Transferon™, a peptide mixture with immunomodulatory properties is not immunogenic when administered with various adjuvants

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ABSTRACT

Transferon, a human dialyzable leukocyte extract (hDLE), is a biotherapeutic that comprises a complex mixture of low-molecular-weight peptides (<10 kDa) and is used to treat diseases with an inflammatory component. Some biotherapeutics, including those composed of peptides, can induce anti-drug antibodies (ADA) that block or diminish their therapeutic effect. Nevertheless, few studies have evaluated peptide-derived drug immunogenicity. In this study, the immunogenicity of Transferon was examined in a murine model during an immunization scheme using the following adjuvants: Al(OH)₃, incomplete Freund’s adjuvant (IFA), or Titermax Gold. The inoculation scheme entailed three routes of administration (intraperitoneal, Day 1; subcutaneous, Day 7; and intramuscular, Day 14) using 200 μg Transferon/inoculation. Serum samples were collected on Day 21. Total IgG levels were quantitated by affinity chromatography, and specific antibodies against components of Transferon were analyzed by dot-blot and ELISA. Ovalbumin (OVA, 44 kDa) and peptides from hydrolyzed collagen (PFHC, <17 kDa) were used as positive and negative controls, respectively, in the same inoculation scheme and analyses for Transferon. OVA, PFHC, and Transferon increased total IgG concentrations in mice. However, only IgG antibodies against OVA were detected. Based on the results, it is concluded that Transferon does not induce generation of specific antibodies against its components in this model, regardless of adjuvant and route of administration. These results support the safety of Transferon by confirming its inability to induce ADA in this animal model.

Introduction

Human dialyzable leukocyte extracts (hDLE) are complex mixtures of low-molecular-weight peptides (<10 kDa). These biotherapeutics are generated from the lysis and subsequent dialysis of the leukocyte fraction (buffy coat) of blood from healthy donors, obtained from a certified blood bank (Medina-Rivero et al. 2014, 2016). hDLE have a demonstrated value as coadjuvants in treating diseases with an inflammatory component (Pizzialonga et al. 1996; Berrón-Pérez et al. 2007; Hernandez et al. 2013), such as herpes zoster infections (Estrada-Parra et al. 1998) and atopic dermatitis (Flores Sandoval et al. 2005), based on their immunomodulatory properties. Though mechanisms of action of hDLE are not fully understood, they have been associated with: toll-like receptor (TLR) stimulation (Garcia-Hernandez et al. 2014); induction of transcription factors and second messengers in γδ T-lymphocytes (Herlin et al. 1981); and, modulation of tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and interleukin (IL)-6 levels in human and mouse serum (Hernandez et al. 2013; Salinas-Jazmín et al. 2015).

Transferon is an hDLE manufactured by Unidad de Investigación, Desarrollo e Innovación Médica y Biotecnológica (UDIMEB) under good manufacturing practices (GMP) (Medina-Rivero et al. 2016). The active substance of Transferon and glatiramer (drugs that comprise four naturally occurring amino acids in a complex co-polymeric mixture) is a hetero-molecule that consists of closely-related peptide entities (Crommelin et al. 2015; Medina-Rivero et al. 2016). Thus, the quality, efficacy, and safety of these products must be examined using well-designed physicochemical and biological analyses that describe their overall components. Though characterizing the physicochemical/biological properties of Transferon has been difficult, studies in our laboratories have determined the peptide nature of Transferon (Medina-Rivero et al. 2016), its batch-to-batch consistency (Medina-Rivero et al. 2014), and biological activity (Salinas-Jazmín et al. 2015). Nevertheless, its immunogenic effects remain unknown.

Immunogenicity refers to an ability of any substance (primarily proteins) to elicit an immune response in a host and involves induction of antibodies against the “antigen” itself. Immunogenicity to protein- and peptide-derived biotherapeutics is undesirable, because this process might generate antidepressant antibodies (ADA) (van de Weert and Horn Moller 2008; Diao and...
Meibohm 2013). The effects of ADA range from decreasing the efficacy of a biotherapeutic to initiating an autoimmune disease; thus, an induction of ADA is a major concern in the development of biotherapeutics (Kessler et al. 2006; Bomprezzi et al. 2011; Wang et al. 2012). To examine and prevent such adverse effects, international health agencies have established guidelines for evaluating the immunogenicity of biotherapeutics (Committee for Medical Products for Human Use (CHMP) 2007; Secretaria de Salud México 2014; U.S. Department and Human Services 2014).

Peptide-derived drugs are a fast-growing segment in the pharmaceutical and food industries. Consequently, analysis of their safety, including their immunogenicity, has become critical for industry and regulatory agencies (Wu et al. 2017). Generally, peptides are regarded as poorly immunogenic due to their small size that causes them to be readily metabolized without triggering immunogenic events (Craik et al. 2013). Two anti-microbial peptides, Bacteriocin TSU4 and BLS P34, do not evoke immunogenicity in a BALB/c mouse model (Vaucher Rde et al. 2011; Sahoo et al. 2017). In contrast, the GLP-1 human analog taspolutide elicits a significant hypersensitivity reaction likely related to ADA induction (Diao & Meibohm 2013). In addition, Copaxone™ – a complex mixture of 5–9-kDa synthetic peptides with immunomodulatory properties and the reference for glatiramoid development, induces ADA in multiple sclerosis patients, although the clinical significance of this effect has not been determined (Brenner et al. 2001; Bomprezzi et al. 2011; Weinstein et al. 2015).

Experimental strategies should be formed to accelerate/maximize ADA induction during development of peptide-derived biotherapeutics. Generally, immunogenicity assays of peptides consider the inoculation of the antigen with only one adjuvant by one route of administration (Vaucher Rde et al. 2011; Sahoo et al. 2017). In this study, the immunogenicity of Transferon using three well-known adjuvants, i.e., Al(OH)₃, incomplete Freund’s adjuvant (IFA), or Titermax Gold in a 21-day immunization scheme - by three routes of administration [intrapерitoneal, subcutaneous, intramuscular], was analyzed.

Material and methods

Transferon samples

Transferon batches were manufactured using a modified version of the Borkowsky and Lawrence (1981) method, as described in Medina-Rivero et al. (2016). Samples were obtained from three standard Transferon batches (13D15, 13E16, 13E17) that met the safety and efficacy criteria of UDIMEB and Mexican Health authorities. These quality controls included: (a) total protein content (0.4 mg/ml ± 15%) by bicinchoninic acid method (Medina-Rivero et al. 2014); (b) measurement of endotoxin levels (< 0.4 UE/ml) using an Endosafe Portable Test System (Charles River Laboratories, Charleston, SC) (Farmacopea de los Estados Unidos Mexicanos 2011a); (c) microbiological content (< 10 CFU), according to the Mexican pharmacopoeia (Farmacopea de los Estados Unidos Mexicanos 2011b); (d) identity test, comparing the SE-UPLC profile of each batch versus that of the reference standard of Transferon (Medina-Rivero et al. 2014), as shown in Figure 1; and, (e) biological activity assessment measured as improvement in survival in mouse model of herpes simplex virus (HSV)-1 infection (Δ survival >40%) (Salinas-Jazmin et al. 2015).

Peptides from hydrolyzed collagen (PFHC)

PFHC were used as a negative control and prepared per an in-house method. Briefly, a solution of bovine collagen (25 g/L) (Sigma, St. Louis, MO) was mixed with a bromelain solution (1 mg/ml) (Sigma) at a ratio of 40:1. Both solutions were prepared in 50 mM citrate buffer [pH 6.5]. The resulting solution was incubated at 37°C for 2h with constant stirring before being ultra-filtered through a 10-kDa Hydro cassette (Sartorius, Goettingen, Germany) in a SartoJet system (Sartorius) and

Figure 1. Identity test of Transferon batches in the immunization scheme. Comparison of SE-UPLC profiles of Transferon batches vs. the standard of the same product. The chromatographic profiles of batches 13E17, 13E16, and 13D15 are the same, and all of them exhibit peaks with a molecular weight below 17 kDa compared with a molecular weight reference (MWR): [1] thyro-globulin (670 kDa), [2] γ-globulin (158 kDa), [3] ovalbumin (44 kDa), [4] myoglobin (17.0 kDa), and [5] vitamin B12 (1.35 kDa).
analyzed by SE-UPLC under the same analytical conditions as the Transferon batches (Medina-Rivero et al. 2014). Figure 2 shows a representative chromatographic profile of the PFHC.

**Sample preparation**

Transferon and PFHC samples were freeze-dried using a freeze dry system (Labconco; Kansas City, MO). Lyophilized samples and OVA (Sigma) were then reconstituted in isotonic saline solution (ISS). Each sample was assayed for endotoxin and total protein content [bicinchoninic acid]. Thereafter, the concentration was adjusted to 10 mg/ml in ISS, and each antigen (Transferon, OVA, or PFHC) was then mixed with a given adjuvant at a 1:1 ratio (v/v).

**Animals**

For the studies, BALB/c mice (male, 6–8-week-of-age, 15–20 g) were obtained from Ferandelh (Mexico City, Mexico). All mice were housed in a P/NC IVC system (Allentown Inc., Allentown, NJ) in a facility maintained at 22 ±2°C with a 55 ±10% relative humidity and a 12-h light/dark cycle. Mice had ad libitum access to standard chow (Harlan Labs, Indianapolis, IN) and filtered water. All procedures were performed according to Mexican guidelines on the use and care of laboratory animals and International Guide for the Care and Use of Laboratory Animals (Secretaría de Salud México 1999; National Research Council of the National Academies 2011). All the procedures were approved by the CIPFT research committee under code FTU/DF 015/001/PRO. All efforts were made to minimize suffering and reduce numbers of mice used.

**Immunization scheme**

Mice were randomly allocated into three blocks of seven experimental groups (n=6 mice/group). Each block was inoculated with aluminum hydroxide [Al(OH)₃], IFA, or Titermax Gold (all adjuvants were purchased from Sigma) per the following scheme: batch 13D15 + adjuvant (G1), batch 13E16 + adjuvant (G2), batch 13E17 + adjuvant (G3), OVA + adjuvant (G4), PFHC + adjuvant (G5), adjuvant alone (G6 control), and ISS (G7 control). Immunogenicity of Transferon, PFHC, and OVA in the mice was analyzed using an immunization scheme (Figure 3) comprised of intraperitoneal (Day 0), subcutaneous (Day 7), and intramuscular (Day 14) administrations of 40 µl antigen + adjuvant mixture (200 µg total protein/mouse/administration). On Day 21 after the first administration, blood samples were collected from the facial vein into Microtainer tubes (BD Biosciences, Franklin Lakes, NJ). Serum samples were obtained by centrifugation (670 x g, 15 min, 4°C) and stored at −70°C until analysis (Note: none of the experimental animals exhibited adverse effects or died during the immunization scheme).

**Total IgG quantification**

Serum total IgG was quantified by affinity chromatography in Day 21 serum samples that were thawed and pooled prior to...
analysis. In brief, pooled serum (20 μl) was diluted with 25 mM phosphate/100 mM NaCl buffer (pH 6.8), then filtered through a 0.10-μm PVDF membrane (Merck Millipore, Germany), and injected [in triplicate] into an Acquity UPLC system (Waters, Milford, MA) using a Poros A/20 affinity column (2.1 mm x 30 mm) (Applied Biosystems, Foster City, CA). IgG from serum proteins was retained at 1.3 ml/min using 25 mM phosphate/100 mM NaCl buffer [pH 6.8] (Phase A) and eluted using a 0.5 M aqueous acetic acid solution [pH 2.8] (Phase B). Column and sample temperatures were maintained at 28°C and 10°C, respectively. Signals were detected by UV/VIS at 280 nm in a UV/vis spectrophotometer (Waters); Empower software (Waters) was used for data analysis. Sample concentrations were estimated against a quantification curve of a murine IgG standard (BD Biosciences, San Jose, CA). The total IgG concentration from the control group that was inoculated with ISS was subtracted from each treatment group to determine absolute increases.

**Measures of ADA by dot-blot**

Specific antibodies against Transferon were detected by dot-blot assay. Because the components of Transferon and PFHC are low-molecular-weight peptides, we use 0.22-μm PVDF Immobilon strips (Millipore). The dot-blot assay consisted of seven strips, which were identified as G1–G7, vide infra. After membrane activation, five dots were placed on each strip: Transferon 50 μg (1), Transferon 100 μg (2), Transferon 200 μg (3), OVA 0.75 μg (4), and PFHC 164 μg (5). Batch 13D15 was used to prepare strip G1, batch 13E16 was used to prepare strips G2 and G4–G7, and batch 13E17 was used to prepare strip G3. The strips were blocked with a 5% skim milk solution (BD Biosciences) for 30 min at room temperature, then incubated with the respective experimental group serum (strip G1 with serum from group G1, strip G2 with serum from group G2, etc.) at 1:6000 for 30 min at room temperature.

Biotinylated goat anti-mouse IgM and IgG (ThermoFisher Scientific, Waltham, MA) and streptavidin-horseradish peroxidase (SAv-HRP) (BD Biosciences) (diluted 1:10,000 and 1:1000, respectively) were incubated with the strips for 30 min at room temperature; the dilution factors for serum and detection antibodies were determined earlier by titration. The strips were washed three times (5 min, room temperature) with 1X TBS Tween solution between each incubation. Finally, the strips were washed four times with 1X OptEIA solution (BD Biosciences). The specific antibodies were then detected by addition of biotin-tagged polyclonal anti-mouse IgG/IgM (H + L) antibody (1:2500, ThermoFisher) followed by streptavidin-horseradish peroxidase (HRP, BD Bioscience). The colorimetric reaction was halted after 5 min by addition of 100 μl stop solution (1 N H2SO4) to each well (BD Biosciences). End-titers were reported as ELISA units (EU/mL) and were estimated as described elsewhere (Glenn et al. 2000; Martínez-Becerra et al. 2012).

Briefly, the quantification range with linear behavior was established from 0.2–1.4 optical density (OD) values at 450 nm/570 nm in an EPOCH plate reader (BioTek, Winooski, VT) using a quantification curve generated with a positive serum. The reciprocal of the dilution of the serum, which had a value of 0.2 by interpolation, was established as the lower limit of quantification and was considered as the cutoff point for a positive response.

**Statistical analysis**

Data were analyzed using Prism software (v.6.0 for Windows, GraphPad, La Jolla, CA). For total IgG quantification, the homogeneity of variance test was used, followed by a one-way analysis of variance (ANOVA) with a Bonferroni’s post-hoc, to compare differences between means. Statistical significance was accepted at p < 0.05. Data are presented as means ± SD.

In the same analysis, comparisons were made: between groups that were administered only adjuvants, in the OVA + adjuvant groups, and in the OVA + adjuvant vs. the respective Transferon + adjuvant group. Total IgG Concentrations were also compared in the Transferon + Titermax Gold vs. the group that was only administered Titermax Gold, because only Transferon + Titermax Gold elicited an increase in total IgG.

**Results**

**Total IgG**

To determine immunogenicity of Transferon, three batches of this hemoderivative were admixed individually with commercial adjuvants and administered to BALB/c mice. Systemic humoral responses were subsequently measured in the host serum by affinity chromatography. Adjuvants nonspecifically increase circulating antibody levels (Apostolico Jde et al. 2016), an effect observed in control groups that were given adjuvant alone (Figure 4). Thus, inoculation with Transferon, OVA, or PFHC with adjuvants also raised total IgG levels with respect to that in the ISS controls. Total IgG peaked in animals that were administered OVA (positive control) with the various adjuvants:IFA, 3.04 ± 0.02 mg/ml; Titermax Gold, 1.91 ± 0.03 mg/ml; and, Al(OH)₃, 1.67 ± 0.04 mg/ml. Induction of total IgG due to inoculation with the same antigen (PFHC and Transferon) also differed between adjuvants (F = 1556; df = 8.2, p < 0.0001). Only Transferon + Titermax Gold increased IgG concentrations vs. its control (1.82 ± 0.10 vs. 0.83 ± 0.03 mg/ml; F = 271.5; df = 17.5, p < 0.001).

**Detection of ADA**

Although total IgG levels rose in all experimental groups (antigen + adjuvant and adjuvant alone), the clinical relevance of this increase depends on a presence of specific antibodies, such as IgG and IgM. Specific antibodies against Transferon were
detected in sera from mice that were treated with Transferon + adjuvants (Day 21) by dot-blot.

Specific antibodies were only observed in groups given OVA + adjuvant, the signal of which was defined as 100% in OD units (Figure 5). In contrast, groups treated with PFHC + adjuvants failed to develop a response, consistent with findings that suggest that PFHC does not induce generation of antibodies against its components (Estrada-Garcia et al. 2016). All groups inoculated with Transferon + adjuvant also lacked specific antibodies, despite experiencing an increase in total IgG concentrations. Considering dots of Transferon and OVA were placed on the same strip, reactivity between anti-Transferon serum and OVA and between anti-OVA and Transferon serum was examined (Supplemental Figure S1). Sera cross-reactivity was not seen.

Specific antibodies against Transferon were also measured by ELISA (Figure 6). The groups inoculated with Transferon + adjuvant and PFHC + adjuvant harbored undetectable titers (0 UE/ml) of specific antibodies, like the ISS control. In contrast, groups that were inoculated with OVA + adjuvant induced high levels of specific antibodies, ranging from 90,000 to 702,000 UE/ml. The OD values of the ELISA analysis, from which UE/ml were calculated, are reported in Supplemental Figure S2. Overall, these results indicate that the low-molecular-weight peptides in Transferon are minimally immunogenic in the current model, even after being administered in repeated doses through routes of administration with various adjuvants.

Discussion

Proteins have myriad activities in living organisms. Today, the use of protein-derived drugs is becoming more significant, because they are effective and specific therapies for chronic degenerative diseases. Therapeutic proteins also have fewer adverse effects than drugs that are generated by chemical synthesis (Flower 2009). Further, proteins are the most immunogenic particles among all biomolecules, and can induce immunogenic responses when administered. This response is characterized by the generation of ADA that recognize components of the active pharmaceutical ingredient (API) and – depending on their titers and affinity – can compromise the safety and efficacy of the therapeutic protein (Fogdell-Hahn 2015).

Peptide-derived drugs have emerged as a new pharmaceutical option for the treatment of various diseases – these biomolecules have high potency and selectivity, a broad range of targets, potentially lower toxicity than small molecules, low accumulation in tissues, and high biological and chemical diversity (Craik et al. 2013). In addition, peptides meet the criteria of poorly immunogenic particles: low molecular weight, low structural complexity, and high solubility (Estrada-Garcia et al. 2016). Nevertheless, there is clinical evidence that certain complex peptide mixtures promote the generation of ADA (Brenner et al. 2001; Bomprezzi et al. 2011). Brenner and colleagues reported the presence of ADA in patients who were treated for at least 6 months with glatiramer acetate, although later reports suggested that its therapeutic efficacy was unaltered (Teitelbaum et al. 2003).

hDLE, such as Transferon, are complex peptide-derived biotherapeutics that are used to treat immunological diseases, but their immunogenicity has not been evaluated. To determine whether the components of Transferon are immunogenic, in a murine model, we administered adjuvants and high doses of Transferon (Tuteja et al. 2007; Bomprezzi et al. 2011; Zhang et al. 2011; Feng et al. 2012), up to 200-times that used in mice. In addition, several routes of administration were used to improve antigenic recognition by antigen-presenting cells (APC), T- and B-cell cooperation, and the inflammatory environment. Intraperitoneal inoculation favors the activation of intraperitoneal macrophages, subcutaneous administration promotes exposure to
Figure 5. Immunization with Transferon + adjuvants does not promote generation of detectable levels of specific antibodies by dot-blot. Induction of specific IgM and IgG antibodies was evaluated using Day 21 serum samples. (A) Design of dot-blot analysis included seven strips (G1-G7) with five dots of antigens each: PFHC, OVA, and three levels of Transferon. Strips were incubated with respective serum from experimental groups: G1 with 13D15 + adjuvant group, G2 with the 13E16 + adjuvant group, G3 with the 13E17 + adjuvant group, G4 with OVA + adjuvant group, G5 with PFHC + adjuvant group, G6 with adjuvant alone group, and G7 with ISS group. The same dot-blot array was used to analyze immunogenicity of Transferon with each adjuvant. Specific antibodies were not detected at 1:6000 after 21 days of inoculation in groups that were immunized with Transferon plus: (B, C) Al(OH)₃, (D, E) IFA, and (F, G) Titermax Gold. PFHC (negative control) also failed to induce specific IgG antibodies in any group. Conversely, OVA (positive control) generated specific antibodies in all groups; its intensity was defined as 100% for the optical density (OD) analysis. Strips were over-exposed intentionally during data acquisition to evince any signal in groups inoculated with Transferon.
dendritic cells, and intramuscular injections facilitate diffusion of a drug into nearby lymphatic nodes for recognition by lymphocytes B (Apostolico Jde et al. 2016). Thus, all cells involved in antigen recognition were stimulated to generate IgM and IgG against Transferon components. Additionally, it has been reported that recombinant human IFNγ (hIFNγ) induces a higher immunogenic response in wild-type (FVB/N) mice than in hIFNγ-immune tolerant mice (Hermeling et al. 2005). Thus, the inoculation scheme proposed using wild-type BALB/c mice has the practicality of favoring immune responses towards OVA, PFHC and the components of Transferon.

OVA was used as a positive control, because its structure and size (≈ 45 kDa) are more complex than the components of PFHC and Transferon, therefore, OVA is an excellent immunogen. As expected, OVA + adjuvants increased the total IgG concentration compared with the respective adjuvants; among the types of IgG generated, specific anti-OVA antibodies were detected. In contrast, no specific antibodies against components of PFHC were observed, although total IgG levels also rose.

Unlike Bomprenzi et al. (2011) who detected antibodies against the components of glatiramer in a mouse model (subcutaneous administration, 7 days), the current study was unable to identify specific antibodies against Transferon using two sensitive immunoassays. This was completely unexpected, considering that our immunization scheme favors the triggering of immunogenic responses against Transferon. Serum interferences with the ELISA assay were also considered. Transferon cannot be described with classic pharmacokinetics since it is composed of several peptides. However, taking into account that the peptide components of Transferon are natural-occurring and lack chemical modifications that could increase their permanence in sera, their half-lives are expected to be on the scale of only hours. As such, collecting serum samples 7 days after the final Transferon immunization ensures an absence of interference between circulating Transferon peptides and the ELISA measurements.

It is worth noting that total IgG increments were observed in all the experimental groups, including those given adjuvant alone. Adjuvants not only decrease the solubility and serve as a depot for antigen but, depending on the adjuvant used, they can activate some pathways of the innate immune system. For example, some aluminum-containing adjuvants activate inflammasome NLRP3/NALP3 complexes, some adjuvants composed of water-in-oil emulsions enhance phagocytosis, leukocyte infiltration, and cytokine production, and some polymer-containing adjuvants can activate various TLR (Apostolico Jde et al. 2016; Scheibelhofer et al. 2017). The mechanisms noted above are likely responsible for the increases in total IgG observed here in all the experimental groups, regardless of the antigen used.

The differences in immunogenic properties between hDLEs and glatiramoids might be attributed to their polydispersity, i.e. whereas the molecular weight of the peptides in Transferon does not exceed 10 kDa, the poly-dispersion of glatiramer acetate ranges from 2 to 20 kDa (Weinstein et al. 2015). In addition, immunogenicity not only depends on the size, but also the structural complexity (i.e. sequence), folding, biological origin, and the content of any aggregates or impurities (Wang et al. 2012; Scheibelhofer et al. 2017). Considering that Transferon did not promote generation of specific IgG despite its human origin and the experimental conditions, we postulate the peptide components of this immunomodulator do not have complex structures and they are not prone to aggregation.

**Experimental limitations**

Non-human primates are among the most suitable models for assessing immunogenicity of human therapeutic proteins. However, they are expensive and their use is restricted by understandably-strict bioethics requirements. Accordingly, to open the field of immunogenicity assessment to a wider array of laboratories, a murine model was used as a first stage for the immunogenic characterization of Transferon. Building on findings here, future studies should and will employ biological models with a genetic background closer to that of humans.

**Conclusions**

The administration to mice of high doses of Transferon with adjuvants elicited an immune response that was mediated by an increase in total IgG. However, specific antibodies against this biotherapeutic were not detected. These results suggest that Transferon, a human DLE, is a poorly immunogenic biotherapeutic. The results of this study results lend support to claims of the safety of this type of immunomodulant.

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Disclosure statement
All the authors declare no conflicts of interest. The authors alone are responsible for the content of this manuscript.

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References
Apostolico Jde S, Lunardelli V, Coirada F, Boscardin S, Rosa D. 2016. Adjuvants: classification, modus operandi, and licensing. J Immunol Res. 2016:1459394
Berron-Perez R, Chavez-Sanchez R, Estrada-Garcia I, Espinosa-Padilla S, Cortez-Gomez R, Serrano-Miranda E, Ondarza-Aguilera R, Perez-Tapia M, Pineda Olivera B, Jimenez-Martinez Mdel C, et al. 2007. Indications, usage, and dosage of the transfer factor. Revista Alergia Mexico (Tecamachalco, Puebla, Mexico: 1993). 54:134–139.
Brompezi R, Schafer R, Reese V, Misra A, Vollmer T, Kala M. 2011. Glutaminyl acetate-specific antibody titres in patients with relapsing/remitting multiple sclerosis and in experimental autoimmune encephalomyelitis. Scand J Immunol. 74:219–226.
Borkowsky W, Lawrence H. 1981. Deletion of antigen-specific activity from leukocyte dialysates containing transfer factor by antigen-coated polystyrene. J Immunol. 126:486–489.
Brenner T, Arnon R, Sela M, Abramsky O, Meiner Z, Riven-Kreitman R, Tarric N, Teitelbaum D. 2001. Humoral and cellular immune responses to Co-polymer 1 in multiple sclerosis patients treated with Copaxone. J Neuroimmunol. 115:152–160.
Committee for Medical Products for Human Use (CHMP). 2007. Guideline on immunogenicity assessment of biotechnology-derived therapeutic proteins. London: European Medicines Agency (EMA); p. 18.
Craig D, Fairlie D, Liras S, Price D. 2013. The future of peptide-based drugs. Chem Biol Drug Des. 81:136–147.
Crommelin D, de Vlieger J, Mühlbacher S. 2015. Introduction: defining the position of non-biological complex drugs. In: Crommelin D, de Vlieger J, editors. Non-biological complex drugs. New York: Springer International Publishing; p. 1–7.
Diao L, Meibohm B. 2013. Pharmacokinetics and pharmacokinetic-pharmacodynamic correlations of therapeutic peptides. Clin Pharmacokim 52:855–888.
Estrada-Garcia I, Ruiz-Sánchez B, Talavera-Paulin M. 2016. Antigens and Immunogens. In: Pavon L, Jimenez-Martinez M, García-Alvarez M, editors. Inmunología Molecular Celular y Translacional. Barcelona: Wolters Kluwer; p. 150–159.
Estrada-Parra S, Nagaya A, Serrano E, Rodríguez O, Santamaría V, Ondarza R, Chavez R, Correa B, Monges A, Cabezas R, et al. 1998. Comparative study of transfer factor and acyclovir in the treatment of herpetic zoster. Int J Immunopharmacol. 20:521–535.
Farmacopea de los Estados Unidos Mexicanos. 2011a. MGA-0316, Determination of bacterial endotoxin. In: Farmacopea de los Estados Unidos Mexicanos. 10th ed. Mexico City: Secretaría de Salud México.
Farmacopea de los Estados Unidos Mexicanos. 2011b. MGA-0571, Determination of bacterial content. In: Farmacopea de los Estados Unidos Mexicanos. 10th ed. Mexico City: Secretaría de Salud México.
Feng X, Liu Q, Cao R, Zhou B, Zhang Y, Liu K, Liu X, Wei J, Li X, Chen P. 2012. Characterization and immunomodulatory function comparison of various bursal-derived peptides isolated from the humoral central immune organ. Peptides. 33:258–264.
Flores Sandoval G, Gomez Vera J, Orea Solano M, Lopez Tiro J, Serrano E, Rodriguez A, Rodriguez A, Estrada Parra S, Jimenez Saab N. 2005. Transfer factor as specific immunomodulator in the treatment of moderate-severe atopic dermatitis. Revista Alergia Mexico (Tecamachalco, Puebla, Mexico: 1993). 52:215–220.
Flower D. 2009. Advances in predicting and manipulating the immunogenicity of biotherapeutics and vaccines. BioDrugs. 23:231–240.
Fogdell-Hahn A. 2015. Anti-drug antibodies: B-cell immunity against therapy. Scand J Immunol. 82:184–190.
Garcia-Hernandez U, Robledo-Avila F, Alvarez-Jimenez V, Rodriguez-Cortes O, Wong-Baeza I, Serdin-Lopez J, Aguilar-Anguiano L, Estrada-Parra S, Estrada GI, Perez-Tapia S, et al. 2014. Dialyzable leukocyte extracts activate TLR-2 on monocytes. Nat Prod Commun. 9:853–856.
Glenn G, Taylor D, Li X, Frankel S, Montemarano A, Alving C. 2000. Transcutaneous immunization: A human vaccine delivery strategy using a patch. Nat Med. 6:1403–1406.
Herlin T, Jensen J, Thstrup-Pedersen K, Zacharias H. 1981. Dialyzable leukocyte extract stimulates cAMP in T γ-lymphocytes. Allergy. 36:337–343.
Hermeling S, Aranha L, Damen J, Slipjer M, Schellekens H, Crommelin D, Jiskoot W. 2005. Structural characterization and immunogenicity in wild-type and immune tolerant mice of degraded recombinant human IFNα2b. Pharm Res. 421:94–98.
Hernandez M, Mendilera D, Perez-Tapia M, Bojalil R, Estrada-Garcia I, Estrada-Parra S, Pavon L. 2013. Effect of selective serotonin reuptake inhibitors and immunomodulator on cytokines levels: An alternative therapy for patients with major depressive disorder. Clin Dev Immunol. 2013:267871.
Kessler M, Goldsmith D, Schellekens H. 2006. Immunogenicity of biopharmaceuticals. Nephrol Dial Transplant. 21:9–12.
Martinez-Becerra F, Kissmann J, Diaz-McNair J, Choudhari S, Quick A, Martinez-Sanchez G, Clements J, Pasetti M, Picking W. 2012. Broadly protective Shigella vaccine based on Type III secretion apparatus proteins. Infect Immun. 80:1222–1231.
Medina-Rivero E, Merchand-Reyes G, Pavon L, Vazquez-Leyva S, Perez-Sanchez G, Salinas-Jazmin N, Estrada-Parra S, Velasco-Velazquez M, Perez-Tapia S. 2014. Batch-to-batch reproducibility of TransferonTM. J Pharm Biomed Anal. 88:289–294.
Rivero E, Vallejo-Castillo L, Vazquez-Leyva S, Perez-Sanchez G, Faviari L, Velasco-Velazquez M, Estrada-Parra S, Pavon L, Perez-Tapia S. 2016. Physicochemical characteristics of Transferon batches. Biomed Res Int. 2016:7935181.
National Research Council of the National Academies. 2011. Guide for the care and use of laboratory animals. 8th ed. Washington (DC): National Academies Press.
Pizzato G, de Vinci C, Fornarola V, Palareti A, Baricordi O, Viza D. 1996. In vitro studies during long-term oral administration of specific transfer factor. Biotherapy. 9:175–185.
Sahoo T, Jena P, Prajapati B, Gehlot L, Patel A, Seshadri S. 2017. In vivo assessment of immunogenicity and toxicity of Bacteriocin TSU4 in BALB/c mice. Probiotics Antimicrob Proteins. For forthcoming. DOI: 10.1007/s12602-016-9249-3.
Salinas-Jazmin N. 2016. Estrada-Parra S, Becerril-Garcia M, Limon-Flores A, Vazquez-Leyva S, Medina-Rivero E, Pavon L, Velasco-Velazquez M, Perez-Tapia S. 2015. Herpes murine model as a biological assay to test dialyzable leukocyte extracts activity. J Immunol Res. 2015:146305.
Scheibholfer S, Laimer J, Machado Y, Weiss R, Thalhammer J. 2017. Influence of protein fold stability on immunogenicity and its implications for vaccine design. Expert Rev Vaccines. 16:479–489.
Secretaría de Salud México. 1999. NOM-062-ZOO-1999, Especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio. Ciudad de México. Mexico City: Diario Oficial de la Federación; p. 1–13.
Teitelbaum D, Brenner T, Abramsky O, Aharoni R, Sela M, Arnon R. 2003. Antibodies to glatiramer acetate do not interfere with its biological functions and therapeutic efficacy. Multiple Sclerosis. 9:592–599.

Tuteja U, Kumar S, Shukla J, Kingston J, Batra H. 2007. Simultaneous direct detection of toxigenic and non-toxigenic Vibrio cholerae from rectal swabs and environmental samples by sandwich ELISA. J Med Microbiol. 56:1340–1345.

U.S. Department and Human Services. 2014. Immunogenicity assessment for therapeutic protein products. Bethesda (MD): Food and Drug Administration (FDA); p. 36.

van de Weert M, and Horn Moller E, editors. 2008. Immunogenicity of biopharmaceuticals: causes, methods to reduce immunogenicity, and biosimilars. In: Immunogenicity of biopharmaceuticals. New York: Springer.

Vaucher Rde A, Velho Gewehr Cde C, Correa A, Sant’Anna V, Ferreira J, Brandelli A. 2011. Evaluation of the immunogenicity and in vivo toxicity of the anti-microbial peptide P34. Int J Pharm. 421:94–98.

Wang W, Singh S, Li N, Toler M, King K, Nema S. 2012. Immunogenicity of protein aggregates-concerns and realities. Int J Pharm. 431:1–11.

Weinstein V, Schwartz R, Grossman I, Zeskind B, Nicholas J. 2015. Glatiramoids. In: Crommelin D, de Vlieger J, editors. Non-Biological Complex Drugs. New York: Springer; p. 110–142.

Wu L, Chen F, Lee S, Raw A, Yu L. 2017. Building parity between brand and generic peptide products: Regulatory and scientific considerations for quality of synthetic peptides. Int J Pharmaceutics. 518:320–334.

Zhang J, Huang B, Yu F, Wei M, Yang G, Fu H, Jin L, Bai L, He X, Lu Z. 2011. Production and characterization of polyclonal and monoclonal Abs against the RNA-binding protein QKI. Appl Biochem Biotechnol. 164:283–293.