated by trapezoidal method.

^b Dosages given were 0.3 U/kg. Data are means $\pm$ S.D., $n = 6$. Values in parentheses are percent bioactivity relative to HumulinR[®].

ous insulin secretion. This is necessary because human and canine insulin have identical primary sequences while the primary sequences of their connecting-peptides are distinct. Representative samples of serum from animals injected with unmodified insulin or its pegylated derivatives were assayed for their concentrations of canine C-peptide. Because there were no distinguishable differences in the levels of serum C-peptide observed between animals injected via iv or sc routes and no differences between animals treated with insulin or its pegylated derivatives were observed, average values of serum C-peptide concentrations were used to account for endogenous insulin secretion. Consequently, serum insulin levels depicted in the figures are truly considered to be the levels of exogenously administered insulin (Hum) or its pegylated derivatives (F750 and F2000).

Serum concentrations of insulin (mPEG–insulin conjugates) after correction for endogenous insulin secretion were used to calculate several pharmacokinetic parameters, including apparent volume of distribution (V_z/F), apparent total systemic clearance (Cl/F), terminal rate constant (λ), and half-life ($t_{1/2}$). In addition, the area under the serum insulin (conjugate) versus time curves (AUCs) and MRTs for insulin and its pegylated derivatives were determined. Calculation of these values was performed using the noncompartmental analysis features of WinNonlin™ (Pharsight, USA). For assessment of statistical significance, a two-tailed Student's *t*-test (paired two samples means) was used and mean values were considered to be statistically different when $P < 0.05$.

Fig. 7 shows the serum insulin (PEG–insulin) levels following intravenous administration. Circulating levels of both conjugates (F750 and F2000) were slightly higher than those of Hum at almost every time point after 15-min postinjection, which indicates that the conjugates may be cleared more slowly than Hum. However, the differences in the terminal portions of these pharmacokinetic curves did not generally result in statistically relevant differences in the calculated pharmacokinetic parameters given in Table 8, except in two cases: (1) F750 had a significantly longer elimination half-life than Hum and (2) the mean residence times (provide measure of the average length of time a molecule resides

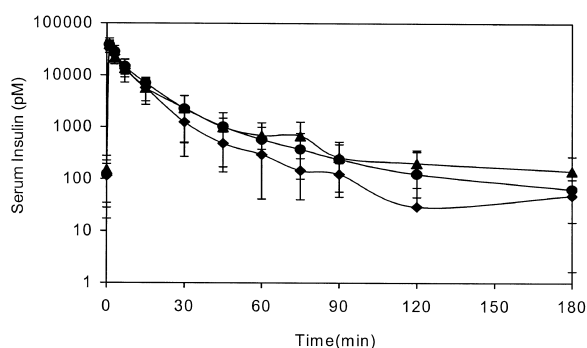


Fig. 7. Serum insulin (mPEG–B1-insulin) levels corrected for endogenous insulin secretion in male beagle dogs following intravenous administration (0.3 U/kg) of HumulinR® (♦), or conjugates F750 (▲), or F2000 (●). Data are means $\pm$ S.D., $n = 5$.

within the body) of both conjugates were significantly longer than Hum after intravenous injection. The increased MRTs of the conjugates relative to Hum might be explained by PEG's ability to interact with cell membranes [6,29]. Caliceti et al. demonstrated that PEG-SOD was able to bind to red blood cells via interaction of the PEG-chains and cell membranes [98]. By interacting with the animals' red blood cells, the conjugates possibly will remain in the circulation longer than unmodified insulin, as seen in our results.

The serum insulin (PEG–insulin derivative) levels following subcutaneous administration of 0.3 U/kg are shown in Fig. 8. This plot provides clear evidence that there are substantial differences in the circulating levels of native insulin (in the form of Hum) and its pegylated derivatives (F750 and F2000) after subcutaneous injection into dogs. Both of the conjugates exhibited increased serum concentrations at most time points tested. As can be seen in Table 9, the conjugates rose to significantly higher C_{max} levels (≈ 1400 pM) than Hum (≈ 1000 pM), and it took longer for the conjugates to achieve their maximum concentrations (T_{max}) than Hum. However, the differences in calculated T_{max} values were not statistically significant. Analysis of the AUC/D values calculated for F750 and F2000 suggest that both conjugates were absorbed to a significantly ($P < 0.02$) greater extent than the Hum insulin hexamers following subcutaneous injection. A clear trend was also observed between the relative availability, defined as $[(AUC/D)_{IV} \div (AUC/D)_{SC}]$, of Hum

Table 8

Dog pharmacokinetic parameters following intravenous administration of 0.3 U/kg insulin or its pegylated derivatives^a

Parameter	Treatment		
	HumulinR [®]	F750	F2000
AUC/dose (min/l)	9.983±1.721	10.051±2.398	10.697±2.282
$t_{1/2}^A$ (min)	12.854±1.899	15.992*±2.183	14.292±2.836
Vss (l)	1.160±0.208	1.645±0.735	1.249±0.287
Cl (l/min)	0.107±0.023	0.099±0.022	0.098±0.026
MRT (min)	12.097±3.447	19.867*±5.537	15.842*±4.730

^a Calculated by noncompartmental methods assuming first-order elimination. Data are means±S.D., $n=5$. $t_{1/2}$ is calculated from the elimination rate constant (λ) using the formula $t_{1/2}=\ln(2)/\lambda$. * denotes $P<0.05$ compared to values calculated for HumulinR[®] using a two-tailed Student's t -test; paired two sample for means.

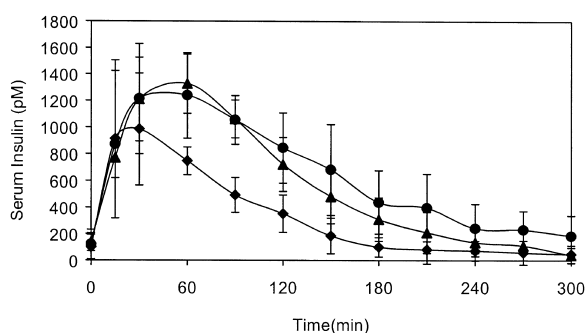


Fig. 8. Serum insulin (mPEG–B1–insulin) levels corrected for endogenous insulin secretion in male beagle dogs following subcutaneous administration (0.3 U/kg) of HumulinR[®] (♦), or conjugates F750 (▲), or F2000 (●). Data are means±S.D., $n=6$.

(0.383), F750 (0.569), and F2000 (0.645) and the molecular weight of PEG attached to insulin. As the molecular weight of PEG attached to insulin increased so did the derivative's relative availability.

These results are not surprising, considering many PEG–protein conjugates have been shown to exhibit improved bioavailability relative to their unmodified forms [29].

Both conjugates had substantially slower terminal clearance rates than Hum and the MRT of F2000 was significantly longer than both F750 and Hum. One of the possible explanations for the differences observed in the clearance rate of the PEG–insulin conjugates and insulin could be that the molecular size of the conjugates is increased relative to insulin, thus decreasing their susceptibility to renal filtration (a process that has been shown to account for nearly 50% of the total clearance of parenterally administered insulin) [99]. Furthermore, it has been reported that the attachment of PEG ($M_r=1500$) to PheB1 of insulin produces conjugates possessing reduced ($\approx 40\%$) in vitro affinities for insulin receptors from rat liver cells and adipocytes [27,48]. The literature clearly states that insulin is primarily cleared through

Table 9

Dog pharmacokinetic parameters following subcutaneous administration of 0.3 IU/kg insulin or its pegylated derivatives^a

Parameter	Treatment		
	HumulinR [®]	F750	F2000
AUC/dose (min/l)	3.822±1.043	5.724*±0.823	6.901*±1.603
$t_{1/2}^A$ (min)	42.31±10.53	53.98±12.47	70.39*#±14.62
V_z/F^b (l)	16.69±4.596	13.57±2.176	15.8±3.527
Cl/ F^b (l/min)	0.282±0.088	0.178*±0.025	0.152*±0.041
MRT (min)	88.25±30.28	97.21±16.62	133.8*#±34.73
C_{max} (pM)	998.8±379.6	1405*±183	1392*±435
T_{max} (min)	40±15.49	50±15.49	75±41.35

^a Calculated by noncompartmental methods assuming first-order elimination.

^b F is the absolute bioavailability of the drug. Data are means±S.D., $n=6$. $t_{1/2}$ is calculated from the elimination rate constant (λ) using the formula $t_{1/2}=\ln(2)/\lambda$. * denotes $P<0.05$ compared to values calculated for HumulinR[®] and # denotes $P<0.05$ compared to values calculated for P750 using a two-tailed Student's t -test; paired two sample for means.

receptor-mediated events and a decrease in the receptor-binding abilities of the mPEG–insulin conjugates used in this study (although not specifically determined) could be another contributing factor to their slower clearance rates following subcutaneous administration. Furthermore, because the conjugates were shown to have minimal antigenicities, clearance via opsonization (antibody binding and consequent recognition of the immune complex by the reticuloendothelial system) mechanisms would be hindered. This effect has been reported in the literature previously for other PEG–protein conjugates [6]. In combination, these three effects of PEG attachment (increased molecular size, decreased receptor-binding ability, and decreased antigenicity) may lead to the statistically significantly ($P < 0.05$) slower clearance rates observed for the conjugates relative to unmodified insulin. Finally, because of PEG's ability to interact with cell membranes, the same arguments can be made for this interaction prolonging the circulation half-lives of these conjugates following subcutaneous administration.

The absorption of insulin from subcutaneous depot is a nonlinear (except for insulin monomers) process that depends on many factors such as the dissolution of insulin oligomers, diffusion of insulin oligomers from the site of injection through capillary walls, local blood flow, and local temperature [66–68]. This makes the analysis of the possible mechanisms responsible for the increased $T_{\max}$ values calculated for PEG–insulin conjugates compared to Humulin very difficult. We assert that the HumulinR[®] formulation used in these studies contains insulin in a hexameric form [68] and the PEG–insulin preparations used in these studies contain insulin conjugates in a dimeric form [53]. However, it is also known that PEG attachment increases the molecular size of the conjugate [29,86] and that PEG substantially interacts with cell membranes [6]. Thus, any increases in subcutaneous absorption rates due to decreases in conjugate association-state can be compensated for by increases in the conjugate's overall size and specific interactions between the PEG portion of the conjugates and the capillary cell membranes. Upon examination of the $T_{\max}$ values in Table 9, it appears (qualitatively) that the latter case overrides the former. That is, the increased molecular

size of the conjugates and the specific interactions of the PEG moiety with capillary cell membranes impede their subcutaneous absorption to a greater extent than the decreased association-state enhances their absorption. Because the differences in $T_{\max}$ values were not statistically significant (due to high levels of variance in the numbers), this argument cannot be proven. However, to reiterate, there does appear to be a clear trend with the value of $T_{\max}$ increasing proportionally with PEG molecular weight.

Helmut Ringsdorf originally proposed using conjugated soluble polymers as drug carriers [100], and by virtue of its low toxicity and FDA approval for use internally, PEG has become one of the most extensively utilized polymers in drug carrier systems today. Specifically, PEG conjugation to therapeutic proteins is a technique commonly used to minimize the effects of these clearance mechanisms and, therefore, increase the proteins' residence times in the circulation without adversely affecting their biological activities. The purpose of this study was to determine the effects of PEG conjugation to human insulin on its biological activity and pharmacokinetic disposition after administration via either intravenous or subcutaneous routes. Results of these studies clearly indicated that the site-specific attachment of low-molecular-weight PEG to insulin at its N-terminal amino acid does not significantly alter the conjugates' biological activities relative to unmodified control samples. Whether the conjugates were administered intravenously or subcutaneously, the ability to lower blood glucose levels in male beagle dogs was within 10% of Lilly's HumulinR[®] rapid-acting soluble insulin preparation. The conjugates did have slightly altered pharmacokinetic profiles compared to Humulin after intravenous injection, and these differences were even more pronounced after subcutaneous injection.

5. Conclusions

Since its introduction almost 80 years ago, insulin still remains the most important therapy for the treatment of diabetes. Attempts to modify insulin to improve its physical, immunological and pharmacological properties have, until recently, achieved

limited success. In addition, it seems an almost insurmountable challenge to obtain efficacious insulin preparations administerable by a nonparenteral route. Many of the problems that insulin therapy encounters are due to the instability intrinsic to insulin. Insulin, along with most other therapeutic peptides and proteins, possesses many unfavorable attributes that make the development of effective therapeutic preparations particularly challenging. These unfavorable attributes include chemical and physical instability, inherent immunogenicity and antigenicity, and rapid clearance from the systemic circulation. The fundamental hypothesis underlying this work was that the site-specific covalent attachment of PEG to human insulin would impart beneficial physical, immunological, and pharmacological properties to this important therapeutic protein. To this end, conjugates with different sites of substitution and molecular weights of PEG attached were prepared. Following purification to homogeneity, a broad-spectrum approach was used to assess the physicochemical, physical, biological, immunological, and pharmacological properties of the conjugates.

Site-specific attachment of low-molecular-weight PEG to insulin via its N-terminal (PheB1) amino group and its penultimate C-terminal ϵ -amino group (LysB29) modified the physical, biochemical, and biological properties of this important therapeutic protein to varying degrees. Specifically, the effects of PEG conjugation on native insulin's conformation, average association-state, and *in vivo* biological activity were minimal. However, when insulin was pegylated at residue PheB1 the resultant conjugates were two orders of magnitude more physically stable.

The conjugates' humoral immunogenicity, cellular immunogenicity, antigenicity, and allergenicity were then examined in two mouse models (A/J and C57BL/10). The attachment of low-molecular-weight PEG to any of the residues resulted in a significant decrease in the conjugates' humoral and cellular immunogenicities, as characterized by circulating IgG and IgE antibody levels and delayed-type hypersensitivity tests, respectively. The PEG–insulin conjugates were unable to elicit any measurable allergic reactions in immunized mice, as determined from circulating IgE antibody levels and the results

of passive cutaneous anaphylaxis tests. We propose that the attachment of PEG to human insulin prevented these conjugates from interacting with anti-insulin antibodies in any substantial manner, as assessed by an ELISA method. These results are very promising in terms of finding a safe method for minimizing the immunogenicity, antigenicity, and allergenicity of recombinant human insulin. Circulating anti-insulin IgG and IgE antibodies can cause unwanted and dangerous problems in diabetes patients and can result in severe systemic anaphylactic reactions. On a more general note, the presence of anti-insulin antibodies negatively affects the clinical efficacy of insulin treatment regimes by causing postprandial hyperglycemia and prolonged hypoglycemia, both of which can lead to more serious complications if left unchecked.

Finally, we investigated the effects of pegylation on insulin's biological activity and pharmacokinetics after intravenous and subcutaneous administration in a dog model. The blood glucose lowering abilities of the conjugates relative to soluble regular human insulin were used to determine the conjugates' bioequivalences. In addition, noncompartmental methods were utilized to calculate several pharmacokinetic parameters, and statistical comparisons were made between the values of the parameters calculated for each treatment group. We found that the blood glucose lowering abilities F750 and F2000 were statistically indistinguishable from insulin after intravenous or subcutaneous administration. Likewise, the terminal clearance rates of F750 and F2000 were also statistically indistinguishable from insulin after intravenous administration, but the pegylated insulin derivatives had significantly slower clearance rates following subcutaneous administration. The elimination half-life of F750 was statistically significantly longer than Humulin after both intravenous and subcutaneous administrations, whereas the half-life of F2000 was significantly longer only in the case of subcutaneous administration. The MRT of F2000 was statistically significantly longer than Humulin after injection via both routes, whereas the MRT of F750 was significantly longer only after intravenous injection. These studies demonstrate there are measurable increases in the circulation half-lives of the PEG–insulin conjugates relative to insulin. However, the data collected in these experi-

ments suffered from a high degree of inter- and intra-subject variability, so more definitive differences in the pharmacokinetic parameters were not observed. Notably, the bioequivalence found between the conjugates and insulin is supported by the work of another group who studied similar conjugates in male Wistar rats and male domestic-bred rabbits [48].

Many unresolved issues remain critical for the development of a clinically feasible insulin substitute in the form of a pegylated derivative. Several issues have not been adequately resolved by studies reported to date, and need to be resolved before these conjugates could be considered for Phase I clinical trials. Some of these issues include: (1) preparation of conjugates with branched PEG species; (2) evaluation of optimized formulation conditions; (3) assessment of chemical stability; and (4) a more comprehensive pharmacological characterization of the optimal conjugate formulations.

Although some beneficial properties were imparted to insulin through covalent attachment of low-molecular-weight PEG species, other conjugates should be prepared using commercially available branched (e.g., PEG₂-Lys) PEG derivatives. These different PEG species, when site-specifically attached to insulin, could conceivably give rise to conjugates with improved physical, immunological, and pharmacological properties compared to the conjugates studied here.

Circulating insulin exists as a complex mixture of monomers, dimers, and hexamers under the influence of a dynamic equilibrium. Furthermore, many variables such as protein concentration, pH, temperature, ionic strength, and the exact composition of the insulin formulation itself influence this dynamic equilibrium. Depending on what properties are desired (prolonged or rapid action, enhanced physical and chemical stability, etc.) in the final product, formulation conditions and compositions will need to be adjusted accordingly.

Another very important issue that *must* specifically be addressed before any candidate PEG–insulin conjugate could be considered for clinical development is chemical stability. Insulin is known to undergo many nonenzymatic reactions including deamidation, β -isomerization, oxidation, covalent dimerization and polymerization, and disulfide ex-

change. It is also known that formulation conditions affect which of these reactions takes place and to what extent. Therefore, careful choosing of optimal formulation conditions will be largely dependent on the findings of real-time chemical stability studies.

Results of this research to date suggest that the site-specific pegylation of recombinant human insulin produces conjugates that are physically more stable, non-immunogenic and nonantigenic, fully bioactive, and cleared from the circulation more slowly following subcutaneous administration. Thus, many of the beneficial properties reported for proteins by the attachment of the water-soluble, nontoxic, and FDA-approved polymer poly(ethylene glycol) were also common to PEG–insulin conjugates. This evidence provides a ‘proof-of-concept’ for the continued development of these conjugates with the hopes that one day their full clinical benefits might be realized.

A recent article by Kozlowski and Harris [101] provides strong testimony that the clinical relevance of pegylated proteins or peptides is finally being realized. Currently two FDA-approved pegylated forms of proteins developed by Enzon (NJ, USA) that are currently on the market: (1) PEG-adenosine deamidase (Adagen[®]) used for the treatment of severe combined immunodeficiency syndrome (SCIDS), and (2) PEG-asparaginase (Oncospar[®]) marketed for treatment of acute lymphoblastic leukemia [101]. In addition, several pegylated proteins are currently in advanced-stage clinical trials and it is expected that some of these conjugates will receive approval within the next year. Examples of these conjugates include soluble PEG-TNF-receptor from Amgen [102], a pegylated antibody fragment that binds TNF- α from Celltech [103] for the treatment of rheumatoid arthritis, PEG-G-CSF from Amgen to be used as an adjuvant for chemotherapy of breast cancer, PEG- α -IFN from Shering-Plough [104,105] and Hoffmann-La Roche [106,107] for the treatment of hepatitis C, and PEG-hGH receptor agonist from Pharmacia-Sensus [108] for the treatment of acromegaly. In light of the recent progress being made in the area of protein pegylation, this technique seems to be gaining acceptance from both the pharmaceutical industry and federal regulatory agencies as an advantageous method of increasing the therapeutic efficacy of protein pharmaceuticals.

Acknowledgements

We wish to thank Drs. Miroslav Baudys, Blanka Rihova, and Feng Liu for their valuable contributions to this project. This work was supported by NIH grant DK-50557.

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