

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO	
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DEP: 2020 DIRECCION DE NUEVAS CREACIONES	EVE: 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS: 17
ACT: 519 PRESENTACION	

DIRECCIÓN DE NUEVAS CREACIONES

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Bogotá, D.C.,

Abogado(a)/Señor(a)
JAIME EDUARDO DELGADO VILLEGAS

Asunto: Radicación: 11-12635- -5
Trámite: 11
Evento: 378
Actuación: 519
Oficio: 1433
Folios: 17

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/US2009/052702				
Fecha de Presentación Internacional	04/08/2009				

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	No. 11-012635-00000-0000	03/Febrero/2011
Reivindicaciones:	No. 11-012635-00000-0000	03/Febrero/2011
Dibujos:	No. 11-012635-00000-0000	03/Febrero/2011
Secuencias:	No. 11-012635-00000-0000	03/Febrero/2011
Prioridad:	US 61/085,980	04Agosto/2008

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del capítulo primero del Título 10 de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las

cubiertas por la tasa pagada. En el presente caso se estudiará la totalidad del capítulo reivindicatorio No. 11-012635-00000-0000 (reivindicaciones 1-35).

Título de la solicitud: “Anticuerpos monoclonales contra el inhibidor de la vía del factor tisular (TFPI)”.

El problema planteado en esta solicitud consiste en la necesidad de desarrollar anticuerpos completamente humanos anti-TFPI para evitar la inmunogenicidad de los anticuerpos y ser usados en el tratamiento de enfermedades hematológicas y cáncer.

Para resolver este problema técnico la solicitud en estudio proporciona anticuerpos monoclonales contra el factor de la vía tisular (TFPI) humano, composiciones farmacéuticas que comprenden los anticuerpos y procedimientos de tratamiento de deficiencias o defectos en la coagulación genéticos y adquiridos.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C07K 16/36, C12P 21/08.

4. No es una invención (Art 15 D 486 literal (c))

El objeto de las reivindicaciones 22-23, 32 no se considera una invención, de acuerdo con el Art citado.

5. Excepción a la Patentabilidad (Art 20 D 486 literal (d))

El objeto de las reivindicaciones 15-18, 20-21, 24-25, 34-35 no es patentable de acuerdo con el Art citado.

6. De la solicitud de patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

- 1- Las reivindicaciones 1-6, 9-11, 13, 22, 32 no son claras y dan lugar a múltiples posibilidades de anticuerpos que no tienen sustento. No se puede reclamar el anticuerpo por referencia exclusiva a una sola CDR como se presenta en la reivindicación 1. El anticuerpo debe ser definido a partir de su estructura completa, esto es, de las secuencias de las regiones variables de la **cadena ligera y cadena pesada o las secuencias de los 6 CDRs**. La alusión a segmentos parciales de la molécula no permite definirla, menos aun cuando dicha alusión no establece ni siquiera todos los segmentos de unión (CDRs) de la misma. Se sugiere restringir la solicitud a los anticuerpos que tienen sustento en la descripción. Se le recuerda al solicitante que al hacer referencia a las secuencias de regiones variables se deben definir por las secuencias completas de la región variable y no por los CDR que contiene.
- 2- La reivindicación 12 se refiere al resultado a alcanzar con el anticuerpo, que sería la disminución del tiempo de coagulación sanguínea en presencia del anticuerpo, medido mediante el tiempo de protrombina diluida, pero no define la(s) características técnicas relacionadas con dicho resultado a alcanzar. Se recomienda eliminar dicha reivindicación.
- 3- El método de producción del anticuerpo debe ser caracterizado a partir de las etapas específicas que posee e indicar las modificaciones específicas realizadas al anticuerpo (reivindicaciones 28-31).

- 4- La molécula de ácido nucleico debe ser caracterizada a partir de la secuencia nucleótida de la misma y hacer referencia a la(s) secuencia(s) del anticuerpo completo (reivindicaciones 26-27).
- 5- La composición de las reivindicaciones 14, 19, 23, 33 debe ser caracterizada a partir de los elementos que la conforman (sus ingredientes activos y excipientes) y las proporciones de los mismos.
- 6- No existe soporte de la obtención de la composición de la reivindicación 19. Se sugiere eliminar dicha reivindicación.
- 7- La reivindicaciones 7, 8, 9 y 10 no son concisas, se sugiere restringir la solicitud a una razonable generalización de anticuerpos que han sido sintetizados ó probados, es decir, que se encuentran debidamente sustentados.
- 8- El capítulo reivindicatorio no es conciso porque contiene un excesivo número de reivindicaciones independientes, lo cual hace difícil definir cuál es el objeto que se busca proteger.
- 9- Los anticuerpos de las reivindicaciones 9-10 utilizan términos ambiguos como “que tiene al menos una identidad del %”, que no permiten definir el alcance de la invención. Estas expresiones además carecen de sustento en la descripción.

Se requiere al solicitante hacer las aclaraciones necesarias.

7. Unidad de invención (Art 25 D 486)

La solicitud no cumple con el requisito de unidad de invención porque se refiere a 44, grupos inventivos.

Los grupos inventivos están definidos por las siguientes características esenciales:

1° - Anticuerpos anti-TFPI que comprenden una secuencia de cadena ligera que comprende CDR1, CDR2, CDR3 de SEQ ID NO: 173, 216, 259, respectivamente y una cadena pesada que comprende CDR1, CDR2, CDR3 de SEQ ID NO: 302, 345 y 388, respectivamente; ácidos nucleicos, células huésped y métodos de producción de los polipéptidos (cuyo concepto común inventivo, anticuerpos de origen humano, están divulgados en los documentos US2004/0171538 y D2): Reivindicaciones 1-14, 19, 22-23, 26-33 parcialmente.

2- 44° Anticuerpos anti-TFPI que comprenden 6 CDRs, ácidos nucleicos, células huésped y métodos de producción de los polipéptidos (cuyo concepto común inventivo, anticuerpos de origen humano, están divulgados en los documentos US2004/0171538 y D2): Reivindicaciones 1-14, 19, 22-23, 26-33 parcialmente.

Se le sugiere al solicitante restringir la materia a patentar en un solo concepto común inventivo especificando el avance tecnológico sobre el estado de la técnica. Esto se logra entre otras formas definiendo las características esenciales de la invención y mostrando las otras características particulares como dependientes, pero sin perjuicio de la concisión de las nuevas reivindicaciones tanto independientes como dependientes.

El solicitante también puede realizar una o más de las divisionales de acuerdo a los grupos de conceptos técnicos inventivos presentados en esta solicitud, esto claro, sin ampliar el objeto inicial de la invención.

8. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: Agosto 04 de 2008 y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	WO9709063	"Uses of TFPI inhibitor for treatment of cancer"	13/Marzo/1997
D2	Welsch, et al., 1991. Thrombosis Research. Vol. 6: 2. Págs. 213–222	"Effect of lipoprotein-associated coagulation inhibitor (LACI) on thromboplastin-induced coagulation of normal and hemophiliac plasmas"	15/Octubre/1991
D3	US 7,015,194	"Pharmaceutical composition comprising factor VIIa and anti-TFPI"	21/Marzo/2006

El documento D1¹ divulga el uso de anticuerpos anti-TFPI para el tratamiento de tumores. El documento sugiere el uso de anticuerpos humanos para el tratamiento en humanos (Pág. 9).

El documento D2² divulga el efecto curativo en pacientes con hemofilia con la administración de anticuerpos policlonales y monoclonales que se unen a TFPI (Resumen).

El Documento D3³ divulga composiciones que comprenden anticuerpos anti-TFPI y factor VIII (Reivindicación 1)

9 . Examen de Patentabilidad (Art 14 D486)

Nivel inventivo (Art18 D486)

La reivindicación 1 consiste en un anticuerpo monoclonal aislado que se une al inhibidor de la vía del factor tisular humano, en el que el anticuerpo comprende una CDR3 que comprende una secuencia de aminoácidos seleccionada del grupo constituido por las SEQ ID NO: 388-430

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1	D2
Anticuerpo monoclonal anti-TFPI con CDR3 con SEQ ID NO: 388-430	Anticuerpo anti-TFPI (Pág 9).	Anticuerpos monoclonales y policlonales que se unen a TFPI (Resumen).

Los documentos D1 y D2 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 1. El documento D1 divulga anticuerpos anti-TFPI para el tratamiento de tumores y sugiere el uso de anticuerpos humanos para el tratamiento en humanos (Pág. 9). El documento D2 divulga el efecto curativo en pacientes con hemofilia con la administración de anticuerpos policlonales y monoclonales que se unen a TFPI (Resumen).

La diferencia entre la invención, definida en la reivindicación 1 y el anticuerpo divulgado en D1 consiste en que el anticuerpo monoclonal tiene la secuencia CDR3 humana.

La diferencia entre la invención, definida en la reivindicación 1 y el anticuerpo D2 consiste en que el anticuerpo monoclonal tiene la secuencia CDR3 humana.

El efecto que logra el incluir la CDR3 humana es que el anticuerpo es menos inmunogénico para su uso en humanos.

1. http://worldwide.espacenet.com/publicationDetails/biblio?CC=WO&NR=9709063&KC=&FT=E&locale=en_EP
2. <http://www.sciencedirect.com/science/article/pii/S004938489190120L>
3. <http://www.google.it/patents/US7015194>

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: “cómo modificar los anticuerpos conocidos en D2 para obtener anticuerpos monoclonales anti-TFPI con menor inmunogenicidad para hacerlos más eficientes en el tratamiento con humanos”.

Sin embargo, el uso en humanos de anticuerpos monoclonales obtenidos de bibliotecas humanas anti-TFPI para disminuir la inmunogenicidad, ya está divulgada en documento D1 que sugiere el uso de anticuerpos humanos para el tratamiento en humanos (Pág. 9).

De manera que el estado de la técnica sugiere a la persona del oficio normalmente versada en la materia la forma en que se resuelve el problema en esta reivindicación. Por lo cual no se considera obvia.

La reivindicación 7 consiste en: *“un anticuerpo que comprende las regiones variables de cadena ligera (...)”*.

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1	D2
Anticuerpo monoclonal anti-TFPI con región variable ligera (SEQ ID NO: 173, 216, 259), y región variable de cadena pesada (SEQ ID NO: 302, 345, 388)	Anticuerpo anti-TFPI (Pág 9).	Anticuerpos monoclonales y policlonales que se unen a TFPI (Resumen).

Los documentos D1 y D2 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 7. El documento D1 divulga anticuerpos anti-TFPI para el tratamiento de tumores y sugiere el uso de anticuerpos humanos para el tratamiento en humanos (Pág. 9). El documento D2 divulga el efecto curativo en pacientes con hemofilia con la administración de anticuerpos policlonales y monoclonales que se unen a TFPI (Resumen).

La diferencia entre la invención, definida en la reivindicación 7 y el anticuerpo divulgado en D1 consiste en las secuencias de los CDRs con región variable ligera (SEQ ID NO: 173, 216, 259), y región variable de cadena pesada (SEQ ID NO: 302, 345, 388).

La diferencia entre la invención, definida en la reivindicación 7 y el anticuerpo D2 consiste en que las secuencias de los CDRs con región variable ligera (SEQ ID NO: 173, 216, 259), y región variable de cadena pesada (SEQ ID NO: 302, 345, 388).

El efecto que logra el incluir las CDRs humanas es que el anticuerpo es menos inmunogénico para su uso en humanos.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: “cómo modificar los anticuerpos conocidos en D2 para obtener anticuerpos monoclonales anti-TFPI con menor inmunogenicidad para hacerlos más eficientes en el tratamiento con humanos”.

Sin embargo, el uso en humanos de anticuerpos monoclonales humanos anti-TFPI para disminuir la inmunogenicidad, ya está divulgada en documento D1 que sugiere el uso de anticuerpos humanos para el tratamiento en humanos (Pág. 9).

De manera que el estado de la técnica sugiere a la persona del oficio normalmente versada en la materia la forma en que se resuelve el problema en esta reivindicación.

Por lo cual no se considera obvia. Lo mismo aplica para la reivindicación 8.

La reivindicación 19 consiste en “una composición que comprende una cantidad terapéuticamente eficaz de (a) un anticuerpo monoclonal (...)”

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1	D3
Composición que comprende	Composición que comprende (Pág 9).	Composición que comprende (Reivindicación 1, resumen)
Anticuerpo monoclonal anti-TFPI con región variable ligera (SEQ ID NO: 173, 216, 259), y región variable de cadena pesada (SEQ ID NO: 302, 345, 388)	Anticuerpo anti-TFPI (Pág 9).	Anticuerpo anti-TFPI (Reivindicación 1)
Factor VIII o Factor IX en el que la composición no contiene factor VII	No menciona	Factor VIIa, Factor VIII (Reivindicación 1)

El documento D3 se considera el estado de la técnica cercano a la invención definida en la reivindicación 19. El documento 3 divulga composiciones que comprenden anticuerpos anti-TFPI y factor VIII (Reivindicación 1)

La diferencia entre la invención, definida en la reivindicación 19 y el anticuerpo divulgado en D3 consiste en las secuencias de los CDRs con región variable ligera (SEQ ID NO: 173, 216, 259), y región variable de cadena pesada (SEQ ID NO: 302, 345, 388) y la exclusión del factor VII.

El efecto que logra el incluir las CDRs humanas es que el anticuerpo es menos inmunogénico para su uso en humanos. No es claro el efecto técnico de la exclusión del factor VII en la composición.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: “cómo modificar los anticuerpos conocidos en D1 para obtener anticuerpos monoclonales anti-TFPI con menor inmunogenicidad para hacerlos más eficientes en el tratamiento con humanos”.

Sin embargo, el uso en humanos de anticuerpos monoclonales obtenidos de bibliotecas humanas anti-TFPI para disminuir la inmunogenicidad, ya está divulgada en documento D1 que sugiere el uso de anticuerpos humanos para el tratamiento en humanos (Pág. 9).

De manera que el estado de la técnica sugiere a la persona del oficio normalmente versada en la materia la forma en que se resuelve el problema en esta reivindicación. Por lo cual no se considera obvia.

10. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	WO9709063	1-14, 19, 26 – 31, 33	Novedad
D2	Welsch, et al., 1991. Thrombosis Research. Vol. 6: 2. Págs. 213–222	1-14, 26 – 31, 33	Nivel inventivo
D3	US 7,015,194	19	Nivel inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



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El anterior requerimiento fue notificado por fijación en lista, de fecha 2014-02-11

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EFFECT OF LIPOPROTEIN-ASSOCIATED COAGULATION INHIBITOR (LACI)
ON THROMBOPLASTIN-INDUCED COAGULATION OF
NORMAL AND HEMOPHILIC PLASMAS

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ABSTRACT

Lipoprotein-associated coagulation inhibitor (LACI) is a plasma-derived protein that inhibits the tissue factor/factor VIIa/calcium/phospholipid complex in a factor Xa-dependent manner. Recombinant LACI (rLACI) added exogenously to plasma prolonged the activated partial thromboplastin time (APTT) and prothrombin time (PT) as a function of rLACI concentration in linear and curvilinear manners, respectively. Under these standard assay conditions, the amounts of rLACI required to double the APTT and PT were approximately 350- and 90-fold the plasma concentration of LACI, respectively. Likewise, addition of antibodies against LACI to pooled normal, factor VIII-deficient, or factor IX-deficient plasma had no effect on their respective APTTs and PTs, demonstrating the insensitivity of these assays to endogenous LACI. The prothrombin time assay was modified by using dilute thromboplastin. Unlike the standard prothrombin time assay, the clotting times were prolonged for factors VIII- or IX-deficient plasma relative to pooled normal plasma in this modified PT assay. Additionally, the degree of factor deficiency, as determined by the APTT assay, was correlated with that determined by the modified PT assay using dilute thromboplastin. When antibodies against LACI were added to pooled normal, factor VIII-deficient, or factor IX-deficient plasma and the prothrombin time assay initiated using dilute thromboplastin, the clotting times for antibody-treated plasma were shorter than for the corresponding plasma in the absence of antibodies. Moreover, the clotting times for factors VIII- and IX-deficient plasmas treated with antibodies raised against LACI were at least as fast as pooled normal plasma in the absence of LACI antibodies when dilute thromboplastin was used to initiate clotting. These results suggest that the prothrombin time assay using dilute thromboplastin may more accurately reflect what occurs *in vivo* and that LACI may play an important role in the prolonged bleeding of those with hemophilia A or B.

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Key words: Blood coagulation, Lipoprotein-Associated Coagulation Inhibitor.

INTRODUCTION

The coagulation of blood results from a series of reactions in which inactive zymogens are sequentially activated to serine proteases. Evidence suggests that initiation of coagulation is a consequence of the formation of a quaternary complex composed of tissue factor (TF), factor VIIa (VIIa), calcium ions (Ca^{++}), and phospholipid (PL) (1). This catalytic complex results in the activation of factors IX and X to factors IXa and Xa, respectively (2-5). The factor IXa produced, in conjunction with its cofactor factor VIIIa, also converts the inactive factor X zymogen to the active serine protease factor Xa. Therefore, active factor Xa can be generated via the TF/VIIa/ Ca^{++} /PL complex either directly by the action of the complex on factor X or indirectly requiring factors VIII and IX.

The clinical manifestations exhibited by those deficient in factors VIII or IX (hemophilia A or B, respectively) suggest that, if coagulation is initiated by the TF/VIIa/ Ca^{++} /PL complex, the indirect pathway of factor X activation is predominant *in vivo*. Furthermore, the relative utility of the direct and indirect pathways of factor X activation has been the subject of numerous reports (5-11).

The identification and mechanistic understanding of a plasma protein which inhibits the TF/VIIa/ Ca^{++} /PL complex suggest that it may be important in the regulation of factor X activation. This inhibitor has been given the name lipoprotein-associated coagulation inhibitor (LACI) (12), tissue factor inhibitor (TFI) (13), or extrinsic pathway inhibitor (EPI) (14). LACI, a protein containing three tandem Kunitz domains (15), acts as a direct inhibitor of factor Xa (12, 16). Furthermore, its unique mode of action allows LACI to act as an inhibitor of the TF/VIIa/ Ca^{++} /PL complex through a feedback mechanism which requires factor Xa (12, 16). Descriptions of coagulation including the proposed effect of LACI have recently been reported (17, 18). Briefly, coagulation is initiated by formation of the TF/VIIa/ Ca^{++} /PL complex and results in the activation of some factor X to factor Xa and some factor IX to factor IXa. Once small amounts of factor Xa have been generated, LACI is able to inhibit the TF/VIIa/ Ca^{++} /PL complex. Thus, additional factor Xa can only be produced through the indirect pathway.

This model of coagulation which includes LACI-induced feedback inhibition of the TF/VIIa/ Ca^{++} /PL complex explains the need for both the direct and indirect pathways of factor X activation. Additionally, the model predicts that in normal hemostasis the TF/VIIa/ Ca^{++} /PL complex produces a temporal burst of factor Xa sufficient to induce local platelet aggregation and activation of factors V and VIII and that persistent hemostasis requires the action of factors VIIIa and IXa. Importantly, this prediction is consistent with the observation that the bleeding seen in hemophiliacs is often delayed in onset (19).

Based on this understanding of the initiation and regulation of coagulation, the effects of inhibitors of LACI on normal, factor VIII-deficient, and factor IX-deficient plasmas were investigated in an attempt to assess the physiological importance of LACI in these plasmas. These studies demonstrated that the prothrombin time (PT) and activated partial thromboplastin time (APTT) assays as routinely used are not sensitive to LACI. Furthermore, these investigations demonstrated that when clotting was initiated with dilute thromboplastin the clotting times were prolonged for factors VIII- or IX-deficient plasma relative to normal plasma and the degree of factor deficiency could be correlated with the APTT. Finally, our results showed that, when clotting was initiated with dilute thromboplastin, antibodies to LACI shortened the time to clot formation for normal, factor VIII-, or IX-deficient plasma and that the clotting time for factor VIII- or IX-deficient plasma in the presence of LACI antibodies was as fast or faster than for normal plasma in the absence of antibodies. These investigations suggest that inhibition of LACI function may be viewed as a route to enhanced factor Xa formation which may be especially useful for those with hemophilia A or B.

EXPERIMENTAL PROCEDURES

Materials. Fresh frozen congenital factor VIII and factor IX deficient plasmas as well as fresh frozen pooled normal plasma were obtained from George King Bio-Medical (Overland Park, Ks). Lyophilized factor VIII and factor IX immunodepleted plasmas were from American Diagnostica (Greenwich, CT). Lyophilized normal pooled plasma was purchased from Organon Teknika (Durham, NC). Immunization of rabbits with purified LACI and purification of the polyclonal

anti-LACI-Ig (PAb) were carried out as previously described (20). Monoclonal anti-LACI-Ig (MAb) was from hybridoma cell line #14 which was a fusion product of Balb/cj myeloma cell line P3x63AG8.653 (American Type Culture Collection) and splenocytes from mice immunized with purified LACI. Monoclonal cell line supernatants were screened for the ability to remove fluorescein labelled LACI from solution after a 20 minute incubation. All other reagents were of the highest purity available and all solutions were prepared with distilled and deionized water.

Expression, purification, and characterization of recombinant LACI. Recombinant LACI (rLACI) was expressed in mouse C127 cells using a bovine papilloma virus vector and the rLACI cell line was grown, harvested, and the rLACI purified as previously described (21). The purified protein yielded a single band by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE), gave only the expected amino acid sequence relative to the sequence deduced from the cDNA sequence of LACI, and the stoichiometry of factor Xa inhibition in an amidolytic substrate assay (20) was approximately 1:1. Protein concentrations were determined by amino acid analysis or by the Bio-Rad Protein Assay since both gave similar results. A molecular weight of 38000 for LACI was used for calculations of molar concentrations.

Prothrombin Time assay (PT). Plasma samples were prepared by mixing plasma with 0.1 volumes of control buffer, rLACI, non-immune Ig (1 mg/ml), polyclonal anti-LACI-Ig (1 mg/ml), or monoclonal anti-LACI-Ig (1 mg/ml). After incubation for 30 min at 25 °C, plasma samples (0.1 ml) were combined with 0.2 ml of appropriately diluted Simplastin Excel as a source of thromboplastin (Organon Teknika) and the clotting time determined using a Coag-A-Mate RA4 instrument (Organon Teknika). Dilutions of thromboplastin were made into 0.05 M Tris base containing 0.10 M NaCl, 0.1% bovine serum albumin, and 0.02 M CaCl_2 at pH 7.5. The standard or routinely used prothrombin time assay refers to the protocol described using thromboplastin reconstituted as per manufacturers instructions.

Activated Partial Thromboplastin Time assay (APTT). Plasma samples were prepared by mixing plasma with 0.1 volumes of control buffer, rLACI, non-immune Ig (1 mg/ml), polyclonal anti-LACI-Ig (1 mg/ml), or monoclonal anti-LACI-Ig (1 mg/ml). After incubation for 30 min at 25 °C, plasma samples (0.1 ml) were incubated with 0.1 ml of automated APTT reagent (Organon Teknika) for 3 min at 37 °C. Clotting was then initiated by the addition of 0.1 ml of 0.025 M CaCl_2 (Organon Teknika) and the clotting time determined using a Coag-A-Mate RA4 instrument (Organon Teknika).

RESULTS

Effect of exogenously added rLACI on the prothrombin and activated partial thromboplastin times. When rLACI was added to plasma the clotting time as determined by the APTT was linearly related to the concentration of exogenously added LACI (Figure 1A). Based on the best linear fit to the data, it was found that about 35 μg of rLACI /ml of plasma was required to double the APTT. Interestingly, when the PT was measured as a function of added rLACI a curvilinear response curve was obtained (Figure 1B). An exponential fit of the data revealed that about 9 μg of LACI/ml of plasma was required to double the prothrombin time of plasma without added rLACI. These concentrations of rLACI are approximately 350- and 90-fold greater than that reported to be present in plasma (13).

Effect of antibodies raised against LACI on the PT and APTT of various plasmas. LACI exists in human plasma at a concentration of approximately 100 ng/ml (13). To further investigate whether endogenous LACI plays a regulatory role in coagulation, the effects of antibodies against LACI on the clotting times determined by standard APTT and PT assays were investigated. Table 1 shows that the PTs and APTTs of various plasmas were unaffected by the presence of LACI antibody as compared to the same plasmas pre-incubated with non-immune rabbit Ig (NIg). This was the case whether polyclonal (PAb) or monoclonal (MAb) antibodies and whether normal, factors VIII-, or IX-deficient plasmas were used. The concentrations of antibodies used in this experiment were the same as subsequent experiments which demonstrated that this level of antibody was saturating with respect to LACI function (see below). These results demonstrated that endogenous levels of LACI do not inhibit the rate of clot formation as determined by the standard prothrombin time and activated partial thromboplastin time assays.

Effect of thromboplastin concentration on the PTs of various plasmas. Prothrombin times were

measured for pooled normal, factor VIII-deficient, and factor IX-deficient plasmas as a function of thromboplastin concentration (Table 2). Additionally, investigations employing factor-deficient plasmas with differing APTTs revealed that the degree of prolongation of the PT, when initiated with dilute thromboplastin, was related to the severity of the factor deficiency as determined by APTT assays (Tables 1 & 2). In fact, when the prothrombin times measured at a given dilution of thromboplastin were plotted as a function of the activated partial thromboplastin time for each plasma a strong positive linear correlation (correlation coefficient = 0.97) was observed at two dilutions of thromboplastin (Figure 2A & B). These results indicated that prothrombin times initiated with diluted thromboplastin depended on factors VIII and IX.

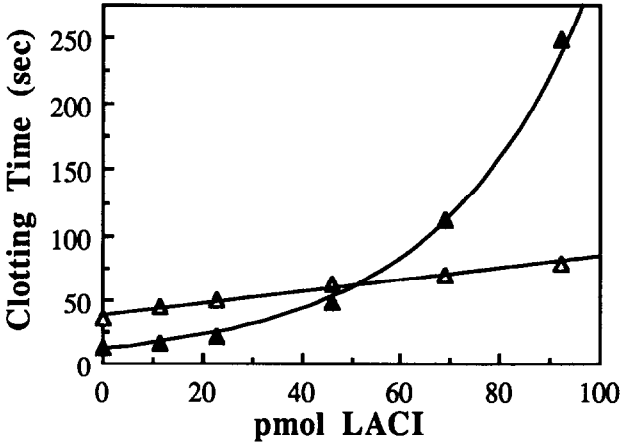


Figure 1. Activated partial thromboplastin (Δ) and prothrombin (▲) times as a function of exogenously added recombinant LACI. Values given are the average clotting times obtained from typical duplicate experiments having a standard deviation of $\leq 2.5\%$ of the mean.

TABLE 1

APTT and PT of Various Plasmas With or Without Antibodies Against LACI

Sample	PT(sec)			APTT (sec)		
	+ NIg	+ PAb	+ MAb	+ NIg	+ PAb	+ MAb
<u>Normal Plasma</u>						
Pool 1	13.3	12.8	13.8	32.3	32.3	32.3
Pool 2	12.7	12.4	12.3	34.5	34.0	34.7
<u>F.VIII deficient</u>						
Patient 1	12.0	11.7	11.7	74.2	71.8	76.1
Patient 2	12.2	12.2	12.3	78.6	75.1	78.0
Patient 3	12.2	12.0	12.4	85.5	85.0	86.1
Immunodepleted	13.6	13.6	13.4	72.5	72.1	74.7
<u>F.IX deficient</u>						
Patient 1	12.1	11.7	12.3	73.7	80.9	83.9
Patient 2	12.2	12.0	12.2	82.0	79.6	85.5
Patient 3	12.5	12.0	12.2	102.7	97.6	99.1
Immunodepleted	13.2	13.2	13.5	106.3	105.7	109.4

Effect of LACI antibodies on the prothrombin times of various plasmas as a function of thromboplastin concentration. Based on the aforementioned results, the ability of LACI antibodies to affect prothrombin times initiated with varying thromboplastin concentrations was investigated. These studies revealed that LACI antibodies significantly shortened the clotting times for pooled normal, factor VIII-deficient, and factor IX-deficient plasmas when clotting was initiated with dilute thromboplastin (Figure 3A-C). For the sake of clarity, only representative pooled normal, factor VIII-deficient, and factor IX-deficient plasmas are shown. The results, however, were qualitatively similar for each class of plasma. The amount of antibody added to plasma was saturating as determined by the fact that additional antibody did not further reduce the clotting times (data not shown). Several monoclonal antibody preparations were screened and some had no effect in this assay. The monoclonal antibody preparation reported was the one showing the strongest inhibition of LACI function as determined in this assay. Clearly, the results revealed that when the PT assay was initiated with dilute thromboplastin endogenous LACI acted as an inhibitor of coagulation. Furthermore, that both monoclonal and polyclonal antibody preparations yielded similar clotting times suggests the complete inhibition of LACI function in both cases.

TABLE 2

Clotting Times for Various Plasmas as a Function of Thromboplastin Concentration

Sample	Thromboplastin Concentration (fold dilution)				
	1	10	100	1000	5000
<u>Normal Plasma</u>					
Pool 1	13.3	28.5	49.5	92.9	141.0
<u>F.VIII deficient</u>					
Patient 1	12.0	28.1	50.3	112.1	163.7
Patient 2	12.2	29.8	55.2	114.1	166.1
Patient 3	12.2	32.8	62.0	124.3	178.4
<u>F.IX deficient</u>					
Patient 1	12.1	29.3	52.5	109.2	168.7
Patient 2	12.2	33.0	52.9	121.4	175.9
Patient 3	12.5	33.5	65.8	131.4	187.9

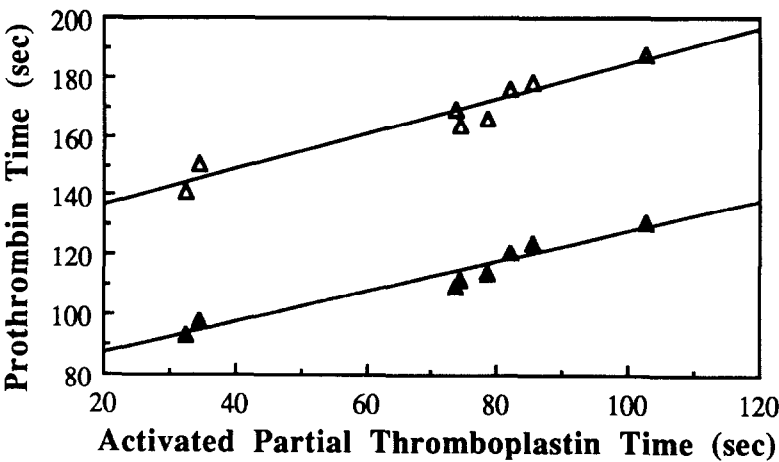


Figure 2. Relationship between activated partial thromboplastin and prothrombin times. Prothrombin times were determined when clotting was initiated with 1000- and 5000-fold dilutions of thromboplastin (▲ and Δ, respectively). The data reported are the average of duplicate determinations having a standard deviation of $\leq 2.5\%$ of the mean. The correlation coefficient for the best linear fit to the data was 0.97 for both dilutions of thromboplastin.

Importantly, these studies demonstrated that when the PT assay was initiated with dilute thromboplastin, LACI antibodies shortened the clotting times of plasmas from congenital factor VIII or IX deficient hemophiliac donors such that they were at least as fast as those which resulted from pooled normal plasma that was not treated with LACI antibodies (Figure 4A & B). For example, when a 1000-fold dilution of thromboplastin was used and samples were incubated with non-immune Ig the time to clot formation for pooled normal and factor VIII-deficient plasmas were 93 and 124 seconds, respectively. At this thromboplastin concentration the clotting times for this factor VIII-deficient plasma in the presence of polyclonal or monoclonal antibody against LACI were 84 and 86 seconds, respectively. The clotting times for the various plasmas treated with LACI antibodies employing dilute thromboplastin may still reflect the severity of the factor deficiency as determined by APTT assays. That is, the prothrombin times using a 1000-fold dilution of thromboplastin and factor VIII-deficient plasmas in the presence of non-immune Ig were 112, 114, and 124 seconds. The corresponding prothrombin times in the presence of polyclonal or monoclonal antibodies against LACI were 76, 81, and 84 seconds and 78, 79, and 86 seconds, respectively. As with the other clotting times reported, these times represent the average of duplicate determinations having a standard deviation of $\leq 2.5\%$ of the mean. This suggested that in the presence of LACI antibodies some factor Xa generation may result from the indirect action of the TF/VIIa/ Ca^{++} /PL complex requiring factors VIII and IX.

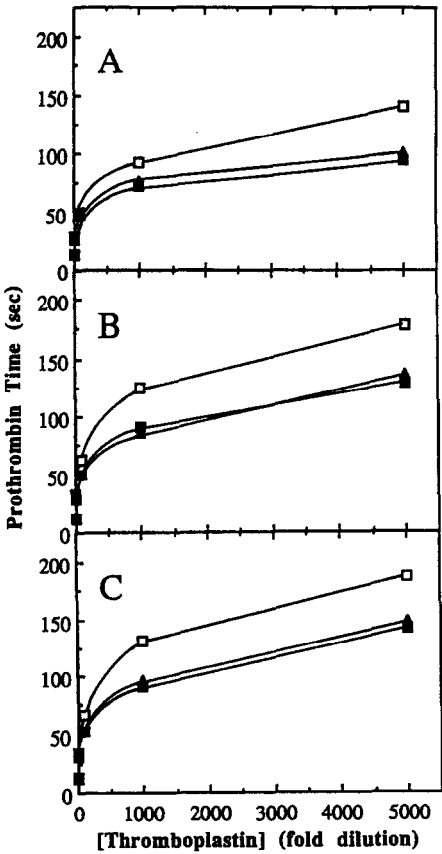


Figure 3. Prothrombin time as a function of thromboplastin concentration in the presence or absence of antibodies against LACI. The prothrombin times for normal (panel A), congenital factor VIII-deficient (panel B), and congenital factor IX-deficient (panel C) plasmas were determined at several concentrations of thromboplastin in the presence of non-immune-Ig (□), polyclonal anti-LACI-Ig (▲), and monoclonal anti-LACI-Ig (■). The clotting times are the average of duplicate determinations which differed by $\leq 2.5\%$ of the mean.

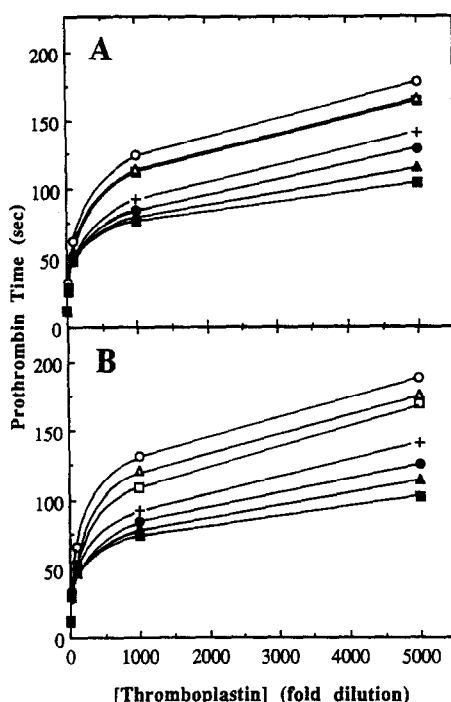


Figure 4. **Comparison of the prothrombin times of various plasmas.** Panel A shows the prothrombin times for pooled normal (+) and factor VIII-deficient plasmas [patient 1 ($\square$, $\blacksquare$), patient 2 (Δ , $\blacktriangle$), and patient 3 ($\circ$, $\bullet$)] as a function of thromboplastin concentration. Panel B shows the clotting times for pooled normal (+) and factor IX-deficient plasmas [patient 1 ($\square$, $\blacksquare$), patient 2 (Δ , $\blacktriangle$), and patient 3 ($\circ$, $\bullet$)] as a function of thromboplastin concentration. Factor deficient samples were assayed in the presence of non-immune Ig (open symbols) or polyclonal anti-LACI-Ig (closed symbols). Pooled normal plasma was assayed in the presence of non-immune Ig. The clotting times are the average of duplicate determinations which differed by $\leq 2.5\%$ of the mean.

DISCUSSION

Previous investigations have demonstrated that a complex of tissue factor, factor VIIa, calcium ions, and phospholipid can catalyze the formation of factor Xa directly by its action on factor X or indirectly by converting factor IX to factor IXa which subsequently, in the presence of factor VIII, converts factor X to factor Xa. The relative importance of each of these reaction pathways to factor X activation has been investigated (5-11, 22, 23). Several of these studies have demonstrated that the effect of an inhibitor of the TF/VIIa/ Ca^{++} /PL complex, LACI, must be addressed when studying the mechanism of factor Xa generation. The aim of the studies presented was to investigate the effect that inhibitors of LACI have on the thromboplastin-initiated clotting times of pooled normal, factor VIII-deficient, and factor IX-deficient plasmas in order to better understand the physiological role of LACI.

Addition of rLACI or antibodies against LACI to various plasmas demonstrated that the standard prothrombin and activated partial thromboplastin time assays were relatively insensitive to LACI function. Exogenously added rLACI prolonged the clotting time of plasma, as determined by the APTT assay, linearly as a function of rLACI concentration. The dose-response curve for rLACI in the APTT assay was similar to that reported for the factor Xa inhibitor known as tick anticoagulant protein (TAP) (24). The concentration of rLACI required to double the APTT was approximately 350-fold higher than the plasma concentration of LACI suggesting that

this assay did not measure the *in vivo* effect(s) of LACI. When rLACI was added to plasma and the standard prothrombin times measured a curvilinear dose-response curve was obtained. This type of response might result from the simultaneous effects of LACI acting as an inhibitor of both factor Xa and the TF/VIIa/Ca⁺⁺/PL complex. Further experiments will be needed to determine if this is in fact the case. Nevertheless, the concentration of rLACI required to double the PT in the absence of exogenously added rLACI was about 90-fold higher than the endogenous LACI concentration. Additionally, antibodies raised against LACI had no effect on the clotting times as determined by the standard PT and APTT assays. Taken together, these investigations demonstrate the insensitivity of the PT and APTT assays to LACI.

The data showed a correlation between the activated partial thromboplastin time and the prothrombin time measured when clotting was initiated with dilute thromboplastin. These results confirmed and extended previous investigations which showed that hemophilic plasma clotting times exceeded those of normal plasma only when dilute thromboplastin was used to initiate clotting (9). Therefore, not only do the clotting times of plasma induced to clot with dilute thromboplastin identify plasmas deficient in factors VIII or IX but they are also sensitive to the degree of factor deficiency. These results suggest that prothrombin times measured for samples induced to clot with dilute thromboplastin may more accurately reflect the *in vivo* situation than prothrombin times determined using undiluted thromboplastin.

The prothrombin time assay initiated using dilute thromboplastin is kinetically related to the rate of formation of the inert TF/VIIa/Ca⁺⁺/PL/factor Xa/LACI complex. The importance of a kinetic assay, as opposed to endpoint assays which measure residual tissue factor activity after preincubation of test samples, has been reported (20). Briefly, an assay which includes the rate of inert complex formation more stringently differentiates various LACIs and is likely to more closely mimic physiological conditions.

Using antibodies raised against LACI in pooled normal, factor VIII-deficient, and factor IX-deficient plasmas and diluted thromboplastin to initiate clot formation, it was demonstrated that neutralization of LACI function resulted in a decrease in clotting time. These antibody results were similar to the results obtained by a comparison of clotting times for various plasmas as a function of tissue factor concentration. That is, an effect was observed only when dilute thromboplastin was used to initiate coagulation. These results further imply that the thromboplastin concentrations routinely used in the prothrombin time assay, while sensitive to several coagulation protein deficiencies, may not be an entirely appropriate mimic for hemostasis. Furthermore, these results would be consistent with LACI being a factor Xa-dependent inhibitor of the TF/VIIa/Ca⁺⁺/PL complex *in vivo*.

Additionally, the data showed that the clotting times for factors VIII or IX-deficient plasmas in the presence of LACI antibodies were at least as fast as those for pooled normal plasma not treated with LACI antibodies.

Determination of factor IX activation peptide concentrations in the plasma of patients with hereditary deficiencies of factors VII, IX, or XI revealed the dependence of factor IXa generation *in vivo* on the TF/VIIa/Ca⁺⁺/PL complex (11). Additionally, investigations have demonstrated that under a variety of cofactor conditions the activation of factor IX by the TF/VIIa/Ca⁺⁺/PL complex may be an important reaction in normal hemostasis (10). An experiment employing a tubular reactor and physiological protein concentrations to approximate *in vivo* conditions demonstrated that factors VIII and IX are required for optimal factor Xa production when coagulation is initiated by the TF/VIIa/Ca⁺⁺/PL complex (22). In these studies, the presence of factors VIII and IX led to a 2- to 3-fold enhancement in factor Xa production in the absence of LACI and to approximately a 20-fold enhancement in factor Xa production in the presence of 1 nM LACI (22). Finally, it has been shown that in the presence of plasma concentration of factors VIII and IX at least ten times the plasma concentration of LACI is required to reduce factor VIIa-dependent factor X activation one order of magnitude *in vitro* and that in the absence of functional factor VIII and IX a plasma level of LACI was sufficient to be a potent inhibitor of this factor X activation (23). Our results are consistent with the above studies and extended them to the study of hemophilic plasma.

In summary, our studies showed that the standard prothrombin and activated partial thromboplastin time assays were not sensitive to endogenous LACI and extended the very old observation that the standard PT assay does not give prolonged clotting times for plasmas from patients deficient in factors VIII or IX (25). They identified a thromboplastin-dependent assay

that gave prolonged clotting times for plasmas deficient in factors VIII and IX that could be correlated with activated partial thromboplastin times. They demonstrated that, using this assay, antibodies raised against LACI shortened the clotting times of pooled normal, factor VIII-deficient, and factor IX-deficient plasmas. They also demonstrated that under these assay conditions the clotting times of plasmas from patients with hemophilia A or B in the presence of antibodies against LACI, acting as inhibitors of LACI function, were at least as fast as the clotting times for normal plasma in the absence of such antibodies. The development of an inhibitor of LACI function and its efficacy in the treatment of hemophilias A and B are the subject of current investigations.

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