Señor Director

NUEVAS CREACIONES

Superintendencia de Industria y Comercio

E. S. D.

Trámite: 011 **Evento:** 378 **Actuación:** 523

Referencia:	Expediente No. 14.138.326
Asunto:	Solicitud de patente para: *"MÉTODO DE OBTENCIÓN DE EXTRACTO DIALIZABLE DE LEUCOCITOS"*
Solicitante:	INSTITUTO POLITECNICO NACIONAL
Fecha de solicitud:	26 de Junio de 2014

RESPUESTA A PRIMER EXAMEN DE PATENTABILIDAD

ANDRÉS RINCÓN USCÁTEGUI, identificado como aparece al pié de mi firma, en mi condición de apoderado de la sociedad solicitante de la patente de la referencia, doy respuesta al Oficio **4086**, notificado por fijación en lista el 05 de Abril de 2016, para el cual solicité plazo el día 27 de junio de 2016.

I. MODIFICACIONES AL PLIEGO REIVINDICATORIO

En respuesta al examen de patentabilidad el solicitante decide aportar un nuevo capítulo reivindicatorio conformado por 05 reivindicaciones en las que se han incluido las siguientes modificaciones:

1. Se ha modificado la reivindicación 1 de manera que en la nueva reivindicación 1 se indica que la muestra que será sometida al proceso es una muestra de concentrado leucocitario.

2. Se ha modificado la reivindicación 2 de manera que se ha dirigido a un factor de transferencia caracterizado por su perfil cromatográfico, de acuerdo al proceso de la reivindicación 1. El soporte para dicha modificación se encuentra en la Figura 1.

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3. Se han introducido las nuevas reivindicaciones 3 a 5, las cuales se encuentran debidamente soportadas en la reivindicación 1 originalmente presentada y en la descripción de la presente solicitud originalmente presentada, por lo tanto dicha modificación no corresponde a ampliación de materia.

Estas modificaciones se ajustan al artículo 34 de la Decisión 486 de 2000 y no constituyen una ampliación de la protección que correspondería a la divulgación contenida en la solicitud inicial.

A pesar de eliminar materia del pliego reivindicatorio originalmente presentado, mi representada no renuncia a la protección que pueda obtener sobre dicha materia y se reserva el derecho de presentar una solicitud divisional que la contenga durante el trámite de la presente solicitud.

II. EN CUANTO A LA SUPUESTA FALTA DE CLARIDAD

1. Con respecto a las objeciones formuladas frente a la reivindicación 1, respecto a la falta de concisión, cabe resaltar que esta reivindicación ha sido modificada en el nuevo pliego reivindicatorio. Adicionalmente es importante mencionar que es inapropiado corregir "cromatografía líquida de ultra-resolución por exclusión molecular (SE-UPLC)" por cromatografía de líquidos de alta resolución (HPLC, dado que la cromatografía descrita y soportada por la presente solicitud en la etapa de identificación es cromatografía líquida de ultra-resolución por exclusión molecular (SE-UPLC) y no HPLC como lo señala el Examinador. Por lo tanto, no es necesario realizar dicha modificación. Adicionalmente, tal y como se ha modificado la reivindicación 1 es claro para un técnico en la materia el uso de dos tamaños de corte (10kDa y luego 1KDa) en el paso de ultrafiltración tangencial. Por lo tanto dicha objeción ha quedado superada.

2. Con respecto a las objeciones formuladas frente a la reivindicación 1 respecto a la menciona la frase "se obtiene el producto graficado en la figura 1" es considerada como un resultado a alcanzar y que no define al método, el Solicitante considera que dicha objeción ha quedado superada ya que la materia referente al producto graficado en la figura 1 se ha eliminado de la nueva reivindicación 1 y que la materia de dicha reivindicación 1 es clara porque los pasos de dicho proceso se encuentran definidos de una manera más clara.

3. Con respecto a las objeciones formuladas frente a la reivindicación 2 por mencionar un "producto", si bien es cierto que un producto usualmente se caracteriza por sus componentes,

más que por su proceso de obtención. Sin embargo el producto reclamado en la presente solicitud está intrínsecamente ligado a su proceso de fabricación: cada operación unitaria selecciona a las moléculas que conformarán al producto, las cuales son responsables de la actividad biológica que se le atribuye. Una forma de evidenciar sus componentes es, efectivamente, por los perfiles de SE-UPLC. Adicionalmente, la figura 1 de abajo muestra una evidencia de que las operaciones unitarias tienen un gran impacto en la composición del producto, haciéndolo único y asignándole sus características fisicoquímicas y biológicas distintivas; diferente orden y condiciones de operaciones unitarias generan productos distintos con propiedades fisicoquímicas y biológicas también distintas. Considerando la información de la figura 1, el producto reclamado en la reivindicación 2 (factor de transferencia) se ha definido por su proceso de obtención y su perfil cromatográfico. Por lo tanto, se manifiesta que la claridad de dicha reivindicación ha quedado completamente superada.

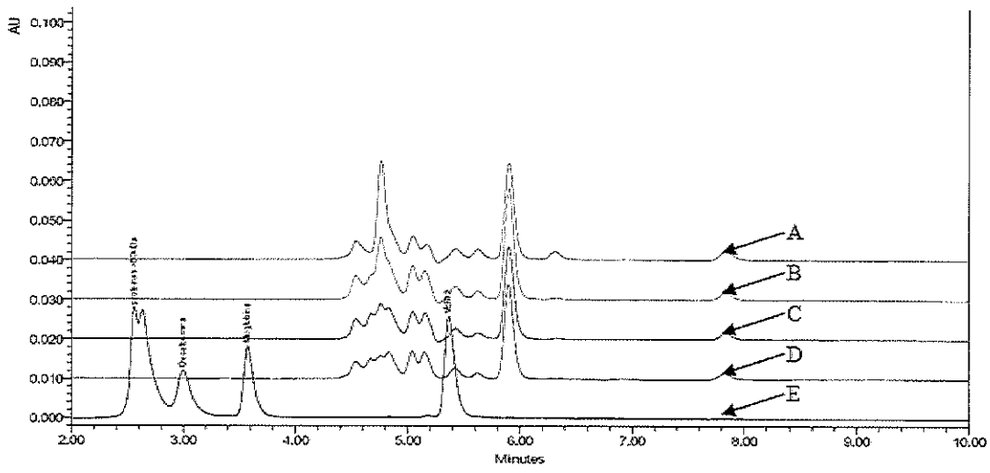

Figura 1. Efecto del número de ciclos de congelación – descongelación en el perfil de SE-UPLC de un factor de Transferencia. Se muestran los cambios en el perfil cromatográfico de un factor de transferencia al someterse a 2 ciclos (D), 3 ciclos (C), 4 ciclos (B) y 5 ciclos (A) de congelación descongelación. Se muestra el perfil de un marcador de peso molecular como referencia (E; Vit. B12 - 1.5 kDa, myoglobin – 17 kDa). Al incrementarse el número de ciclos de congelación – descongelación se incrementa la cantidad de moléculas presentes en el extracto, principalmente de la región de mayor peso molecular.

III. ESTADO DE LA TÉCNICA

Su despacho determinó dos documentos como relevantes para realizar el análisis de patentabilidad de la presente patente de invención:

1. D1, referido al documento del Instituto Politécnico Nacional escuela nacional de ciencias

biológicas- Uriel GarcíaHernández titulado *"Análisis de poblaciones celulares que responden a extractos dializables de leucocitos (Transferón)"* que divulga los extractos dializables de leucocitos o EDL (Pág. 1).

2. D2, referido al documento del Instituto Politécnico Nacional escuela nacional de ciencias biológicas- Frank Hanz Robledo Avila titulado *"Efecto del extracto dializado de leucocitos en citosinas de respuesta temprana en un modelo murino de endotoxemia"* que divulga un método para la obtención de extractos dializable de leucocitos (Pág. 18).

EN CUANTO A LA SUPUESTA FALTA DE NOVEDAD Y NIVEL INVENTIVO

1. El documento D1 no describe ni sugiere el perfil cromatográfico típico del producto como se reclama en la nueva reivindicación 2 ni que el producto se haya obtenido con un proceso de fabricación como el que se reclama en la nueva reivindicación 1. Por lo tanto, se afirma que la materia de la nueva reivindicación 2 es novedosa con respecto al documento D1.

EN CUANTO A LA SUPUESTA FALTA DE NIVEL INVENTIVO

1. El documento D2 describe un proceso para obtener DLE murino que consiste básicamente de los siguientes pasos: obtención de bazo de ratones, disgregación del bazo, lisis por ciclos de congelación y descongelación (10 ciclos), concentración y determinación de proteínas, prueba de endotoxina e inocuidad. La presente invención se diferencia del arte previo más cercano (D2) en que el material de partida es diferente, en D2 el factor de transferencia de murino se obtiene de células de bazo, mientras que en la presente solicitud, el producto reclamado se obtiene a partir de concentrados leucocitarios. Otra diferencia es que en el proceso descrito en D2 se emplea un método de concentración, mientras que el proceso reclamado en la presente solicitud no considera una operación unitaria similar; los ciclos de congelación/descongelación son 10 en D2 y 5 en la presente invención, que D2 no describe un paso de ultrafiltración tangencial con dos límites de membrana uno de 10 kDa y luego uno de 1 kDa; y que no existe un descripción del producto por SE-UPLC. D2 tampoco describe que con el método descrito ahí se obtenga un factor de transferencia que tenga el perfil característico de 11 picos como se define en la nueva reivindicación 2.

2. El documento D1 hace referencia al HPLC como técnica de identificación y cuantificación con el fin de obtener extractos dializables de leucocitos. Si bien es cierto que D1 menciona el uso de HPLC como técnica para caracterizar extractos dializables leucocitarios, lo hace como

parte del estado del arte referenciando un producto y un trabajo completamente ajeno al descrito en la presente solicitud. En ningún momento profundiza en la ejecución del método ni mucho menos la caracterización por HPLC del extracto descrito en la presente solicitud.

3. La materia del nuevo capítulo reivindicatorio que se presenta no sería obvia a partir D2 solo ni en combinación con D1 ya que una persona normalmente versada en la materia, a partir de la información de D2 y D1, no habría sido capaz de modificar el proceso de D2 para llegar al proceso reclamado en la nueva reivindicación 1 con los pasos particulares como se describen en dicha reivindicación, el cual proporciona un producto consistente lote a lote, tanto en su perfil de distribución de masa por SE-UPLC *Medina-Rivero E. et al., 2014*, (Batch-to-batch reproducibility of TransferonTM, J. Pharm. Biomed. Anal, 88, 289-294, **Anexo 1**, adjunto a este documento), como en las pruebas de actividad biológica, las cuales comprenden la producción de interferón gamma en líneas celulares Jurkat (Medina-Rivero et al., 2014) y el incremento de la supervivencia en un modelo murino de infección con virus del Herpes simple (*Salinas-Jazmin N. et al., 2015* Herpes Murine Model as a Biological Assay to Test Dialyzable Leukocyte Extracts Activity, Journal of Immunology Research, **Anexo 2**, adjunto a este documento).

4. Con respecto al documento adjunto de *Medina-Rivero E. et al., 2014*, se desea poner de presente que el extracto dializable leucocitario (EDL) obtenido y probado en dicha referencia de Medina-Rivero E. et al., 2014 se obtuvo utilizando el proceso reclamado en la presente solicitud y que la diferencia del perfil cromatográfico entre el EDL de Medina-Rivera, 2014 que presenta 8 picos principales y el EDL de la presente solicitud que presenta 11 picos principales radica en la columna Acquity BEH utilizada en el análisis de caracterización (SE-UPLC), columna Acquity BEH 200 en la presente solicitud y columna Acquity BEH 125 en la referencia Medina-Rivero et al., 2014.

5. Con la finalidad de evidenciar que los perfiles cromatógraficos como se describen en la presente solicitud y *Medina-Rivero et al., 2014*, de los EDLs producidos con el proceso de la invención son equivalentes cuando se utiliza una columna de exclusión de tamaño molecular Acquity BEH 200 o una columna de exclusión de tamaño molecular Acquity BEH 125, los perfiles cromatográficos de los lotes 13C08, 13C09 y 13C10 empleando muestras de retención, se obtuvieron. Como lo muestra la figura 1 del **Anexo 3** (adjunto a la presente documento), cuando los lotes se analizaron por una columna Acquity BEH 200, 4.6X150 mm, dichos lotes exhibieron un perfil cromatográfico con 11 picos principales, lo que concuerda con lo descrito por la solicitud de patente. Por otra parte, la figura 2 del Anexo 3 muestra los perfiles cromatográficos de los mismos lotes mostrados en la figura 1, pero evaluados en una prueba

cromatográfica con una columna Acquity BEH 125, los cuales presentaron un perfil cromatográfico con 8 picos principales similar al descrito por *Medina-Rivero et al., 2014.*

6. Considerando los resultados mostrados en el anexo 3, se concluye que los perfiles cromatográficos del EDL descritos en la presente solicitud (11 picos) y el descrito en *Medina-Rivero et al., 2014* (8 picos) son equivalentes y pertenecen al mismo producto; siendo entonces que la diferencia en el número de picos observados en el perfil cromatográfico del producto depende de la columna de Exclusión de tamaño molecular empleada. Adicionalmente, el Solicitante desea llamar la atención para que note que los lotes empleados para las pruebas anteriormente descritas (13C08, 13C09 y 13C10) son parte de los lotes utilizados en el artículo Salinas-Jazmin N. et al., 2015, en donde se describe una actividad anti-herpética consistente en un modelo de supervivencia murino. En vista de lo anteriormente expuesto, las referencias *Medina-Rivero et al., 2014* y *Salinas-Jazmin N. et al., 2015*, son presentadas como evidencia experimental que demuestra las ventajas del proceso reclamado en la presente solicitud.

7. El efecto del método de fabricación en las propiedades biológicas del producto no es fácilmente evidente para una persona normalmente versada en la materia tomando en cuenta lo que se describe en la literatura. Así por ejemplo, y como los muestra la Figura 1 anteriormente discutida en el apartado de claridad, el número de ciclos de congelación-descongelación determina la cantidad y diversidad de componentes presentes en el producto final, los cuales son identificados por un perfil cromatográfico. Asimismo, se desea resaltar que el efecto biológico del producto descrito en la presente solicitud varía con respecto al perfil cromatográfico, es decir, con los componentes presentes en el mismo. Es por ello, que el nivel inventivo radica en el descubrimiento de que la variación en las condiciones de congelación-descongelación así como los periodos entre una y otra condición generan un producto con características definidas como el que se reclama en la nueva reivindicación 2.

8. El proceso de la invención así como el producto obtenido con el mismo, tienen nivel inventivo sobre los documentos D1 y D2 citado en su atento oficio, ya que a partir de las enseñanzas de D1 y D2 no habría sido obvio llegar al proceso de la invención con los pasos particulares como se describen en la nueva reivindicación 1 el cual proporciona un producto consistente de lote a lote con un perfil cromatográfico como se describe en la nueva reivindicación 2, asegurando de esta manera la actividad biológica del factor de transferencia obtenido con dicho proceso, lo cual es evidente en los Anexos 1 y 2. Adicionalmente, es importante resaltar que la Oficina Norteamericana de Patentes ha otorgado la correspondiente Patente Norteamericana No. US 9,328,152 B2 correspondiente con la presente solicitud, de la

cual se anexa una copia simple. Si bien es cierto que esta decisión es independiente de lo que la autoridad determine para el trámite de la presente solicitud, sí es un indicio de que los requisitos universales de novedad y nivel inventivo o actividad inventiva han sido reconocidos en Estados Unidos, lo cual refuerza la aplicabilidad de los argumentos arriba vertidos a la luz de la normatividad aplicable a este caso.

IV. PETICIÓN

Puesto que se ha dado respuesta a todas las objeciones formuladas por la Dirección de Nuevas Creaciones, se solicita proceder al otorgamiento de la solicitud de patente.

V. ANEXOS

1. Sustitución de poder otorgada por LUZ CLEMENCIA DE PÁEZ a ANDRÉS RINCÓN USCÁTEGUI.

2. Nuevo pliego reivindicatorio conformado por 05 reivindicaciones.

3. Anexo 1 (*Medina-Rivero E. et al., 2014,* Batch-to-batch reproducibility of TransferonTM, J. Pharm. Biomed. Anal, 88, 289-294).

4. Anexo 2 (Salinas-Jazmin N. et al., 2015 Herpes Murine Model as a Biological Assay to Test Dialyzable Leukocyte Extracts Activity, Journal of Immunology Research).

5. Anexo 3 (Figura 2 de perfiles cromatográficas empleando la columna BEH 125 de los lotes 13C08).

Señor Director,

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COLO-21260-0841-0001-6
DPG\DPG\ID-289804\WPC21260
No. M-2016-289804\DPG

REIVINDICACIONES MODIFICADAS

1. Un proceso para la producción de un factor de transferencia, caracterizado porque comprende las siguientes etapas:

a. someter un concentrado leucocitario a cinco ciclos de congelación/descongelación, en donde, en cada ciclo, el concentrado se congela a -20 °C durante una semana y, después se descongela completamente, para obtener un producto congelado-descongelado;

b. someter el producto congelado-descongelado obtenido en el paso (a) a diálisis empleando una membrana de diálisis con un corte de 12 kDa, para obtener un producto dializado;

c. someter el producto dializado obtenido en el paso (b), a un proceso serial de ultrafiltración tangencial, primero empleando un tamaño de corte de 10 kDa y luego un corte de 1 kDa, para obtener un producto ultrafiltrado;

d. someter el producto ultrafiltrado obtenido en el paso (c) a cromatografía líquida de ultra resolución por exclusión molecular (SE-UPLC) para su identificación; en donde dicho producto ultrafiltrado obtenido en el paso (c) es un factor de transferencia que exhibe actividad anti-proliferativa.

2. Un factor de transferencia que exhibe actividad anti-proliferativa obtenido por el proceso de conformidad con la reivindicación 1, caracterizado porque presenta once picos cromatográficos en un análisis por SE-UPLC, los cuales eluyen a aproximadamente los siguientes tiempos de retención:

Número de pico	***Tiempo de retención (min)***
1	*8.7*
2	*8.85*
3	*9.4*
4	*9.6*
5	*10.0*
6	*10.2*

7	*10.6*
8	*10.9*
9	*11.1*
10	*11.8*
11	*12.15*

3. El proceso de conformidad con la reivindicación 1, caracterizado porque la actividad anti-proliferativa del factor de transferencia es probada mediante un ensayo de bromuro de bromuro de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolio.

4. El proceso de conformidad con la reivindicación 1, caracterizado porque el factor de transferencia inhibe la proliferación de células de osteosarcoma.

5. El proceso de conformidad con la reivindicación 1, caracterizado porque el factor de transferencia inhibe la proliferación de células AT20.

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Journal of Pharmaceutical and Biomedical Analysis 88 (2014) 289–294

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Batch-to-batch reproducibility of Transferon™☆

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ARTICLE INFO

Article history:
Received 17 June 2013
Received in revised form 4 September 2013
Accepted 6 September 2013
Available online xxx

Keywords:
Dialyzable leukocyte extracts
UPLC
Quality specifications
IFN-gamma
Transferon

ABSTRACT

Human dialyzable leukocyte extracts (DLEs) are heterogeneous mixtures of low-molecular-weight peptides that modulate immune responses in various diseases. Due their complexity, standardized methods to identify their physicochemical properties and determine that production batches are biologically active must be established. We aimed to develop and validate a size exclusion ultra performance chromatographic (SE-UPLC) method to characterize Transferon™, a DLE that is produced under good manufacturing practices (GMPs). We analyzed an internal human DLE standard and 10 representative batches of Transferon™, all of which had a chromatographic profile characterized by 8 main peaks and a molecular weight range between 17.0 and 0.2 kDa. There was high homogeneity between batches with regard to retention times and area percentages, varying by less than 0.2% and 30%, respectively, and the control chart was within 3 standard deviations. To analyze the biological activity of the batches, we studied the ability of Transferon™ to stimulate IFN-γ production *in vitro*. Transferon™ consistently induced IFN-γ production in Jurkat cells, demonstrating that this method can be included as a quality control step in releasing Transferon™ batches. Because all analyzed batches complied with the quality attributes that were evaluated, we conclude that the DLE Transferon™ is produced with high homogeneity.

1. Introduction

Human dialyzable leukocyte extracts (DLEs) are heterogeneous mixtures of low-molecular-weight peptides (<10 kDa) that are released on disruption of peripheral blood leukocytes from healthy donors [1]. Administration of DLEs improves clinical responses in infections [2,3], allergies [4], cancer [5,6], and immunodeficiencies [7].

The therapeutic and adjuvant effects of DLEs are associated with their ability to modulate immune responses in the above mentioned diseases (for detailed reviews see Refs. [8,9]). DLEs alter innate signaling pathways, such as TLRs, NF-κB, and cAMP in cultured immune cells [10]. Consequently, DLEs stimulate the production of proinflammatory cytokines, including TNF-α, IL-6 [11], and IFN-γ [12].

Despite the positive clinical effects of DLEs, their complexity has impeded full characterization of their components and active substances. Thus, characterization, total protein per dose, and efficacy of DLEs have never been reported, necessitating the development and validation of suitable methods that analyze their physicochemical properties. In addition, such methods would allow

Abbreviations: IFN-γ, interferon gamma; HPLC, high-performance liquid chromatography; UPLC, ultra-performance liquid chromatography; SE, size exclusion; RP, reverse-phase; GMPs, good manufacturing practices; SD, standard deviation; RSD, relative standard deviation; DLE, dialyzable leukocyte extract; Da, daltons; NF-κB, nuclear factor kappa B; cAMP, cyclic adenosine monophosphate; TNF-α, tumor necrosis factor alpha; IL-6, interleukin 6; DTH, delayed-type hypersensitivity; Gly, glycine; Ser, serine; Glu, glutamine; BCA, bicinconinic acid; ICH, International Conference of Harmonization; FDA, Food and Drug Administration.

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http://dx.doi.org/10.1016/j.jpba.2013.09.004

establishing the relationship between the physicochemical properties and expected biological activities of DLEs.

The seminal reports on the physicochemical characterization of DLEs have applied chromatographic methods. In 1962, Baram and Mosko used reverse-phase (RP) chromatography to demonstrate that DLEs are a heterogeneous mixture that comprise diverse chromatographic fractions [13]. This group also observed that DLEs generated 10 peaks by size exclusion chromatography—the fifth peak, containing molecules under 10 kDa, harbored most of the biological effects [14,15].

Similarly, Vandenbark and colleagues [16], Burger and colleagues [17], and Khan and colleagues [18] noted that specific subfractions, obtained by high-performance liquid chromatography (HPLC), RP-HPLC, and anion-exchange chromatography, respectively, mediated the effects of DLEs. The active fraction was susceptible to proteases [17], and its physicochemical properties suggested high protein content [18]. On further analysis of the active fractions of the DLEs, the most abundant amino acids were Gly, Ser, and Glu, whereas Lys and aromatic amino acids were the least abundant [19].

Kirkpatrick and colleagues provided definitive evidence that the peptidic content mediates the biological activity in DLEs. They fractionated DLEs by RP-HPLC and isolated peptides that contained the sequence MXLLYAQDL/VEDN. Administration of blocking peptides inhibited the characteristic transfer of delayed type hypersensitivity (DTH) by DLEs [20]. Collectively, these reports demonstrate that DLEs are a heterogeneous mixture of polar and hydrophilic peptides with molecular weights below 10 kDa.

DLEs are produced and commercialized worldwide. Transferon™ is a DLE manufactured by the National School of Biological Sciences (ENCB), National Polytechnic Institute (IPN), Mexico, at GMP facilities. Transferon™ is registered by Mexican health authorities as a drug and commercialized nationally. Similarly, other DLEs are registered as drugs in China, Cuba, and Czech Republic [21–23]. Based on the significance of chromatographic methods in developing and characterizing DLEs and the lack of a standard physicochemical method to analyze them, we aimed to develop and validate an UPLC approach to examine Transferon™. We identified a typical chromatographic profile with eight main peaks.

We also assessed the batch-to-batch reproducibility of 10 representative batches of Transferon™ by analyzing their physicochemical properties and their ability to effect IFN-γ secretion *in vitro*. All batches met the quality control standard with regard to peptide concentration, molecular weight rate, time of retention of main peaks, and efficacy.

2. Materials and methods

2.1. Analytical samples and peptide quantification

Samples of Transferon™ were obtained from 10 typical batches (10K02B, 10K04A, 11A01E, 11A01C, 11A02D, 11A02E, 11A02F, 11A02G, 11E01, and 12A01) produced by UDIMEB at GMP facilities using a modified version of the method of Borkowsky and Lawrence [24]. Briefly, leukocytes were lysed with 5 freeze-thaw cycles, and dialysis was performed using a 12-kDa membrane to obtain low-molecular-weight peptides in the permeate. The peptide concentration was measured by bicinchoninic acid (BCA) method using the Pierce BCA kit (Thermo Fisher Scientific; Massachusetts, USA) according to the manufacturer's instructions [25]. Internal batch pattern of Transferon™ was defined as an internal batch that has been approved satisfactorily all the quality control tests specified for the product (endotoxin content, microbiologic test, characterization, protein content and biological activity).

2.2. Chromatographic methods

We performed SE-UPLC on an H class Acquity™ UPLC system (Waters™; Massachusetts, USA) that was controlled by Empower™ (Waters™) using an Acquity BEH 125 molecular size exclusion column (1.7 μm × 4.6 × 150 mm) (Waters™) at 30 °C. The chromatograms were generated on a tunable ultraviolet (TUV) detector (Waters™) at 280 nm with an isocratic workflow of 0.4 ml/min of monobasic/dibasic sodium phosphate-buffered solution (pH 6.8, 50 mM; Mallinckdrodt Baker; Pennsylvania, USA), sodium chloride 150 mM (Mallinckdrodt Baker) and ultrapure Milli Q water (Millipore, Darmstadt, Germany). The run time was 15 min.

2.3. Characterization analysis: SE-UPLC

SE-UPLC is considered as a characterization method per the International Conference of Harmonization (ICH) [26]. The method was validated by testing system suitability, precision, and specificity. We used a gel filtration standard (Bio-Rad™; California, USA) spiked with L-tryptophan BioUltra (Sigma Aldrich; Missouri, USA) as molecular weight standard. To analyze the batch-to-batch reproducibility, Transferon™ samples were compared against an internal batch pattern (1.5 mg/ml); each sample was injected in duplicate (1 μl for standards and 3 μl for Transferon™ samples). The chromatographic profiles were analyzed using Empower™ (ApexTrack method) to obtain the area percentage and retention time for each peak.

2.4. Biological activity

The efficacy of Transferon™ was determined in Jurkat clone E6-1 cells (ATCC; Manassas, VA, USA). Briefly, 3×10^5 cells were plated in 24-well plates and maintained in RPMI 1640 medium (ATCC; Virginia, USA) that was supplemented with fetal bovine serum (Gibco™, Life Technologies; New York, USA). Cultures were stimulated with Transferon™ (0.1, 1, or 10 μg/ml) or type IV-S concanavalin A (25 μg/ml; Sigma–Aldrich; Missouri, USA) as a positive control. After 72 h, IFN-γ was measured in the supernatant using the BDOptEIA-IFN-γ ELISA kit (Becton Dickinson Biosciences; California, USA) by triplicate. After stimulation, 100 μl of standard or sample were mixed with 50 μl of PBS with 10% of heat inactivated FBS and incubated for two hours in capture antibody-coated wells. After extensive washing, 100 μl of a solution including a biotinylated detection antibody and streptavidin-horseradish peroxidase were added to each well and incubated for one hour. After washing, the reactions were developed with 3,3′5,5′-Tetramethylbenzidine (TMB) plus hydrogen peroxide, stopped, and read immediately at 450 nm [27]. IFN-γ quantification above the detection limit (4.7 pg/ml) was considered as positive, since basal production of this cytokine in Jurkat cells was undetectable.

2.5. Data analysis

To analyze the homogeneity in chromatographic profiles between Transferon™ batches, we calculated the average, standard deviation, and percentage standard deviation for the peak areas and retention times. We also calculated the average and standard deviation for peptide content and IFN-γ.

3. Results and discussion

DLEs and their clinical activity have been described since 1954 [28], but their batch-to-batch reproducibility in GMP facilities has not been examined. We analyzed 10 batches of Transferon™ with

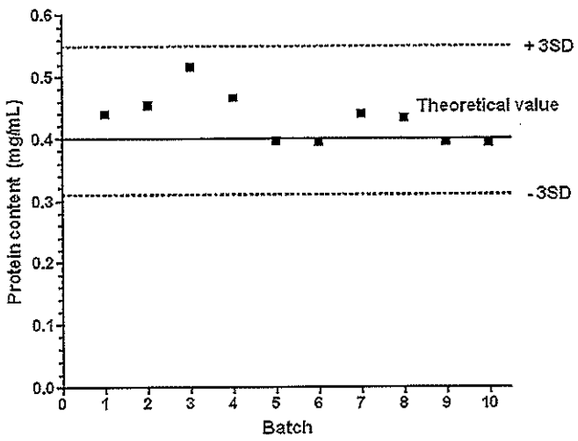

Fig. 1. Analysis of protein content between Transferon™ batches. Protein concentration, quantified by BCA method, is specified for each batch (squares). Theorical value (solid line) and ±3SD interval (dashed lines) are shown.

Table 1
Validation parameters for the SE-UPLC identity method developed for Transferon™.

Parameter	Acceptance criteria	Result
System suitability	Tailing factor less than 1.75	Tailing factor 1.44–1.51
	Relative retention time (against B12 vitamin) of:	Relative retention time:
	Mioglobulin 0.67 ± 0.05 SD	Mioglobin 0.67 ± 0.00
	Ovoalbumin 0.56 ± 0.05 SD	Ovoalbumin 0.57 ± 0.00
Method accuracy	Variation of areas between batches lectures are less than 3.0%	%RSD 0.45–1.76
System accuracy	Variation of areas between three standard lectures are less than 3.0%	%RSD 1.15–2.28
Specificity	Correspondence between internal batch pattern and batches of Transferon™	Chromatographic profiles of batches are equal to internal batch pattern standard

regard to protein content, characterization by SE chromatography-UPLC, and activity in an *in vitro* model of IFN-γ production. The batches were typical production batches from 2010 and 2011.

Early reports obtained DLEs on a low scale using cells from patient-related or DTH-positive donors [13]. In these studies, dose was calculated in arbitrary units that were related to the number of leukocytes of origin [29]. In contrast, each Transferon™ batch is produced from a pool of 1000 healthy donors in order to reduce donor-to-donor variability [30]. Further, the active principle per dose in Transferon™ is quantified as protein content, as reported [31,32]. Transferon™ contains 2 mg of peptides in 5 ml of excipient. By analysis of protein content in 10 batches, Transferon™ had an average content of 0.433 mg/ml, which represent a 108.2% regarding to the theoretical expected value of 0.40 mg/ml. However, this percentage meets the criteria established in the specification for Transferon™, which is from 0.34 to 0.46 mg/ml. The variation in concentrations between batches was within ±3 SD (Fig. 1), demonstrating that the process by which Transferon™ is produced consistently generates batches that are homogeneous with regard to protein content.

3.1. SE-UPLC characterization method

According to the FDA's Guide for Biological Products and Mexican Pharmacopeia, biological drugs for clinical use require characterization of the active principle during production and as part of the final quality control step [33,34]. Since Transferon™ is currently as project to be included in the Mexican Pharmacopeia, we used chromatography, which is a general method for drug characterization [33,34]. We developed a characterization method for Transferon™ using SE-UPLC. We choose to use columns with a 125 Å pore size particles (suitable for low molecular weight peptides separation) and tested two column lengths: 150 and 300 mm. The resolution factor (α) for peaks 3 and 4 was 1.02 and 1.08 respectively for 150 mm column, and 1.45 and 1.50 for 300 mm column. Despite of the better resolution in the 300 mm column, peaks 1, 3 and 4 were not base resolved. Because of that, size exclusion column of 150 mm was established for routine release analysis in quality control laboratory.

To validate the assay, we analyzed its suitability, accuracy, and specificity (Table 1). The system's suitability was examined by comparing the retention times for molecules with known molecular weights (standards) and calculating the tailing factor. To study the accuracy of the method and system, we calculated the variations in peak areas between batches and between 3 standard lectures. In both cases the variation was less than 3 SD. Specificity was evaluated by comparing samples with our internal standard of Transferon™. The chromatographic profiles of the batches were the same as that of the internal batch pattern. These data demonstrate that our characterization assay meets the minimum requirements for qualitative physicochemical methods, as indicated by the International Conference on Harmonization [26].

With this validated method, we analyzed 10 typical batches of Transferon™ at the moment of release. Because DLEs are a complex mixture of peptides, they generate multiple chromatographic peaks. Other studies have reported 4, 7, and 13 peaks for DLEs that have been produced by similar processes [35–39]. In our assay, Transferon™ generated 8 main peaks, as did the internal batch pattern. The difference between our data and the earlier reports can be explained by disparities in the sensitivity of the chromatographic methods [40].

All peaks of Transferon™ were shown in the range of 17 kDa (equine myoglobine) to 0.2 kDa (tryptophan) (Fig. 2A). The fifth peak had the largest area (28.09%), while the seventh peak had the minor area (0.77%). These properties are consistent with a product that has been dialyzed against a 12 kDa membrane, such as DLEs [24].

Next, we compared the area percentages and retention times of the 8 main peaks to analyze batch-to-batch reproducibility. The %RSD for all area percentages was below 30% (Table 2) and varied within 3 SD (data not shown). This variation is accepted as a

Table 2
Comparison of area percentages of the eight main peaks in Transferon™ chromatographic profile between batches.

	BATCH	P1	P2	P3	P4	P5	P6	P7	P8
1	10K02B	4.2	14.75	3.45	17.55	23.1	13.7	1.35	21.4
2	10K04A	5.4	24.55	2.85	14.3	24.15	9	0.9	17.7
3	11A01B	8.85	26.15	2.5	11.95	24.9	10.8	0.75	13.6
4	11A01C	8.85	14.65	3.3	15.05	24.2	12.35	0.7	20.5
5	11A02D	5.7	22.5	2.95	12.45	27.4	9.9	0.6	17.45
6	11A02E	5.45	23.35	2.3	11.2	31.15	10.25	0.75	14.35
7	11A02F	6.3	25.65	2.7	10.05	29.4	10.25	0.7	13.75
8	11A02G	5.35	23.7	2.3	10.85	32.05	10.35	0.7	13.7
9	11E01	3.4	20.2	1.55	10.45	35.3	15.55	0.65	11.45
10	12A01	7	28.35	1.35	12.1	29.2	8.6	0.6	10.65
	MEAN	6.05	22.39	2.53	12.6	28.09	11.08	0.77	15.46
	%RSD	29.41	20.56	27.07	18.76	14.35	19.58	28.75	23.59

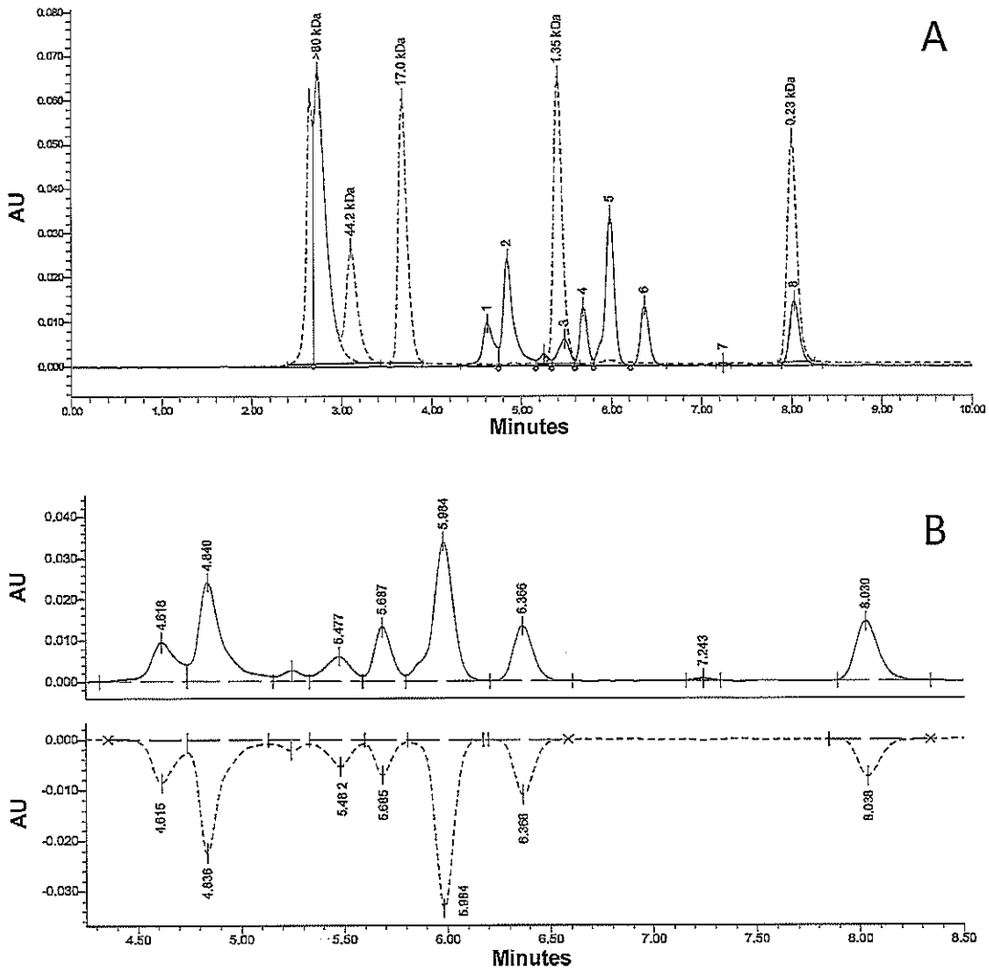

Fig. 2. SE-UPLC chromatographic profile of Transferon™. A. Comparison between a representative batch of Transferon™ (solid line) and molecular weight standards (dashed line), showing that the eight main peaks of Transferon™ have retention times between those of equine myoglobin (17.0 kDa) and tryptophan (0.23 kDa). Other peaks in the standard correspond to tryglobulin/gamma-globulin (>80 kDa), ovoalbumin (44.2 kDa), and B12 vitamin (1.35 kDa). The chromatographic profiles were analyzed using Empower™ (ApexTrack method) to obtain the area percentage and retention time for each peak. B. Representative identity test for Transferon™ (above) compared against an internal human DLE standard (below). All batches showed homogeneity in the test. AUs: Absorbance Units.

quality specification of a biological product by regulatory agencies and allows the product to have a biological activity. For example, the European Pharmacopeial specification of erythropoietin isoform 6 has a range from 10% to 35% area, showing 50% variation [41]. Similarly, the variation in retention times for each peak from batch to batch was below 0.2% (Table 3) and varied within 3 SD (data not shown). Area percentages and retention times depend on the physicochemical attributes of a product; thus, our results indicate that the homogeneity between Transferon™ batches can be tracked for at least 2 years, complying with the minimum quality control standards for the pharmaceutical industry [42].

3.2. Biological activity

Efficacy is an essential attribute of a therapeutic drug, and its activity is expected to be preserved between commercial batches [33]. DLEs have demonstrated activity in clinical trials from the past 50 years [2,8,43–45], but only recently they have begun to be produced by GMPs, necessitating the development of tests that evaluate their activity and batch release.

To this end, animal models and *in vitro* assays are preferred for ethical, timing, and commercial reasons. For example, the activity of DLEs has been demonstrated by inducing DTH in mice [46] or analyzing their effects on leukocyte migration [47]. Although

Table 3
Comparison of retention times of the eight main peaks in Transferon™ chromatographic profile between batches.

	BATCH	P1	P2	P3	P4	P5	P6	P7	P8
1	10K02B	4.7	4.9	5.49	5.76	5.94	6.42	7.19	8.04
2	10K04A	4.68	4.9	5.5	5.76	5.94	6.43	7.2	8.05
3	11A01B	4.66	4.89	5.49	5.76	5.95	6.43	7.21	8.07
4	11A01C	4.66	4.9	5.49	5.76	5.95	6.43	7.21	8.06
5	11A02D	4.68	4.9	5.49	5.75	5.93	6.42	7.21	8.06
6	11A02E	4.68	4.9	5.49	5.75	5.94	6.43	7.21	8.06
7	11A02F	4.68	4.9	5.48	5.75	5.94	6.42	7.21	8.06
8	11A02G	4.68	4.9	5.49	5.75	5.94	6.42	7.22	8.06
9	11E01	4.7	4.9	5.48	5.75	5.94	6.43	7.22	8.07
10	12A01	4.67	4.89	5.49	5.76	5.94	6.42	7.2	8.05
	MEAN	4.68	4.90	5.49	5.76	5.94	6.43	7.21	8.06
	%RSD	0.21	0.08	0.11	0.17	0.07	0.07	0.13	0.11

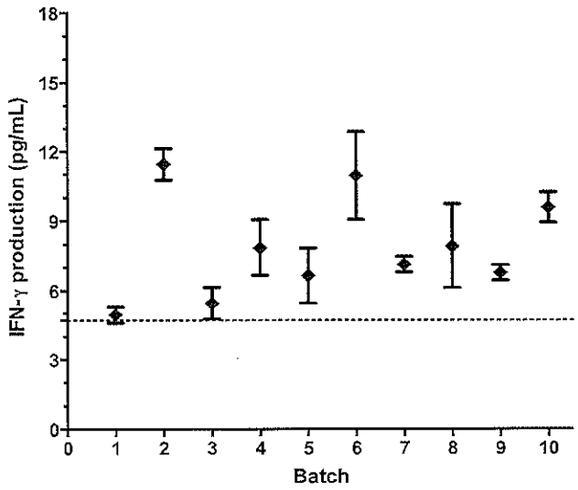

Fig. 3. Effect of Transferon™ on IFN-γ secretion by Jurkat cells. Cells were treated for 72 h with different Transferon™ batches, and the concentration of IFN-γ in supernatants was quantified by ELISA (diamonds). Each experiment included positive (25 μg/ml concanavalin A) and negative (vehicle) controls (not shown). All batches induced IFN-γ secretion above the method's detection limit (dashed line). Data are shown as mean ± SEM (triplicates).

animal models reflect the complexity of a drug response in a whole organism, we developed an *in vitro* assay as a quality control for the release of Transferon™ batches to avoid the variability between animal models, minimize the number of animals that is employed during Transferon™ production, and reduce the analysis time.

DLEs upregulate IFN-γ in lymphocytes [48], and our assay analyzed the ability of a lymphocytic cell line (Jurkat clone E6-1) to produce IFN-γ in response to Transferon™. Our positive and negative controls were concanavalin A- and vehicle-treated cells, respectively. All batches induced IFN-γ production to levels above the detection limit (4.7 pg/ml; Fig. 3) and within 3 SD (data not shown). These results contrast previous attempts to develop *in vitro* assays to evaluate DLE activity, such as leukocyte proliferation [49].

Thus, all analyzed batches shared physicochemical properties and consistently generated a biological response—i.e., IFN-γ production.

4. Conclusions

Our analysis of 10 batches of Transferon™, produced in GMP facilities, demonstrates that is possible to produce a mixture of peptides that have been extracted from complex raw materials, such as lysed human leukocytes, with high batch-to-batch reproducibility. The 10 batches complied with quality standards for their attributes, such as peptide concentration in the final dose, molecular weight, time of retention of the main peaks, and activity. Thus, Transferon™ is a DLE that observe the government normativity required for hemoderivatives used for clinical use, including batch-to-batch reproducibility.

Authors' contributions

SV-L, GP-S, and NS-J performed the experiments. GM-R, LP, SE-P, MV-V, and EM-R analyzed the data and wrote the manuscript. SMP-T, MV-V, and EM-R designed the experiments. All authors read and approved the final manuscript.

Role of funding source

All authors declare that the funding sources had no impact on the study design; the collection, analysis, and interpretation of data; the writing of the report, or the decision to submit the paper for publication.

Conflict of interest

SV-L, GP-S, NS-J, SE-P and SMP-T are employees or haven compensated for their work at "Proyecto Factor de Transferencia/UDIMEB" the producer of Transferon™. All other authors declare no competing interests.

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Journal of Immunology Research
Volume 2014, Article ID 146305, 9 pages
http://dx.doi.org/10.1155/2014/146305

Research Article

Herpes Murine Model as a Biological Assay to Test Dialyzable Leukocyte Extracts Activity

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Received 28 July 2014; Revised 22 September 2014; Accepted 23 September 2014; Published 20 October 2014

Academic Editor: Oscar Bottasso

Human dialyzable leukocyte extracts (DLEs) are heterogeneous mixtures of low-molecular-weight peptides that are released on disruption of peripheral blood leukocytes from healthy donors. DLEs improve clinical responses in infections, allergies, cancer, and immunodeficiencies. Transferon is a human DLE that has been registered as a hemoderivate by Mexican health authorities and commercialized nationally. To develop an animal model that could be used routinely as a quality control assay for Transferon, we standardized and validated a murine model of cutaneous HSV-1 infection. Using this model, we evaluated the activity of 27 Transferon batches. All batches improved the survival of HSV-1-infected mice, wherein average survival rose from 20.9% in control mice to 59.6% in Transferon-treated mice. The activity of Transferon correlated with increased serum levels of IFN-γ and reduced IL-6 and TNF-α concentrations. Our results demonstrate that (i) this mouse model of cutaneous herpes can be used to examine the activity of DLEs, such as Transferon; (ii) the assay can be used as a routine test for batch release; (iii) Transferon is produced with high homogeneity between batches; (iv) Transferon does not have direct virucidal, cytoprotective, or antireplicative effects; and (v) the protective effect of Transferon *in vivo* correlates with changes in serum cytokines.

1. Introduction

Governments worldwide have established standards for the production and release of biotechnological and biological drugs. For example, the US Food and Drug Administration (FDA) and Japanese Ministry of Health & Welfare (MOHW) modified their guidelines regarding biologics in 2007, and the European Medicines Agency (EMA) did so in 2008 [1–3]. In 2012, the Sanitary Risk Authority of Mexico (COFEPRIS) released the Official Mexican Standard NOM-EM-001-SSA1-2012.

A key element in the quality control of the production of biological drugs is the demonstration of their activity, and their efficacy must be preserved between commercial batches. Due to the unique nature and production of biological drugs, specific methods must be developed to evaluate their quality and attributes, including efficacy.

Human dialyzable leukocyte extracts (DLEs) are heterogeneous mixtures of low-molecular-weight peptides (<10 kDa) that are released on disruption of peripheral blood leukocytes from healthy donors [4]. DLEs are produced and commercialized worldwide. In certain countries, such as México, China, Cuba, and the Czech Republic, DLEs are registered as drugs [5–8] because they improve clinical responses in infections, allergies, cancer, and immunodeficiencies (see Berrón-Pérez et al. [9] and Viza et al. [10] for extensive reviews). Their complexity, however, has impeded an extensive characterization of their components, active substances, and biological activities.

Transferon is a human nonspecific DLE that is manufactured by the National School of Biological Sciences (ENCB), National Polytechnic Institute (IPN), Mexico, at GMP facilities. Transferon is registered by Mexican health authorities as a drug and is commercialized nationally. To establish an assay that could be used routinely as a quality control test for Transferon, we aimed to standardize and validated a method to determine its efficacy in animals. Other studies have demonstrated that the activity of DLEs can be measured by assessing the induction of delayed type hypersensitivity (DTH) in mice [11] and *in vitro* by analyzing their effects on leukocyte migration [12] or IFN-γ secretion [8].

DLEs are effective for treating parasitic infections (acute leishmaniasis [13] and alveolar echinococcosis [14]) and viral infections (herpes simplex virus-1 (HSV-1) and herpes zoster). Clinical trials have shown that DLEs mitigate the duration of the acute phase, the frequency of recurrences, and pain in herpes zoster patients better than acyclovir [15, 16]. These effects correlate with increased IFN-γ levels and CD4+ cell counts [17]. Considering the clinical effects of DLEs against herpetic infections and because animal models reflect the complexity of a drug response in an entire organism, we selected a murine model that has been reported to emulate the natural form of HSV-1 infection [18]. This murine model was standardized, validated, and used to evaluate the biological activity of 27 batches of Transferon.

Table 1: Quality attributes of evaluated batches of Transferon.

Batch	Protein (mg/mL)	MAB[#] (CFU/mL)	FF&Y[&] (CFU/mL)	Endotoxin (EU/mL)
12A01	0.392	<1	<1	<0.05
12C01	0.438	<1	<1	0.197
12C03	0.432	<1	<1	<0.05
12C04	0.445	<1	<1	<0.05
12C05	0.419	<1	<1	0.167
12C06	0.394	<1	<1	<0.125
12C07	0.428	<1	<1	<0.125
12D08	0.427	<1	<1	<0.05
12F09	0.427	4	<1	<0.05
12G11	0.347	<1	<1	<0.05
12H14	0.421	<1	<1	0.098
12H15	0.406	<1	<1	<0.05
12K20	0.430	<1	<1	<0.125
12K21	0.436	<1	<1	<0.125
12L22	0.432	<1	<1	<0.125
12L23	0.448	<1	<1	<0.125
13A03	0.417	<1	<1	<0.125
13B07	0.423	<1	<1	<0.25
13C08	0.408	<1	<1	<0.25
12M24	0.397	<1	<1	0.125
12M25	0.422	<1	<1	<0.125
13A02	0.425	<1	<1	<0.125
13C09	0.445	<1	<1	<0.25
13A04	0.398	<1	<1	1.0
13C10	0.397	<1	<1	<0.25
13B05	0.389	<1	<1	<0.25
13B06	0.389	<1	<1	<0.25

[#]Mesophilic aerobic bacteria; [&]Filamentous fungus and yeast.

2. Materials and Methods

2.1. Quality Control of Transferon. We tested Transferon batches that were produced between 2011 and 2013 using a modified version according to Borkowsky et al. [19]. Briefly, leukocytes from 1000 healthy donors were lysed with 5 freeze-thaw cycles and dialyzed against a 12-kDa membrane to obtain low-molecular-weight peptides.

The quality control of Transferon comprised (A) endotoxin content, quantified using the Endosafe-Portable Test (Charles River Laboratories, Charleston SC, USA) according to the manufacturer's instructions; the specification for endotoxin was established in Mexican Pharmacopeia, Section MGA-0316 (≤4.0 EU/mL) [20]; (B) microbiological tests, according to Mexican Pharmacopeia, Section MGA-0571 [21]; (C) physicochemical characterization by a validated ultraperformance liquid chromatography (UPLC) method [8] that analyzes molecular weights and the time of retention of the main peaks compared with those of an internal batch pattern. (D) Peptide content per final dose was measured by bicinchoninic acid (BCA) method using the Pierce BCA kit (Thermo Fisher Scientific, Waltham MA, USA) according to the manufacturer's instructions.

2.2. Herpes Simplex Murine Model. HSV-1 (KOS strain) was obtained from American Type Culture Collection (ATCC; Manassas VA, USA). The virus was propagated in African green monkey kidney (Vero) cells (ATCC CCL-81) that were cultured in Eagle's minimal essential medium (EMEM; ATCC) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Carlsbad CA, USA). Cutaneous infection of herpes was performed by inoculating 5-week-old male BALB/c mice (Ferandelh, Mexico City, Mexico) with HSV-1, as reported [18, 22, 23]. Briefly, mice were anesthetized, and 10 μL of a viral suspension that contained 5×10^6 plaque-forming units (PFU)/mL was administered by cutaneous scarification on 1 cm^2 of plucked dorsal skin [23–25]. Mice

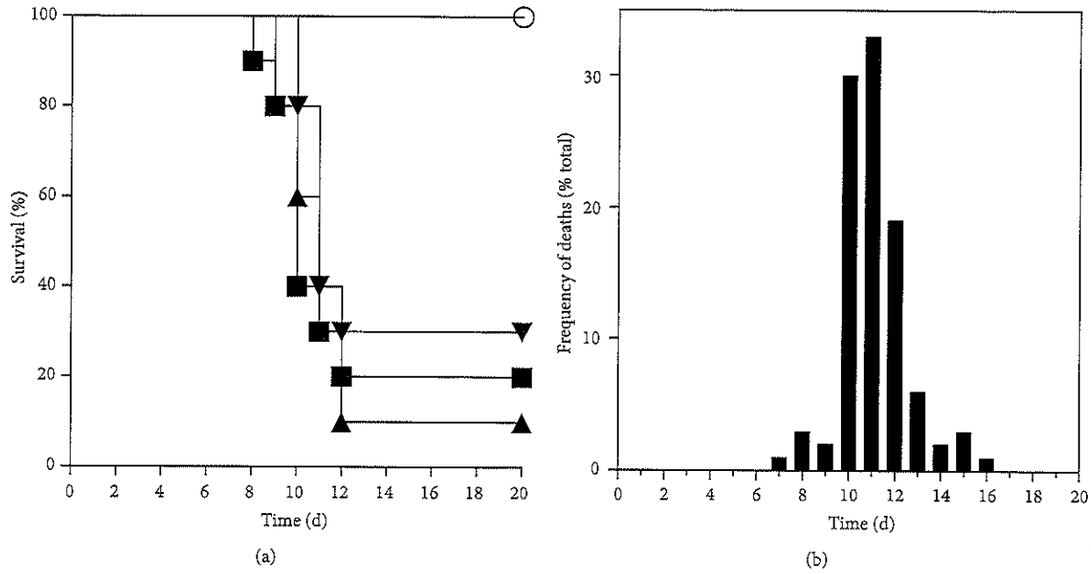

FIGURE 1: Standardization of mouse cutaneous herpes model. (a) Three different experiments performed with 5.0×10^4 PFU of HSV-1 (closed symbols). In each experiment, infected mice showed significantly lower survival than controls (open circles; log-rank Mantel-Cox test; $P < 0.001$). (b) Frequency of deaths from the experiments shown in (a).

were monitored daily for 20 days to identify infection-associated symptoms, such as paralysis of the lower extremities, reduced mobility, and weight loss. When the animals experienced total loss of mobility, they were euthanized and counted as deaths for the survival analysis.

Mice had free access to food (Harlan Laboratories, Indianapolis IN, USA) and water during the experiment. All experiments were performed blindly by trained researchers.

The administration of Transferon began on day 2 after infection and continued every other day until day 10. Doses employed were 12.5 ng, 0.125 μg, 0.25 μg, 0.5 μg, 0.75 μg, 1.0 μg, or 1.50 μg per mouse (each weighing 14–16 g at day 0) and perorally administered in 200 μL. Each experimental group comprised 10 mice. All experiments included a control group of mice that received placebo (pyrogen-free water; PISA Pharmaceutical, Mexico City, Mexico). Survival per group was plotted as Kaplan-Meier graphs and analyzed by Mantel-Cox test ($\alpha = 0.05$) using Prism 5 Project, V 6.0 (GraphPad Software Inc, San Diego CA, USA).

2.2.1. In Vitro Effects on HSV-1. We studied the possible direct antiviral effect of Transferon by mixing equal volumes (50 μL) of Transferon (0.4 mg peptide/mL) and HSV-1 (5.17×10^7 PFU7mL) and incubating the mixture for 60 minutes at 37°C. The infectivity of Transferon-treated virus was determined by analyzing the induction of a visible cytopathic effect (CPE) on Vero cells (10^4 cells/well) at 120 h, as reported [26]. Fifty percent tissue infective dose ($TCID_{50}$) was calculated by Spearman-Kärber method [27]. Cytopathology induced by HSV-1 (100 PFU) in presence of Transferon (10 pg/mL–10 μg/mL) or acyclovir (Zoviraz, GlaxoSmithKline, Mexico City, Mexico) was determined using MTS (CellTiter 96 Aqueous One Solution cell proliferation assay reagent; Promega, Madison WI, USA) as previously reported [28, 29]. The cytoprotective effect of Transferon on target cells was evaluated by preincubating Vero cells (10^4 cells/well) with Transferon (20 μg/mL) for 24 hours before addition of HSV-1 (viral stock 5.17×10^7 PFU/mL). CPE was evaluated 120 h after infection [26]; $TCID_{50}$ calculation was performed as reported [27]. To assess the effect of Transferon on virus replication, 2×10^5 Vero cells were infected with HSV-1 (2000 PFU) and incubated with Transferon (20 μg/mL) or Acyclovir (10 μg/mL) for 24 h at 37°C. Subsequently, viruses were recuperated from cultures through one thaw/freeze cycle and sonication. After removing cell debris by centrifugation (5000 g), the total virus yield on each well was titrated by diluting samples and incubating them with Vero cells for 120 h. We analyzed the presence of CPE at the microscope and by MTS assay. As reported by Heldt et al. [28], the $TCID_{50}$ was defined as the concentration at which absorbance was 50% of the average absorbance from uninfected cells and was determined by nonlinear regression using Prism 6 for Mac OS X (GraphPad Software Inc).

2.2.2. Cytometric Bead Array. To measure cytokine concentrations, blood samples from the orbital sinus were collected from anesthetized mice on days 7 and 9 after infection. Serum was obtained, and samples were stored at −20°C until analysis. Cytokine concentrations were determined using a cytometric bead array (CBA) mouse inflammation kit (BD

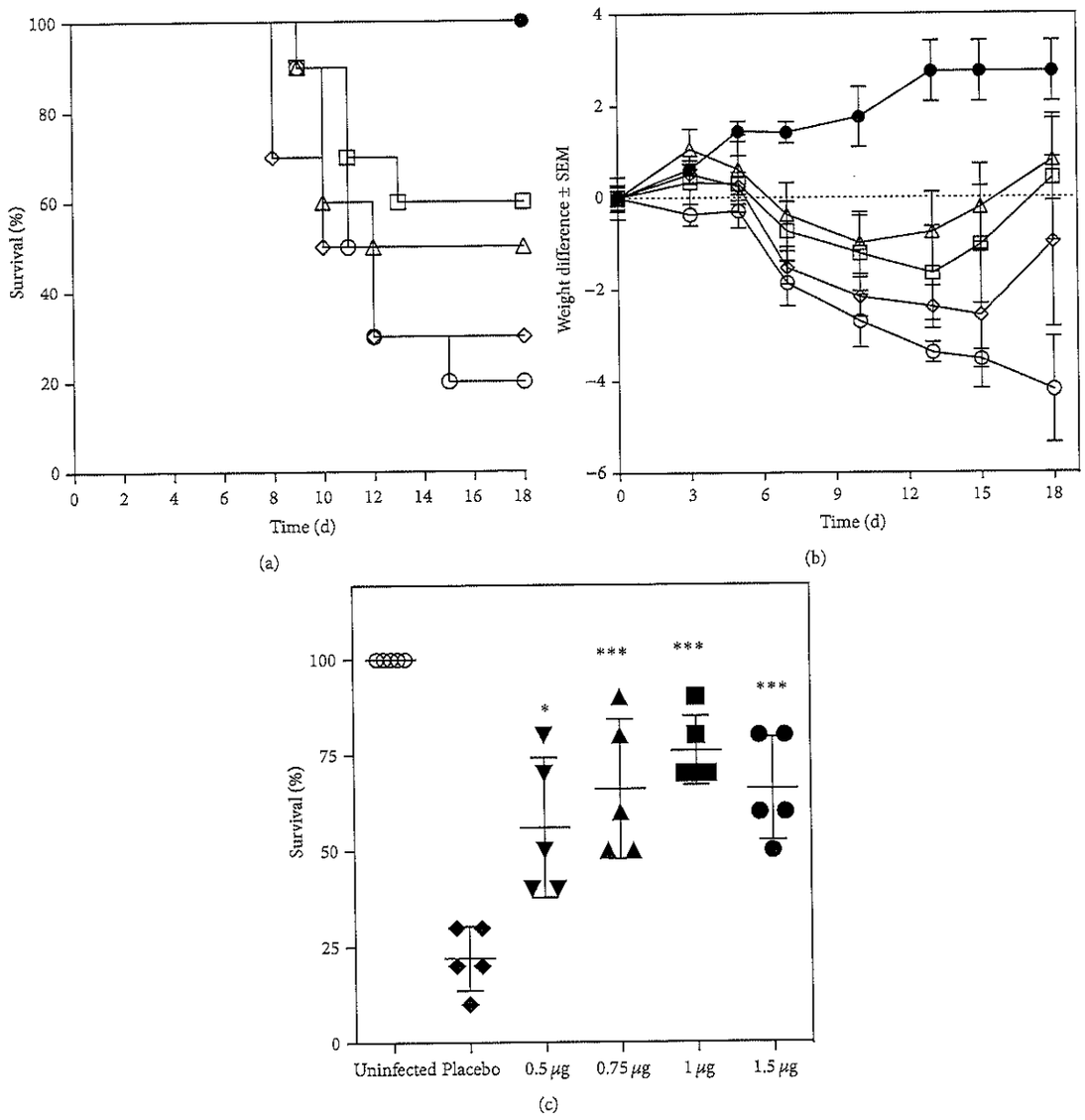

FIGURE 2: Validation of the murine herpes model for the evaluation of Transferon activity. (a) Survival curves for HSV-1 infected mice. Treatments with 1 μg (open triangles) or 0.125 μg (open squares) of Transferon improved survival over placebo (open circles; Log-rank Mantel-Cox test; $P < 0.01$). In contrast, 12.5 ng of Transferon (open diamonds) had no significant effect. A group of mice was left uninfected (closed circles) as control. (b) Weight changes in HSV-1 infected mice. Transferon partially protects mice from the weight loss induced by HSV-1 (symbols are as in (a)). (c) Survival induced by five different Transferon batches was evaluated during a validation protocol. All batches improved survival versus placebo (Bonferroni t test; $^{*}P < 0.05$, $^{**}P < 0.01$; $^{***}P < 0.001$).

Biosciences, San Diego CA, USA) according to the manufacturer's instructions. We used a FACS Aria flow cytometer and BD CBA software (BD Biosciences) for data acquisition and analysis.

2.2.3. Ethics Statement. All experimental procedures with animals were performed according to the Mexican Guidelines for the Production, Care, and Use of Laboratory Animals (NOM-062-ZOO-1999) and the International Guide for the Care and Use of Laboratory Animals [30]. All efforts were made to minimize animal suffering and reduce the number of animals that were used. The experimental procedures were approved by the Ethical Committee of the Transfer Factor Project in "Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional" (protocol FTU/IB/012/010/PRO).

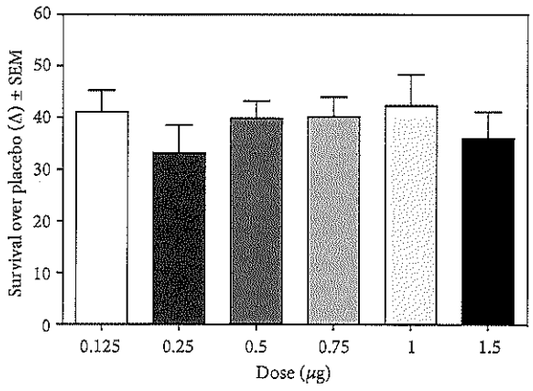

FIGURE 3: Evaluation of biological activity of 27 Transferon batches in the validated murine model of herpes. Statistical analysis (ANOVA, Bonferroni's Multiple Comparison Test) showed no differences between doses.

3. Results

3.1. Quality Control of Batches. All batches had endotoxin levels below 0.05 EU/mL and met the microbiological specifications in Mexican Pharmacopeia (aerobic mesophile bacteria <100 colony-forming units (CFU)/mL, filamentous fungi <10 CFU/mL, and yeasts <10 CFU/mL (Table 1)). By UPLC, we performed a physicochemical characterization of the batches. In the chromatographic profiles of the Transferon batches, we noted 8 peptidic fractions between 17 to 0.2 kDa, as previously reported [8]. The retention times of the main peaks corresponded with those of an internal pattern, defined as a batch of Transferon that satisfied all quality control tests for this product, as previously reported [8]. We also measured peptide concentrations in the Transferon samples. All batches had peptide concentrations that met the established specification of 0.400 ± 0.06 mg/mL. The average concentration in the 27 batches was 0.416 ± 0.023 mg/mL (Table 1).

3.2. Herpes Murine Model. To develop a murine model of cutaneous herpes, we first studied the effect of an HSV-1 inoculum on mouse survival. We inoculated 3.5×10^4, 5.0×10^4, and 5.5×10^5 PFU of HSV-1 and analyzed mouse survival (Figure 1). In subsequent experiments, we used 5.0×10^4 PFU of HSV-1, which was the minimum inoculum that produced a decrease in survival within 70% to 100%, as reported [23–25].

Then, we determined the reproducibility of the effect of the infection by repeating the assay 3 times and measuring survival. The survival of infected animals was 10% to 30% (Figure 1(a)). Using data from the same experiments, we calculated the frequency of deaths (euthanizations) over time, noting that 85% of deaths occurred before day 13 after infection, rising to 100% by day 16 (Figure 1(b)). This information allowed us to establish an endpoint for subsequent assays.

3.3. Biological Evaluation of Transferon. To determine the doses of Transferon that were to be used to evaluate the commercial batches, we examined a ≈100-fold range of doses (12.5 ng–1.50 μg per mouse). Doses above 0.125 μg/mouse were equally efficacious in reducing HSV-1-induced mortality and weight loss. In contrast, 12.5 ng was ineffective, indicating that the activity of Transferon is dose-dependent. As expected, uninfected mice gained weight and showed 100% survival (Figures 2(a) and 2(b)).

The model was validated using 5 batches that were produced in 2011 and tested at 4 doses (Figure 2(c)). We evaluated (i) system specificity, by analyzing responses in placebo- and Transferon-treated animals; the average survival in controls was 23.3% (range 10% to 30%), whereas all Transferon doses induced a significant increase in survival (average increase versus placebo (Δ survival) 43.5%); (ii) system precision, by calculating the relative standard deviation (RSD) percentage for the results with each Transferon dose; % RSD was below 30% in all cases (range: 11.7% to 28.3%); (iii) system suitability, by corroborating that at least 1 dose produced a Δ survival ≥ 40%. All parameters were within the limits that were established in the validation protocol.

Eighteen batches that were produced in 2012 and 9 that were generated in 2013 were examined in the validated murine HSV-1 model. In certain batches, we evaluated 2 additional doses (0.125 and 0.25 μg per mouse). All batches improved the survival of HSV-1-infected mice by an average of 37.8% (range: 30.5% to 43.3%) (Figure 3). There were no differences in average survival between doses, indicating that any of these doses could be used in future quality control assays.

3.4. Effects of Transferon on HSV-1 Infectivity. We measured the antiviral activity of Transferon by (i) preincubating HSV-1 with the drug before adding it to the target (Vero) cells (Figure 4(a)) and (ii) simultaneously incubating target cells with HSV-1 and various Transferon concentrations (10 pg/mL–10 μg/mL) (Figure 4(b)). Transferon did not show direct antiviral effect on either experimental system. Preincubation of target cells with Transferon for 24 h before the viral challenge did not prevent HSV-1 infection (Figure 4(c)), suggesting that Transferon does not modify the target cell-susceptibility to infection. Additionally, we evaluated the effect of Transferon on virus replication. The titration of HSV-1 on Transferon-treated samples showed that the $TCID_{50}$ is not different from that of control samples. Values obtained from nonlinear regression fit were $-\log_{10} = 2.711$ versus $-\log_{10} = 2.752$, respectively (Figure 4(d)). These results, obtained by a colorimetric assay, correlate with the visual analysis of CPE (Supplemental Figure 1 available online at http://dx.doi.org/10.1155/2014/146305), as reported for others viruses [28], and demonstrate that Transferon has no effect on HSV-1 replication.

3.5. Effects of Transferon on Systemic Cytokines. Blood (serum) cytokine levels were measured at days 7 and 9, before the period of high mortality. TNF-α, IL-6, and IFN-γ levels rose in HSV-1-infected and placebo-treated mice versus uninfected mice (Figures 5(a)–5(c)). In contrast, Transferon did not change the cytokines levels in uninfected mice. Treatment of HSV-1-infected mice with Transferon

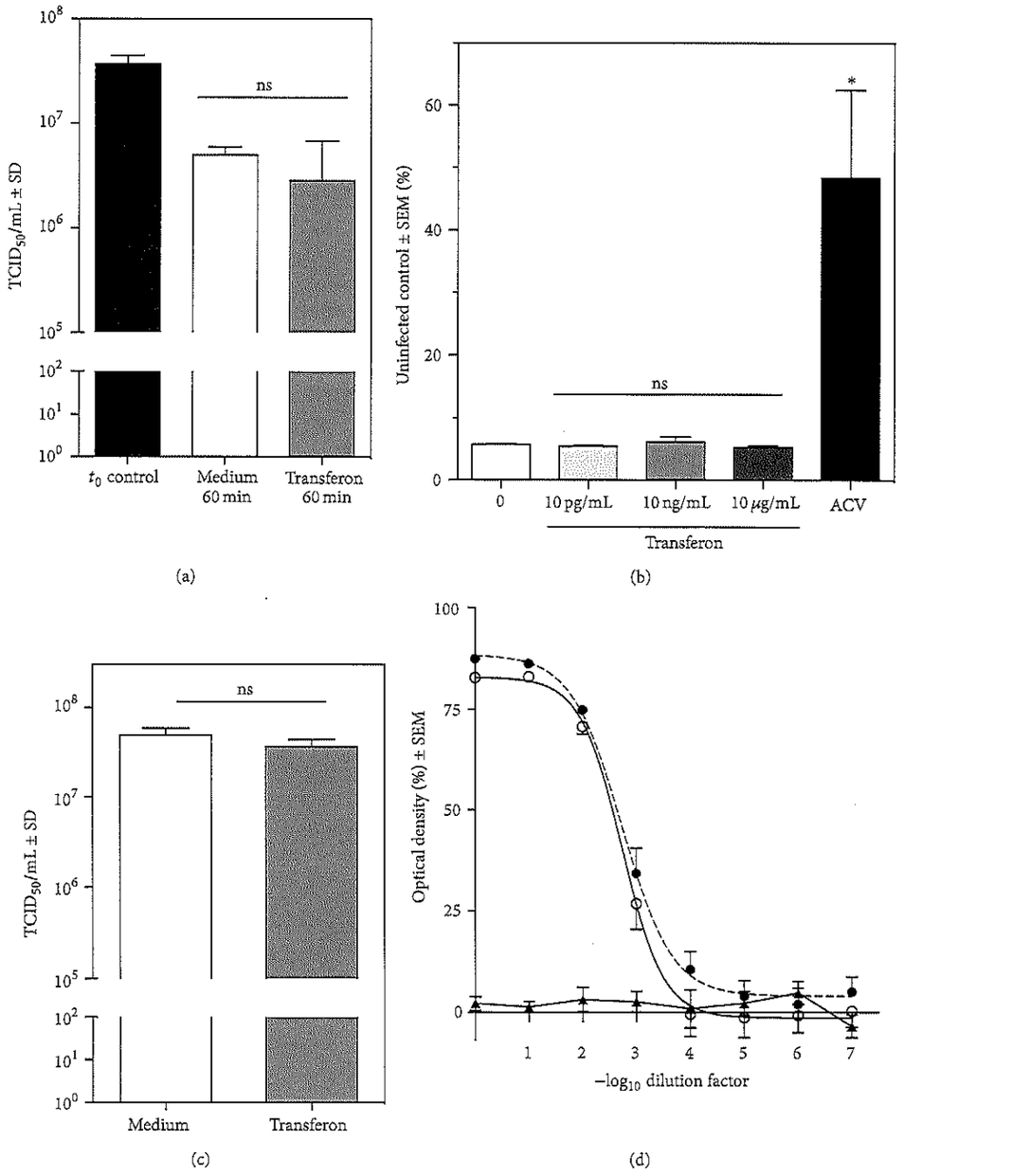

FIGURE 4: *In vitro* evaluation of antiviral activity of Transferon. (a) HSV-1 was preincubated for 60 min with Transferon (20 μg/mL) or medium before evaluation of visible cytopathic effect (CPE) on Vero cells. Viruses with no preincubation (t_0) were used as control (ANOVA, Bonferroni's Multiple Comparison Test; ns: nonsignificant). (b) Effect of Transferon (10 pg/mL–10 μg/mL) on HSV-1-induced cytopathology, evaluated by MTS assay. Acyclovir (ACV; 10 μg/mL) was included as a positive control. The graph represents data from 2 independent experiments (ANOVA, Bonferroni's Multiple Comparison Test; $^*P < 0.0001$). (c) $TCID_{50}$ obtained from Vero cells preincubated for 24 h with Transferon (20 μg/mL) or medium prior to HSV-1 infection (Student's t test). (d) Titration of samples obtained from Vero cells infected 24 h with HSV-1 and simultaneously treated with medium (open circles) or 20 μg/mL of Transferon (closed circles). ACV (10 μg/mL; triangles) was included as a positive control. Sum-of-squares F test showed no differences between the $TCID_{50}$ from medium- and Transferon-treated cells ($P = 0.7527$). The graph represents data from 2 independent experiments.

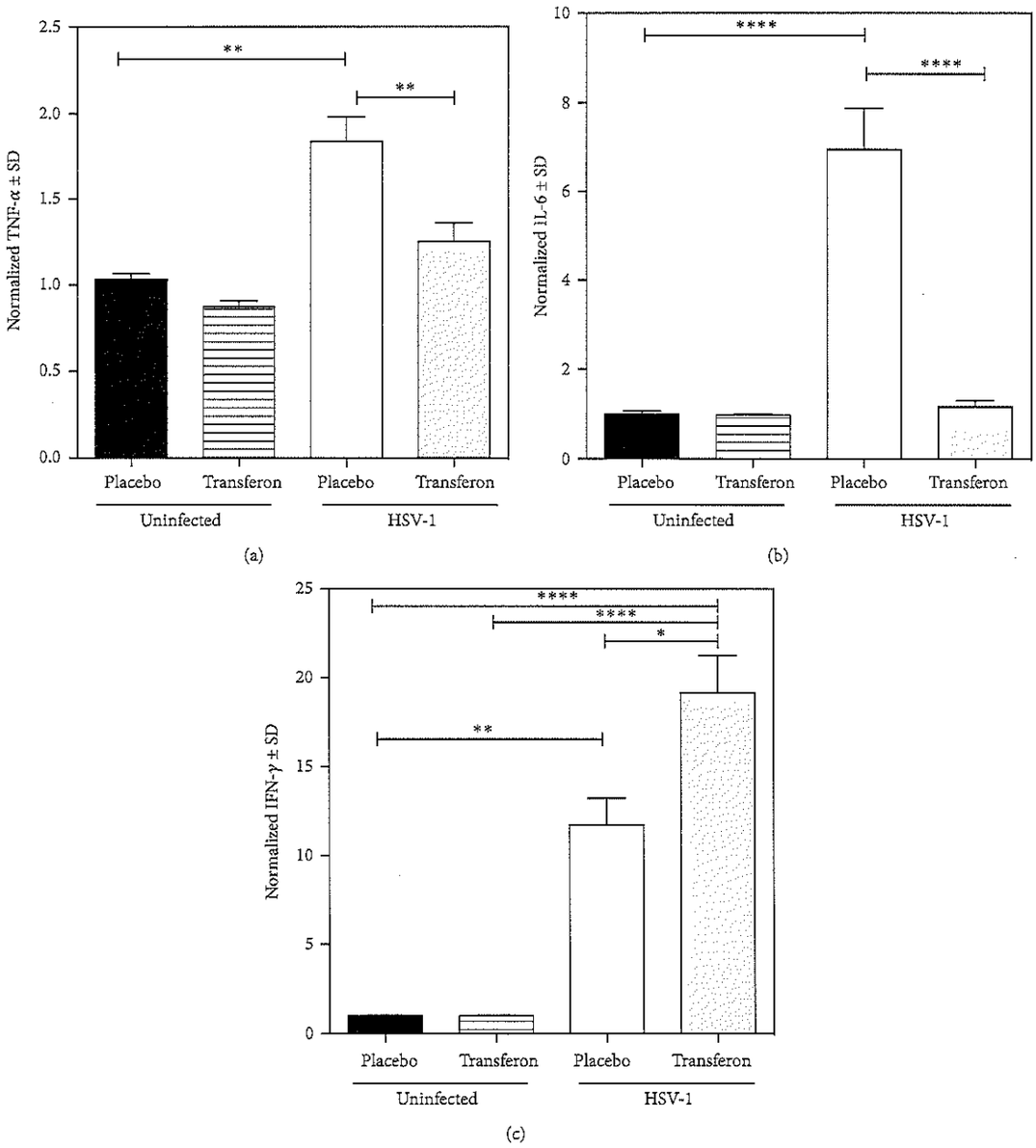

FIGURE 5: Quantitative analysis of blood (serum) cytokine levels. Transferon (Batch 12C04; 0.75 μg/mouse) significantly reduced the concentrations of TNF-α (a) and IL-6 (b) at day 9 postinfection, but it increased that of IFN-γ at day 7 (c). Transferon administration had no effect on uninfected mice. All measurements were normalized to placebo-treated, uninfected controls. The graphs represent data of 2 independent experiments (ANOVA, Bonferroni's Multiple Comparison Test; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$).

significantly decreased TNF-α and IL-6 levels at day 9, compared with placebo-treated animals (Figures 5(a) and 5(b). In contrast, Transferon further increased IFN-γ levels at day 7 (Figure 5(c)).

4. Discussion

All 27 batches of Transferon in this study complied with quality standards with regard to their attributes. As shown for batches that were produced in 2010-2011 [8], we noted high homogeneity in microbial content, peptide concentration, molecular weight, and time of retention of the main peaks by UPLC. These results demonstrate that is possible to produce

a mixture of peptides that have been extracted from complex raw materials, such as lysed human leukocytes.

To evaluate the activity of these batches, a murine model of herpes was standardized and validated. Transferon partially protected animals from the HSV-1-induced weight loss, which correlated with greater survival. This model was chosen because DLEs are effective in clinical studies of herpetic infections. DLEs significantly reduce the average duration of the acute phase and the frequency of recurrences in herpetic infections as successfully as antiviral drugs [15, 16]. All batches of Transferon significantly increased survival dose-independently, indicating that it is biologically active over a wide range of doses (10-fold). Thus, our murine model of herpes can be used to measure efficacy—a fundamental attribute of biological drugs—in DLEs.

However, the herpes murine model has several disadvantages. The variability that is intrinsic to a whole-animal model is significant; such a model requires the use of many animals. Also, more time is needed to evaluate activity than for an *in vitro* experiment.

Transferon has no direct virucidal or antireplicative effects on HSV-1 nor cytoprotective effects on target cells, suggesting that the *in vivo* activity of Transferon is mediated by its effects on the immune system. We found that HSV-1-infected mice had higher serum concentrations of TNF-α, IL-6, and IFN-γ compared with uninfected controls. Consistent with these findings, in astrocyte cultures, HSV-1 infection upregulates TNF-α, IL-6, and NF-κB in a Toll-like receptor (TLR)-3-dependent manner [31]. Administration of Transferon to HSV-1-infected mice downregulates TNF-α and IL-6, suggesting that it suppresses innate immune responses. The modulation of proinflammatory cytokines by DLEs has been associated with reduced inflammation-associated tissue damage by pathogens [32, 33].

Conversely, Transferon increased IFN-γ, a cytokine that is produced during the adaptive phase of immunity, suggesting that Transferon indirectly stimulates the activation of T lymphocytes that are specific for HSV-1. IFN-γ effects resistance against HSV-1 infection [34], and its upregulation in DLE-treated herpes patients favors a positive clinical response [17] and limits relapses [16]. Although the mechanisms of action of DLEs have not been determined completely, our results indicate that they have differential effects on innate and adaptive immunity.

5. Conclusions

Our analysis of 27 batches of Transferon demonstrate that (i) the cutaneous model of herpes can be used to evaluate the biological activity of DLEs, such as Transferon; (ii) the assay can be used as a routine test for batch release; (iii) Transferon is produced with high homogeneity between batches, meeting the standards that are required for hemoderivatives that are intended for clinical use; (iv) Transferon does not have direct virucidal, cytoprotective, or antireplicative effects; and (v) the protective effects of Transferon in our murine model correlate with changes in serum cytokine levels.

Conflict of Interests

Nohemí Salinas-Jazmín, Sergio Estrada-Parra, Miguel Angel Becerril-García, Alberto Yairh Limón-Flores, Said Vázquez-Leyva, and Sonia Mayra Pérez-Tapia are employees or have been compensated for their work at "UDIMEB" the producer of Transferon. All other authors declare no competing interests.

Acknowledgments

This work was partially supported by FTU/IB/12/010/PRO (Nohemí Salinas-Jazmín) and CONACYT INFR-2014-01 225313 (MAV-V). Authors are grateful for the valuable help of Natanael Valtierra-Botello and Leonardo López-Juárez (animal housing), Emiliano Hisaki-Itaya (flow cytometry data acquisition and analysis), Gilberto Pérez-Sánchez (validation protocol), and Antonio Ortega-Roque (cell and virus culture).

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Anexo 3

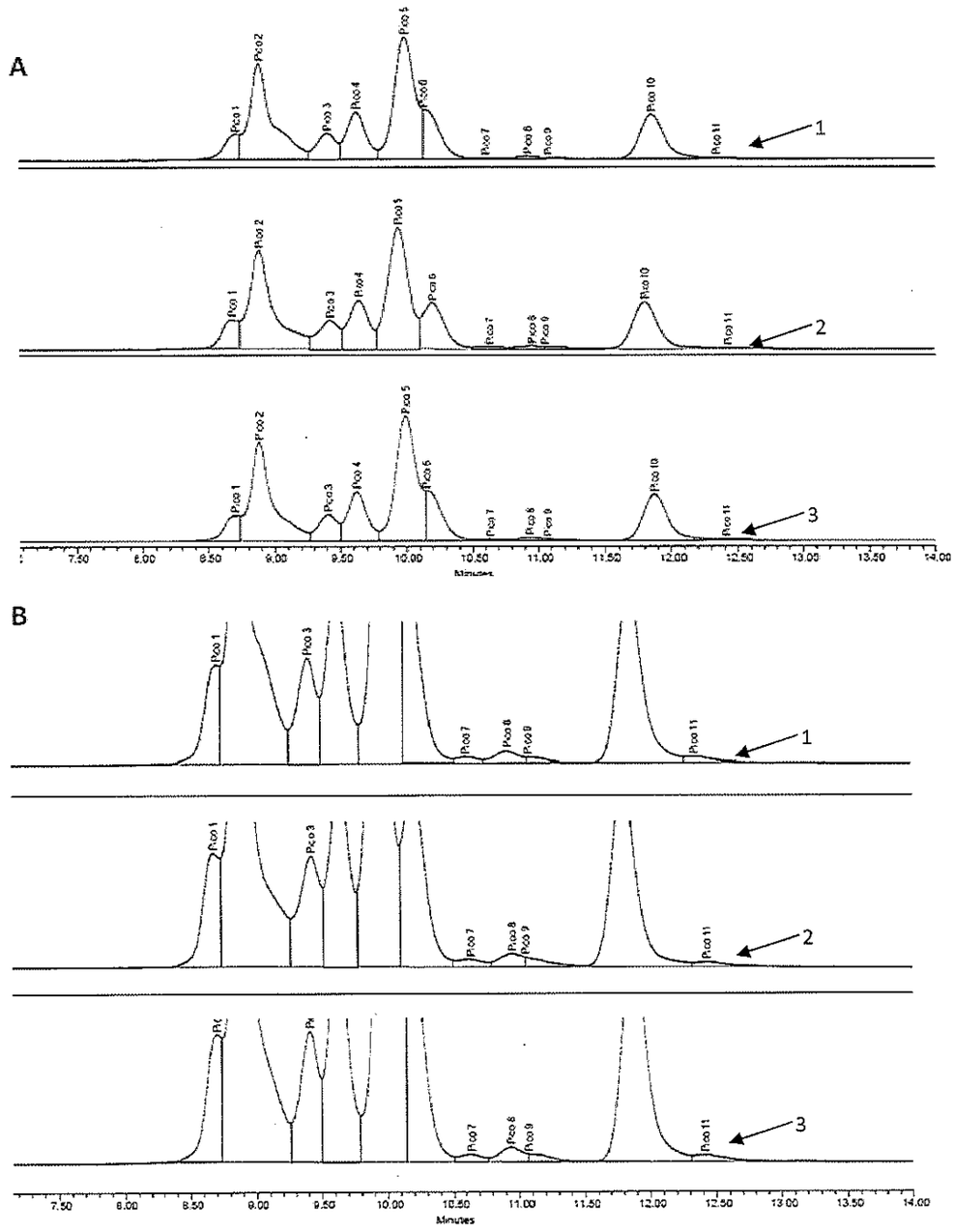

Figura 1. Perfiles cromatográficos empleado la columna BEH 200 de los lotes 13C08 (1) ,13C09 (2) y 13C10 (3). Los perfiles cromatográficos de los lotes valuados muestran 11 picos principales (A). La sección B es una ampliación de los cromatogramas mostrados en A.

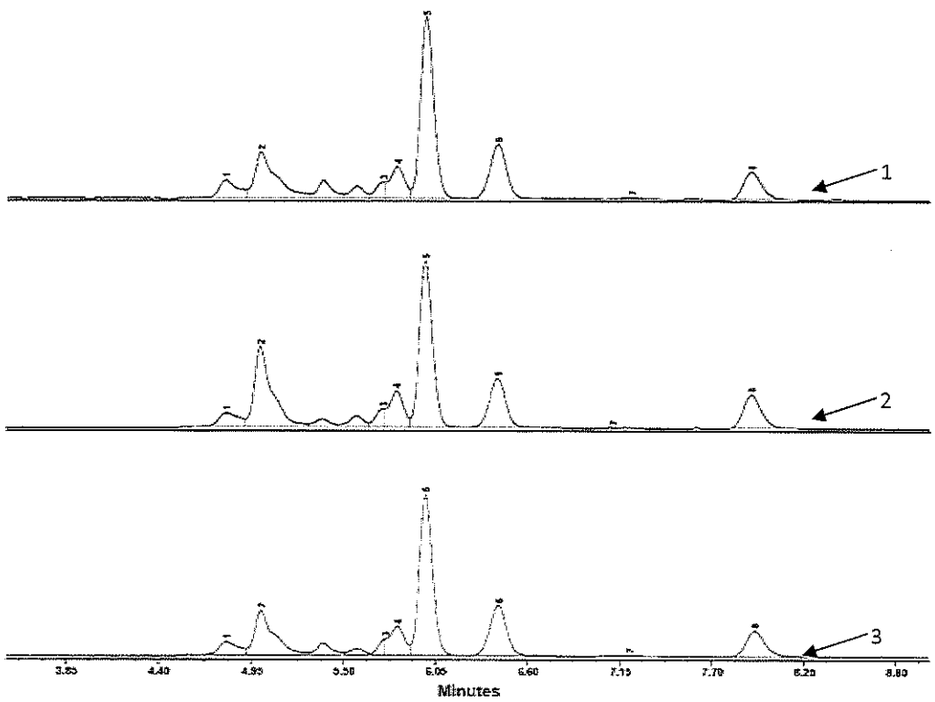

Figura 2. Perfiles cromatográficos empleando la columna BEH 125 de los lotes 13C08 (1), 13C09 (2) y 13C10 (3). Estos perfiles cromatográficos muestran 8 picos principales, resultado similar al mostrado por Medina et. al., 2014.