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Señora Jefe de la  
**DIVISION DE NUEVAS CREACIONES**  
Superintendencia de Industria y Comercio  
E. S. D.

**Trámite : 011 Evento : 379 Actuación : 444**

Referencia : Expediente No. 02.112.910  
Asunto : Solicitud de Patente para **INMUNIZACION DE GANADO LECHERO CON PROTEINA GapC CONTRA LA INFECCION DE ESTREPTOCOCOS.**  
Solicitante : UNIVERSITY OF SASKATCHEWAN  
Fecha de Solicitud : 12 de diciembre de 2002

1. Yo, Emilio FERRERO, obrando como apoderado del solicitante en el asunto antes identificado, me refiero al Oficio No. **14200** notificado por fijación en lista del 9 de noviembre de 2007.

2. Mediante mi memorial de fecha 05 de febrero de 2008 solicité un plazo de 30 días para atender el Oficio No. **14200**.

3. Mediante el Oficio No. **14200** se puso en conocimiento del solicitante el informe que contiene las observaciones hechas por su Despacho sobre la presente solicitud de patente.

Atiendo a las observaciones de la manera siguiente:

3.1. CLARIDAD DE LA SOLICITUD Y ES UNA INVENCION.

Para subsanar las objeciones que sobre claridad y por no ser una invención hizo su Despacho, presento nuevo pliego reivindicatorio con 3 reivindicaciones en el cual se han

hecho cambios que permiten apreciar claramente el objeto reivindicado en la presente solicitud de patente. Las modificaciones al capítulo reivindicatorio fueron las siguientes:

- El nuevo pliego reivindicatorio esta dirigido a composiciones de vacuna para mastitis que comprenden un adyuvante y una proteína GapC o un polinucleótido.
- La expresión "cantidad terapéutica eficaz" ha sido incluida en el nuevo pliego reivindicatorio. Así mismo, las expresiones "inclusive" y "al menos un 80% de identidad" se han eliminado de este pliego.
- Las reivindicaciones referentes a la proteína asilada han sido eliminadas de este pliego reivindicatorio.

De esta manera es claro que con las anteriores modificaciones del capítulo reivindicatorio quedan subsanadas las objeciones que por claridad y por no ser una invención hizo su Despacho, ya que ahora, dicho pliego reivindica un producto en los términos sugeridos por su Despacho, permite determinar el alcance de la protección reivindicada y compararla frente al estado del arte, de acuerdo con las disposiciones de los artículos 15 y 30 de la Decisión 486 y adicionalmente, revela materia no exceptuada de patentabilidad.

### 3.2. NOVEDAD Y NIVEL INVENTIVO

Ese Despacho afirma que los documentos **D1 a D3** afectan la novedad y el nivel inventivo de la presente invención, toda vez que en dichas anterioridades se encontraban todas las sugerencias para emplear dichas proteínas en composiciones de vacunas, con lo cual estamos en desacuerdo por los argumentos expuestos a continuación. Veamos:

Primero que todo, respetuosamente ponemos de presente que no solo ninguno de los documentos citados como anterioridades describen ni sugieren composiciones de vacuna para mastitis que incluyan GapC en una cantidad efectiva, y por lo tanto no describen

todas las características técnicas de la invención; sino que adicionalmente, dicha materia objeto de protección no antes divulgada representa un avance inesperado en el estado de la técnica.

A este respecto, es importante aclarar que la mastitis se define como un incremento en el conteo de células somáticas en leche. Dicho incremento se debe a una respuesta inflamatoria en la glándula mamaria. Es del conocimiento de un técnico medio que la respuesta inmune posterior contra el patógeno invasor al inmunizar un sujeto utilizando un antígeno particular, tal como una proteína GapC, podría en sí mismo originar una reacción inflamatoria, incrementándose por lo tanto, a diferencia de reducir, los conteos de células somáticas (ver Anexo 5.2.).

Por lo tanto, los resultados obtenidos utilizando las vacunas de proteína *Streptococcus* GapC de la presente invención son inesperados, toda vez que los conteos de células somáticas disminuyen, al contrario de los conocimientos de un técnico medio, para quien no sería factible desarrollar una vacuna efectiva contra la mastitis originada por bacterias gram-positivas, tal como *streptococcus*.

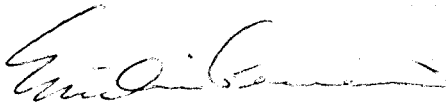
Por consiguiente, toda vez que (i) la presente invención ha sido limitada a composiciones de vacuna para mastitis que comprenden un adyuvante y una proteína GapC o un polinucleótido en una cantidad terapéutica eficaz; (ii) que no existe sugerencia en D1 a D3 con respecto a tal materia; y (iii) que dichas composiciones de vacuna permiten obtener una disminución en el conteo de células somáticas en leche; se puede concluir, que ninguno de los documentos de la técnica anterior (es decir, D1 a D3) solos o en combinación, enseñan y/o sugieren la materia objeto del nuevo conjunto de reivindicaciones, y por lo tanto, dichos documentos no afectan ni la novedad ni el nivel inventivo de la presente solicitud.

4. Puesto que se han atendido a cabalidad las observaciones hechas por su Despacho sobre la presente solicitud de patente, atentamente solicito que se continúe su tramitación.

5. **Anexos:**

- 5.1. Nuevo pliego reivindicatorio con 3 reivindicaciones;
- 5.2. Anderson, J.G., Br. Vet. J. (1978) 134: 412-420;
- 5.3. Formulario para modificaciones;
- 5.4. Recibo por valor de \$103.000 por concepto de modificaciones en solicitud pendiente;

Señora Jefe,



**EMILIO FERRERO**  
T.P.A. No. 31.929

**FORMULARIO UNICO DE CORRECCIONES Y MODIFICACIONES**

|                                                  |                                                                                                                                                                                                                                                                                                                                                                                                                  |                                                                                                                                                                                                    |
|--------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>1</b>                                         | <b>SOLICITUD DE:</b><br><input type="checkbox"/> Corrección de Error Material <input checked="" type="checkbox"/> Modificación a una solicitud                                                                                                                                                                                                                                                                   |                                                                                                                                                                                                    |
| <b>2</b><br><b>SOLICITANTE</b>                   | <b>Nombre:</b> UNIVERSITY OF SASKATCHEWAN<br>Sociedad constituida y existente bajo las leyes de Canadá.<br><br><b>Dirección:</b> 120 Veterinary Road,<br>Saskatoon,<br>Saskatchewan S7N 5E3,<br>Canadá<br><br><b>Domicilio:</b> Saskatoon,<br>Saskatchewan,<br>Canadá<br><br><b>Teléfono:</b> 546 09 70 <b>Fax:</b> 249 40 70<br><b>E-mail:</b> <a href="mailto:clientes@cavelier.com">clientes@cavelier.com</a> | <b>IDENTIFICACION</b><br><br>C.C. <input type="checkbox"/> NIT <input type="checkbox"/><br>C.E. <input type="checkbox"/> Otro <input type="checkbox"/><br><br>Número: _____                        |
| <b>3</b><br><b>REPRESENTANTE<br/>O APODERADO</b> | <b>Nombre:</b> Emilio FERRERO<br><b>Dirección:</b> Carrera 4ª No. 72-35<br><b>Teléfono:</b> 347 36 11 <b>Fax:</b> 217 92 11<br><b>E-mail:</b> <a href="mailto:cavelier@cavelier.com">cavelier@cavelier.com</a>                                                                                                                                                                                                   | <b>IDENTIFICACION</b><br><br>C.C. <input checked="" type="checkbox"/> NIT <input type="checkbox"/><br>C.E. <input type="checkbox"/> Otro <input type="checkbox"/><br><br>Número: 438.019 TP 31.929 |
| <b>4</b>                                         | <b>Número de expediente:</b> <u>02.112.910</u>                                                                                                                                                                                                                                                                                                                                                                   |                                                                                                                                                                                                    |
| <b>5</b>                                         | <b>Descripción de la corrección o modificación solicitada:</b><br>Presento nuevo capítulo reivindicatorio compuesto por 3 reivindicaciones con el fin de superar las objeciones hechas por Su Despacho a la presente solicitud y pido que sean tenidas en cuenta al momento de efectuar el examen de fondo.<br><br>_____<br><br>_____                                                                            |                                                                                                                                                                                                    |
| <b>6</b>                                         | <b>Comprobante de pago No.</b> <u>08-20.868</u> <b>Fecha:</b> <u>10 de marzo de 2008</u>                                                                                                                                                                                                                                                                                                                         |                                                                                                                                                                                                    |
| <b>7</b>                                         | <b>Anexos</b><br><input checked="" type="checkbox"/> Comprobante de pago de la tasa por \$103.000.00 por concepto de modificaciones.<br><input type="checkbox"/> Poderes, si fuere el caso<br><input type="checkbox"/> Documento que acredita la existencia y representación legal de las partes, cuando sean personas jurídicas.                                                                                |                                                                                                                                                                                                    |
| <b>8</b>                                         | <b>NOMBRE:</b> EMILIO FERRERO                                                                                                                                                                                                                                                                                                                                                                                    | <b>FIRMA:</b>                                                                                                  |
| C.C. 438.019 de Usaquén T.P.A. 31.929            |                                                                                                                                                                                                                                                                                                                                                                                                                  |                                                                                                                                                                                                    |
| NNJ                                              |                                                                                                                                                                                                                                                                                                                                                                                                                  |                                                                                                                                                                                                    |

### Reivindicaciones

1. Una composición de vacuna para mastitis que comprende un adyuvante y una proteína GapC o un polinucleótido que codifica una proteína GapC en una cantidad terapéutica eficaz, en donde la proteína GapC se selecciona del grupo que consiste de:
  - (a) Una proteína GapC *streptococcus dysgalactiae* que comprende la secuencia de aminoácido mostrada en las posiciones de aminoácido 1 a 336 de la SEQ ID No. 4;
  - (b) Una proteína GapC *streptococcus agalactiae* que comprende la secuencia de aminoácido mostrada en las posiciones de aminoácido 1 a 336 de la SEQ ID No. 6;
  - (c) Una proteína GapC *streptococcus uberis* que comprende la secuencia de aminoácido mostrada en las posiciones de aminoácido 1 a 336 de la SEQ ID No. 8;
  - (d) Una proteína GapC *streptococcus parauberis* que comprende la secuencia de aminoácido mostrada en las posiciones de aminoácido 1 a 336 de la SEQ ID No.10; y
  - (e) Una proteína GapC *streptococcus iniae* que comprende la secuencia de aminoácido mostrada en las posiciones de aminoácido 1 a 336 de la SEQ ID No.12.
2. La composición de vacuna de la reivindicación 1, en donde el adyuvante es un adyuvante basado en aceite.
3. La composición de vacuna de la reivindicación 2, en donde el adyuvante es VSA3.

## THE PROBLEM OF IMMUNIZATION AGAINST STAPHYLOCOCCAL MASTITIS

By J. G. ANDERSON

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### INTRODUCTION

The possibility of immunizing the dairy cow against mastitis has claimed the attention of research workers for most of this century. In his survey of the literature to the end of 1935 Munch-Petersen (1935) listed 57 reports of attempts to immunize cattle against mastitis. The success of these and subsequent attempts has been qualified (Mellenberger, 1977) and currently the attitude is of exasperated optimism: the cow should be able to mount a significant immune response in the mammary gland if only an effective antigen is given by the correct route at the proper stage in the lactation cycle (Faukey, 1977).

The purpose of this paper is not to review the numerous attempts that have been made to immunize against staphylococcal mastitis, but to bring together the knowledge of the way in which staphylococci cause infection and the way in which the mammary gland responds to the presence of staphylococci, in order to identify and evaluate the problems inherent in immunization.

### THE DEFENCE SYSTEM

The premise is that the principal mechanism by which the lactating mammary gland removes staphylococci that have entered the mammary tissue is phagocytic killing by neutrophils. A virulent staphylococcus is one that resists phagocytic killing and though the way in which this occurs is not clear (Anderson, 1976), it remains true that phagocytic killing by neutrophils is the method of host defence. Successful phagocytosis involves recruitment of neutrophils, opsonization and killing.

#### *Recruitment of neutrophils*

Neutrophils accumulate in the alveolar lumen of the mammary gland by recruitment from the systemic circulation and this occurs in response to the generation of chemotactic factors by the staphylococci. Chemotactic factors may act directly on the corresponding cell (cytoactive) or may, by acting on enzyme systems or complement, cause the release of chemotactic peptides (cytoactive) (Keller & Sorkin, 1967). In addition, complement activation results in the formation of anaphylatoxins which, through histamine release, increase the permeability of blood vessels. The

products of staphylococcal growth have been shown to possess little direct chemotactic activity and some inhibition of neutrophil locomotion. However indirect chemotactic substances are generated by the combination of staphylococcal growth products and plasma components (Russell *et al.*, 1976). Although these mixtures have been demonstrated only *in vitro* it is tempting to suggest that *in vivo* the cytotoxic factors attract neutrophils to the site of staphylococcal infection and that the inhibitory activity immobilizes the neutrophils in the vicinity of the staphylococci.

*Opsonization*

Ingestion of staphylococci by neutrophils is facilitated by opsonization of the organisms. The first of the two major systems in serum which are opsonic for staphylococci is heat-labile (56°C for 30 min) and involves the activation of complement through the alternate (properdin) or classical (IgG or IgM) pathways so that the opsonically active fragments of complement are deposited on the surface of staphylococci and bind them to neutrophils. The other system is heat-stable and involves the attachment of IgG to the organisms through the Fab fragments; there are receptors for the Fc portion of the antibody molecule on the surface of the neutrophil so that recognition and ingestion are promoted (Winkelschtein, 1973; Sussel, 1974a).

Apart from the first few days after parturition in the cow when the colostrum is rich in IgG, IgM and IgA, the predominant immunoglobulin in normal milk is IgG1 (Bader, 1978). The transfer of IgG1 into the milk is a selective process which is a function of the glandular epithelium (Brandon, Watson & Lancelles, 1971), the complement content of normal milk is variable (Reiter & Brock, 1975). Thus there are limitations on the antibody and complement content of normal milk. However if the integrity of the epithelium is broken, as by inflammation, then there is a transudation of serum proteins and therefore of immunoglobulins and complement into milk.

*Ingestion and killing*

During ingestion the cell membrane of the neutrophil invaginates at the point of contact with the opsonized staphylococcus to form a phagosome and the cytoplasmic granules begin to fuse their membranes with that of the phagosome to form a phagolysosome. The secondary granules discharge their lactoferrin and lysozyme into the phagolysosome. Myeloperoxidase is discharged from the primary granules and together with  $H_2O_2$  provided by the respiratory burst associated with ingestion, and  $Cl^-$ , completes the bactericidal myeloperoxidase-hydrogen peroxide halide system. Lysozyme, clausase and radicle proteins are also derived from the primary granules and function as oxygen independent bactericidal systems. There are therefore a number of bactericidal systems in the neutrophil that can act separately or in concert (Sussel, 1974b; Spitznagel, 1977). Once a neutrophil has degranulated no new granules are formed, and though the life-span of neutrophils once they have entered the milk is not known with certainty, it seems likely that it is no more than a few hours. The cells with their ingested material degenerate and are removed by macrophages or by milking the udder.

Recent studies *in vitro* have shown that neutrophils in milk ingest and kill fewer staphylococci than neutrophils in serum. This is not due to a lack of opsonin, since neutrophils in whey ingest staphylococci as efficiently as in serum (Russell & Reiter,

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1975), but is due to the presence in milk of casein micelles and fat globules which are ingested by neutrophils and exhaust the cells by diverting the discharge of granules into phagolysosomes that contain casein or fat (Paape *et al.*, 1975; Russell, Brunker & Reiter, 1976). It is probable that this abortive discharge of granules occurs *in vivo* but the detrimental effect of the phenomenon may be overstated. The short life-span of neutrophils in the udder means that these cells can only be efficient for a few hours after extravasation. Since newly extravasated neutrophils are as efficient as circulating neutrophils in their bactericidal capacity the udders with casein and fat primarily indicate that efficient neutrophil phagocytosis requires a constant flow of new mature neutrophils from the alveolar haemata. The studies with casein have also shown that there is no defect in degranulation following ingestion of staphylococci (Russell, Brunker & Reiter, 1977). Degranulation occurs normally. It appears to be characteristic of virulent staphylococci that they survive in phagosomes into which the contents of granules have been discharged.

The consequences of ingestion of staphylococci by a neutrophil are illustrated in Fig. 1.

#### IMMUNIZATION

Protection by immunization is always achieved by utilizing and enhancing the natural method by which a host would defend itself against disease. An understanding of the way in which a disease is produced and knowledge of the host defence mechanisms are therefore prerequisites of any rational attempt at immunization. For example, since tetanus is caused by a neurotoxin produced by growth of *Cl. tetani* in an area of tissue in which there is a reduced oxygen potential, immunization can be effected by presenting the toxin to the host in a non-toxic but immunogenic form so that antibodies are made against the toxin and are immediately available to neutralize the toxin should it be produced in the host. The method by which the mammary gland defends itself against staphylococci has been outlined: effective immunization can operate only by enhancing some stage in the natural mechanism of host defence.

There is, however, a conundrum. In the development of a definition of mastitis stress has been increasingly laid on the presence of potentially pathogenic bacteria and elevated milk somatic cell counts in aseptically sampled foremilk, often in the absence of clinical signs. Thus the International Dairy Federation says 'in all these definitions (normal udders, latent infection, subclinical, clinical and chronic mastitis) the cell count of the milk is the most important criterion, as the threshold value of normal foremilk is recognized as being no higher than 500 000 cells/ml' (Ann. Bull., 1971). This is not an arbitrary figure but is the upper value of two standard deviations about a mean cell count which was the point at which rising cell count coincided with deviations from normal values of sodium, potassium, chloride, lactose and whey nitrogen in milk and of milk yield, and which indicated secretory disturbances in the mammary parenchyma (Reichmuth, 1975). While there may be objections to the emphasis laid on somatic cell counts in diagnosing mastitis in individual cows (Giesecke & van den Hoven, 1974) the value of bulk-milk somatic cell counts, despite their limitations (Wright, 1977), should not be underestimated in monitoring a mastitis control programme.

However, if mastitis is 'a condition of the mammary gland such that there has been an evocation of the cytological defence mechanism with a consequent rise in somatic



Fig. 1. A column of three cells and a phagosome are each.

Reiter, 1977

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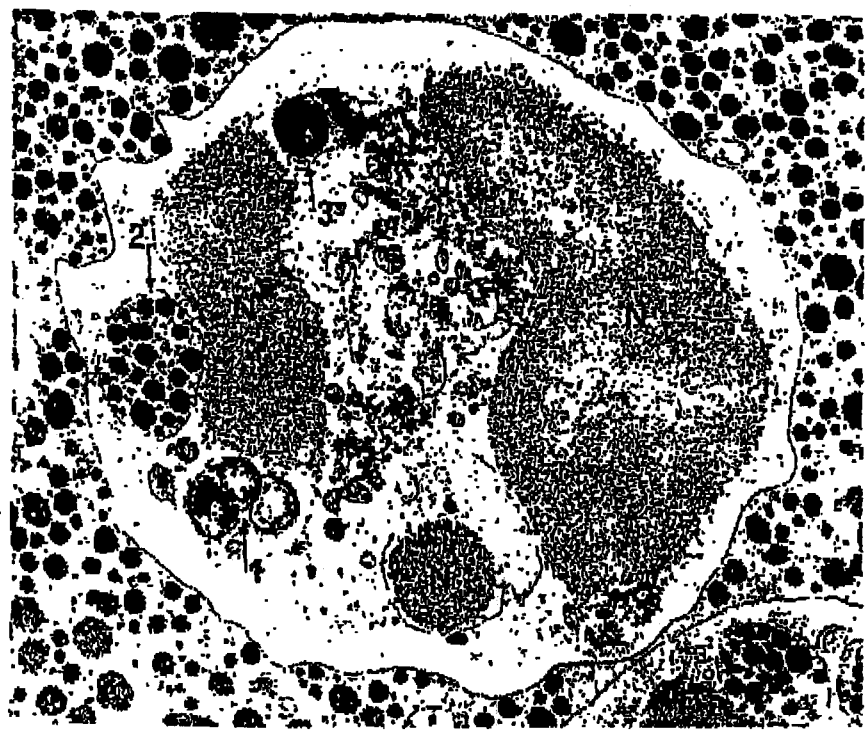


Fig. 1. Neutrophil from a mouse subcutaneous gland inoculated with *Staphylococcus aureus*. There is a group of three staphylococci undergoing destruction in a phagosome (1). There is a phagosome full of cocci (2) and a phagosome containing a single staphylococcus (3). The neutrophil has degranulated and there are visible degenerative changes in the nucleus (N). (x 14,000.)

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IMMUNIZATION AGAINST STAPHYLOCOCCAL MASTITIS

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cell levels' (Reichmann, Tolle & Whidstone, 1976), then it becomes evident that the natural defence mechanism of the mammary gland cannot be used, whether or not enhanced by immunization, to prevent mastitis since it is the defence system that is defined as the disease. Immunization, which cannot operate other than through the natural defence system, must of necessity function, when the mammary gland is challenged by staphylococci, by creating the response (anaphis) it is directed to prevent.

The problem is not removed by redefining mastitis; the distinction between 'infection' and 'inflammation' of the udder is not at issue. The emphasis on somatic cell numbers in the definition of mastitis has merely high-lighted the problem. The only inflammatory response under consideration is that due to invasion of the mammary gland by staphylococci. Frank & Newbould (1968) showed that when small numbers of staphylococci were infused into the udder inflammation invariably resulted and, following infusion of staphylococci, there was never inflammation without infection (Newbould & Neave, 1965). If mastitis is defined only in terms of isolation of staphylococci (Neave, 1975) then the defence system is no longer defined as the disease, but an inflammatory response and isolation of bacteria are so inextricably bound that the argument still holds that challenge of the mammary gland of immunized animals now result in mastitis.

Current definitions of mastitis, whether in terms of somatic cell numbers (Ayo, Bull, 1971), bacterial isolation (Griffin *et al.*, 1977) or a combination of both (Pearson & Green, 1974), indicate the high standards demanded of normal milk and which immunization must satisfy to be successful.

POSSIBLE TYPES OF IMMUNITY

There are five stages in the defence system that might be enhanced by immunization.

1. Neutrophil recruitment

Since recruitment of neutrophils is dependent upon the elaboration of chemotactic factors by multiplying staphylococci there is a delay between the entry of the organisms into the gland and the arrival of neutrophils. Targowski & Bernau (1975) observed that the leucocyte response to a first intramammary infusion of staphylococcal antigens in lactating cows, previously parenterally vaccinated with killed cells in oil-water adjuvant, was comparable to the leucocyte response to a second intramammary infusion in non-vaccinated cows. This increased and more rapid neutrophil response in sensitized cows was interpreted as an expression of cell mediated immunity and suggests a method by which neutrophil recruitment may be accelerated. It has also been argued that activation of the reticulo-endothelial system by staphylococcal antigens should be beneficial in defence against infection (Jones, 1974). Jones recommends the elicitation of cell-mediated immunity in the dry period since macrophages are more numerous in the involuted udder. However an immunologically elicited neutrophil response in lactating cows, though it may succeed in limiting the development of infection, is nevertheless mastitis. In addition, cell-mediated immunity, as it can be harmful rather than beneficial as has been elegantly demonstrated in *Staph. aureus* infections in mice (Barnum & Chynn, 1975), and how

Targowski & Herman (1975) and Jones (1974) are quoted in their recommendation of this form of therapy.

## 2. Anti-toxin immunity

Toxins, especially alpha toxin, play an important part in staphylococcal mastitis and it would seem logical to incorporate them in toxoided form in a vaccine. However, our understanding of the role of alpha toxin is that its production is a terminal event in the pathogenesis of the disease (Anderson, 1976). Alpha toxin is produced and released only towards the end of logarithmic bacterial growth in the mammary gland, that is, at a time when mastitis already exists. Anti-alpha toxin neutralizes alpha toxin; it can therefore only ameliorate the terminal pathological changes in the disease and can have little or no effect on the establishment and early development of mastitis. This view is supported by the observation that in older cows, that have higher levels of serum anti-alpha toxin, the incidence of acute clinical episodes is lower (Schulz, Carroll & Jain, 1971).

Anti-alpha toxin is present in normal milk in lower concentration than in the blood (Edwards & Smith, 1959) and it reaches the milk in the early stages of disease only when the epithelial barrier is damaged by the products of bacterial multiplication (Symons & Wright, 1974). This results in high levels of anti-toxin in milk (Derbyshire, 1960a), but since the epithelium is breached there is also a neutrophil infiltration, and so mastitis exists in terms of high somatic cell count and of staphylococcal excretion.

Although complicated by the inclusion of cell walls with alpha toxin, the vaccination experiments of Derbyshire (1960b) illustrate the problem of immunization in relation to anti-toxin immunity. When non-vaccinated cows were challenged with staphylococci a gangrenous mastitis with complete loss of secretion developed whereas only a mild transient reaction occurred in the vaccinated animals. It is probable that the anti-alpha toxin was responsible for abrogating the gangrenous reaction in the vaccinated cows. Nevertheless, because of the elevated neutrophil response and the continued excretion of staphylococci, the reaction in these vaccinated cows must be classified as mastitis. When rabbits that were immunized with highly purified alpha toxin were challenged with *Staph. aureus* it was found that they were protected against the fatal bacteraemic peritonitis form of the disease but they were not protected against the chronic abscess form (Adlam *et al.*, 1977). Conversely, when goats that were vaccinated with a preparation that did not contain alpha toxin were challenged with staphylococci, severe clinical and gangrenous reactions were obtained (Lepper, 1967). Alpha toxin should be incorporated in any vaccine against staphylococcal mastitis, but it cannot rationally be expected to do any more than prevent acute clinical flare-ups, and can have no effect on chronic or subclinical infections.

## 3. Anti-bacteria immunity

Bactericidal substances exist in milk in the form of lactoferrin and the lactoperoxidase-thiocyanate-hydrogen peroxide system but, though these systems are active against staphylococci (Reiter & Brandley, 1975), they are not antibodies and therefore outside the influence of immunization. Similarly, the substances in serum which are bactericidal for staphylococci are generally considered to be non-antibody in nature, though it has been suggested that natural antibody is involved (Kleinbaum,

Elliot & Zalusky, 1975).  
Thus strong evidence is  
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#### IMMUNIZATION AGAINST STAPHYLOCOCCAL MASTITIS

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Ellison & Zan G, 1971). The antibacterial activity of serum is inhibited by coagulase thus strongly coagulase positive strains of staphylococcus grow luxuriously in serum (Ekschell, 1956).

As regards specific antibody, it is generally accepted that Gram-positive organisms are not readily killed by the complement mediated bactericidal activity of antibodies; both antibody or complement and neutrophils are required. It seems unlikely, therefore, that a protective mechanism can be developed which depended only on specific antibacterial antibody, even if that antibody could be transported across an intact mammary epithelium.

##### 4. Anti-coagulant immunity

The extracellular proteins of staphylococci, many of which have enzymic properties, have been implicated in the pathogenesis of staphylococcal mastitis (Anderson, 1976). Knowledge of the properties of coagulase suggests that it should be one of the most promising of antigens. It is produced early in the logarithmic phase of growth and if it is essential for growth of staphylococci *in vivo* its neutralization at this stage might render staphylococci more susceptible to non-antibody bactericidal substances, and so prevent the establishment of infection. Antibodies can be raised against purified coagulase (Blöbel, Bernan & Shanon, 1960) but in order to protect the mammary gland the antibody would require to lie in IgG1 so that it would reach the milk without a simultaneous neutrophil response.

No record seems to exist of an attempt to immunize against mastitis using only coagulase as antigen. Coagulase was incorporated in a vaccine that was tested in goats but Derbyshire and Helliwell (1962) were unable to attribute any protective effect to that antigen. A vaccine which contained coagulase was tested in cattle and, though the criteria on which mastitis was diagnosed were not clearly stated, a reduction in the incidence and clinical severity of mastitis caused by the homologous strain was recorded (Blöbel & Bernan, 1962). There was no protective effect against heterologous strains of staphylococci.

##### 5. Opsonin immunity

The available evidence indicates that staphylococci are killed in the mammary gland most effectively by the opsonin-neutrophil system. Detailed studies in the mammary gland of the mouse (Anderson & Cloninger, 1975) and *in vitro* studies with bovine leucocytes (Russell *et al.*, 1977) show that there is no defect in ingestion of staphylococci by neutrophils but that degranulation occurs without killing of ingested staphylococci. To be effective immunization must elicit antibodies which not only facilitate ingestion but also render the staphylococci susceptible to the action of the contents of the neutrophil granules.

Two recent attempts to achieve such an immunity have been reported. Watson (1975) showed *in vitro* that neutrophils from ewes that had been infected with staphylococci as well as inoculated with killed staphylococci were more phagocytic for staphylococci than neutrophils from ewes that had received only the killed staphylococci. The enhanced phagocytosis (killing) was later shown to be due to a cytoplasmic opsonin which entered the neutrophils and which belonged to the class IgG2 (Watson, 1976). As yet it is not clear what antigen in the replicating staphylococci is



response which is insufficient to constitute subclinical mastitis, will immunization against bovine mastitis have succeeded.

REFERENCES

- ANDAM, C., WARD, P. D., MCCARTNEY, A. C., ARBUTHNOTT, J. P. & THORLEY, C. M. (1977). *Infect. Immunity* 17, 250.
- ANDERSON, J. C. (1976). *Br. vet. J.* 132, 229.
- ANDERSON, J. C. & CHANDLER, R. I. (1975). *J. comp. Path.* 85, 499.
- ANNEAL, NULLETON (1971). *Int. Dairy Fed. Publ.* 1.
- BLOOM, H. & BUNDMAN, D. T. (1962). *Am. J. vet. Res.* 25, 7.
- BLOOM, H., BUNDMAN, D. T. & SIMON, J. (1960). *J. Bact.* 79, 807.
- BRANDON, M. R., WATSON, D. L. & LASCELLES, A. K. (1971). *Adv. J. exp. Biol. med. Sci.* 49, 613.
- BRUCE, J. H., STEEL, F. D. & REITER, B. (1975). *Res. vet. Sci.* 19, 152.
- BRUCE, J. H., TURVEY, A. & REITER, B. (1973). *Infect. Immunity* 7, 865.
- BRUCE, J. H. (1973). *J. Am. vet. med. Ass.* 163, 795.
- CHRYSTIE, J. B. (1960a). *Res. vet. Sci.* 1, 830.
- CHRYSTIE, J. B. (1960b). *J. comp. Path. Ther.* 70, 222.
- CHRYSTIE, J. B. & HELLINGWELL, D. I. (1962). *Res. vet. Sci.* 3, 56.
- EDMOND, C. S. P. & CLYNN, A. A. (1975). *Immunology* 29, 75.
- EDWARDS, S. J. & SMITH, C. S. (1959). *J. comp. Path. Ther.* 69, 208.
- ELIENKAM, N. J., ELLIOTT, D. F. & ZARCO, R. (1971). *Infect. Immunity* 3, 664.
- ELIENKAM, R. D. (1956). *J. Bact.* 72, 167.
- GRISSECK, W. H. & VANDEN HURVE, L. W. (1974). *Quaterly J. vet. Res.* 41, 169.
- GRIFIN, T. K., DODD, F. H., NEAVE, F. K., WESTGARTH, D. R., KINGWILL, R. G. & WILSON, C. D. (1977). *J. Dairy Res.* 44, 25.
- JONES, R. L. (1974). *Proc. Ann. Meet. U.S. Anim. Hlth Ass.* 74, 148.
- KRIEGER, H. U. & SORMIN, E. (1967). *Int. Arch. Allergy appl. Immun.* 31, 505.
- LEPPER, A. W. D. (1967). *Br. vet. J.* 123, 182.
- MELLENBERGER, R. W. (1977). *J. Dairy Sci.* 60, 1016.
- MURPHY-THOMSON, E. (1958). *Imp. Bur. Anim. Hlth, Res. Ser. No. 1.*
- NEAVE, F. K. (1975). *Proc. Seminar on Mastitis Control, Int. Dairy Fed.* 85, 19.
- NEWMAN, F. H. S. & NEAVE, F. K. (1966). *J. Dairy Res.* 33, 168.
- PAAR, M. J., CHURCH, A. J., KIRK, S. T. & BOIT, D. J. (1975). *Am. J. vet. Res.* 36, 1757.
- PANNEY, J. W. (1977). *J. Am. vet. med. Ass.* 170, 1233.
- PARSON, J. K. L. & GIBBS, D. O. (1974). *Vet. Rec.* 95, 252.
- PARSON, J. K. L. & NEWMAN, F. H. S. (1968). *Can. vet. J.* 9, 170.
- RECHMUTH, J. (1975). *Proc. Seminar on Mastitis Control, Int. Dairy Fed.* 85, 85.
- RECHMUTH, J., THOMAS, A. & WHITTESTONE, W. G. (1976). *N.Z. vet. J.* 24, 47.
- REITER, B. & BRUCE, J. H. (1974). *Proc. Seminar on Mastitis Control, Int. Dairy Fed.* 85, 270.
- REITER, B. & BRUCE, J. H. (1975). *Immunology* 28, 71.
- REITER, B. W. & REITER, B. (1975). *J. Reticuloendothel. Soc.* 18, 1.
- REITER, B. W., REITER, B. E. & REITER, B. (1976). *Br. vet. Sci.* 20, 80.
- REITER, B. W., REITER, B. E. & REITER, B. (1977). *J. comp. Path.* 87, 43.
- RUSSELL, K. J., WILKINSON, P. C., MCNEILLY, R. J., MCKAY, S., MCCARTNEY, A. C. & ARBUTHNOTT, J. P. (1976). *J. med. Microbiol.* 9, 435.
- SARAN, G. W., CARROLL, E. J. & JAIN, N. C. (1971). In *Bovine Mastitis*. Philadelphia: Lea & Febiger.
- SIMPSON, L., REID, G. W., STEFAN, R. A. & CHANIN, E. V. (1967). *J. comp. Path.* 77, 279.
- SIMPSON, J. K. (1977). In *The Hummoleptic Function and Clinical Utilization*, p. 103. New York: Alan R. Liss Inc.
- STONES, T. P. (1971a). *New Engl. J. Med.* 280, 717.
- STONES, T. P. (1971b). *New Engl. J. Med.* 280, 774.



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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

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RECIBO OFICIAL DE CAJA : 08 - 20,860  
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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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| DEPOSITANTE       | TIPO PAGO    | BANCO           | CUENTA    | No. PAGO | FECHA PAGO | Vr. PAGO      |
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| CAVELIER ABOGADOS | CONSIGNACION | BANCO DE BOGOTA | 062754387 | 127023   | 10/03/2008 | 38,184,000.00 |

\*\*\*\*\* CONCEPTO \*\*\*\*\*

| CANT. | RENTISTICO  | CONCEPTO                       | TOTAL CONCEPTO |
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| 1     | 50005-01-03 | CAMBIO DE MODALIDAD Y MODIFICA |                |
|       |             | 12 MARCAS Y LEHAS COMERCIALES  | 103,000.00     |
|       |             | TOTAL :                        | \$ 103,000.00  |

SON: CIENTO TRES MIL PESOS

RESPONSABLE :

RECIBO DE CAJA APLICADO AL EXPECIENTE No. \_\_\_\_\_

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US005328996A

**United States Patent** [19]  
**Boyle et al.**

[11] **Patent Number:** **5,328,996**  
[45] **Date of Patent:** **Jul. 12, 1994**

- [54] **BACTERIAL PLASMIN RECEPTORS AS FIBRINOLYTIC AGENTS**
- [75] **Inventors:** Michael D. P. Boyle, Whitehouse, Ohio; Richard Lottenberg, Gainesville, Fla.; Christopher Broder, Rockville, Md.; Gregory Von Mering, Gainesville, Fla.
- [73] **Assignee:** University of Florida Research Foundation, Inc., Gainesville, Fla.
- [21] **Appl. No.:** 928,462
- [22] **Filed:** Aug. 10, 1992

**Related U.S. Application Data**

- [63] Continuation-in-part of Ser. No. 524,411, May 16, 1990, Pat. No. 5,237,050, which is a continuation-in-part of Ser. No. 330,849, Mar. 29, 1989, abandoned.
- [51] **Int. Cl.<sup>5</sup>** ..... C07H 17/00  
**U.S. Cl.** ..... 536/23.1; 536/23.7;  
424/94.64; 435/172.3; 530/350; 530/388.25;  
530/381; 530/825
- [58] **Field of Search** ..... 536/23.1, 23.7

**References Cited**

**PUBLICATIONS**

- Lottenberg et al J. of Bact. 174(16):5204, 1992.
- Pancholi, V., and V. A. Fischetti (1992) "A Novel Multifunctional Surface Protein (MF6) of group A Streptococci" Abstract of the General Meeting Abstract No. B-252 p. 68.
- Blobel, Gunter (1980) "Intracellular protein topogenesis" Proc. Natl. Acad. Sci. USA 77(3):1496-1500.
- Ferguson, Michael A. J., and Alan F. Williams (1988) "Cell-Surface Anchoring of Proteins Via Glycosyl-Phosphatidylinositol Structures" Ann. Rev. Biochem. 57:285-320.
- Hekman, W. E., D. T. Dennis, and J. A. Miernyk (1990) "Secretion of *Ricinus communis* glyceraldehyde-3-phosphate dehydrogenase by *Escherichia coli*" Molecular Microbiology 4(8):1363-1369.
- Allen, Robert W., Kathleen A. Trach, and James A. Hoch (1987) "Identification of the 37-kDa Protein Displaying a Variable Interaction with the Erythroid Cell Membrane as Glyceraldehyde-3-phosphate Dehydro-

- genase" The Journal of Biological Chemistry 262(2):649-653.
- Goudot-Crozel, Veronica, Daniele Caillol, Malek Djabali, and Alan J. Dessein (1989) "The Major Parasite Surface Antigen Associated With Human Resistance to Schistosomiasis is a 37-kD Glyceraldehyde-3-P-Dehydrogenase" J. Exp. Med. 170:2065-2080.
- Allen, Robert W., and Beverly A. Hoover (1985) "Characterization of the Processed Form of a Ubiquitous Protein Displaying a Variable Membrane Organization in Erythroid Cells" Blood 65(5):1048-1055.
- Michels, Paul A. M., M. Marchand, L. Kohl, S. Albert, R. K. Wierenga, and F. R. Opperdoes (1991) "The cytosolic and glycosomal isoenzymes of glyceraldehyde-3-phosphate dehydrogenase in *Trypanosoma brucei* have a distant evolutionary relationship" Eur. J. Biochem. 198:421-428.
- Tso, J. Yun, Xiao-Hong Sun, Teh-hui Kao, Kimberly S. Reece, and Ray Wu (1985) "Isolation and characterization of rat and human glyceraldehyde-3-phosphate dehydrogenase DNAs: genomic complexity and molecular evolution of the gene" Nucleic Acids Research 13(7):2485-2502.

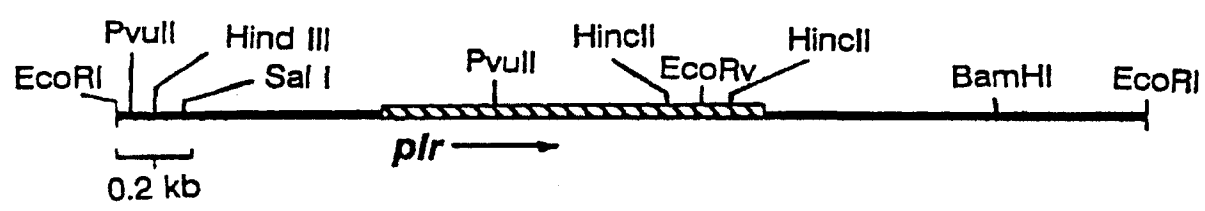
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**Primary Examiner**—Suzanne E. Ziska  
**Attorney, Agent, or Firm**—Saliwanchik & Saliwanchik

[57] **ABSTRACT**

The subject invention concerns novel methods and compositions for thrombolytic therapy. More specifically, a receptor with high affinity for plasmin has been characterized, purified, cloned, and expressed. This receptor can be used in combination therapies where it is administered prior to, concurrently with, or after a plasminogen activator. Also, this receptor can be bound to plasmin and administered to humans or animals in need of thrombolytic therapy. Additionally, the invention pertains to a novel immobilized form of plasmin which advantageously accumulates at the point where antifibrinolytic activity is needed.

**2 Claims, 1 Drawing Sheet**



# BACTERIAL PLASMIN RECEPTORS AS FIBRINOLYTIC AGENTS

## CROSS-REFERENCE TO A RELATED APPLICATION

This is a continuation-in-part of our co-pending application Ser. No. 07/524,411, filed May 16, 1990; now U.S. Pat. No. 5,237,050 which is a continuation-in-part of application Ser. No. 330,849, filed on Mar. 29, 1989, now abandoned.

## BACKGROUND OF THE INVENTION

Ten years ago, the food and drug administration first approved the use of plasminogen activators for thrombolytic therapy. It was originally recommended for the treatment of deep-vein thrombosis and serious pulmonary embolisms. This approach is now also used for treating acute peripheral arterial thrombosis and acute coronary thrombosis and for solubilizing clots in catheters and shunts.

With the development of recombinant DNA technology and the cloning and expression of tissue plasminogen activator (TPA), we have now entered a new age for thrombolytic agents with unique physiological properties and therapeutic promise. The original plasminogen activators that have been used clinically were streptokinase and urokinase. These agents produced in the patients a generalized lytic state which had a variety of side effects that were not directly targeted at solubilizing the fibrin clot. Tissue plasminogen activator, because of its fibrin binding capacity, enhances the selectivity of these agents for fibrin degradation. It is thus being greeted with great fanfare as the new generation of fibrinolytic agents.

Tissue plasminogen activator is not without its potential side effects, and, furthermore, its cost is prohibitive for use in many settings. For example, the V.A. in Gainesville will not authorize tissue plasminogen activator therapy over streptokinase therapy because of its enormous cost. Furthermore, the application of this fibrinolytic therapy to domestic animals or food production animals is limited by the enormous cost.

It is clear to most experts that the wonder drug nature of tissue plasminogen activator has been highly overrated. TPA has been found to have a very short half life in the body. Also, pharmacological doses of tissue plasminogen activator produce a significant bleeding risk for the patient, and in the case of coronary artery thrombosis, re-occlusion of the blood vessel following successful clot lysis occurs in a significant number of patients. The need for an inexpensive, and perhaps even better form of treatment is clearly evident. The use of combination therapies whereby existing compositions and methods are integrally linked in novel ways with new materials and procedures could enhance the effectiveness of plasminogen activators and possibly reduce the amount of plasminogen activator needed to achieve the desired results.

Recently, we have described in our laboratory the presence of a selective, high affinity receptor for human plasmin and other species of plasmin on the surface of certain group A streptococci (Lottenberg, R., C. C. Broder, M. D. P. Boyle [1987] *Infect. Immun.* 55:1914-1918). Group A streptococci cause pharyngitis and invasive infections such as cellulitis and bacteremia (Bisno, A. L. [1991] *New Engl. J. Med.* 325:783-793). There has been a recent increase in invasive group A

streptococcal infections occurring in healthy individuals. Also, group B streptococci are important pathogens for pregnant women. *Staphylococcus aureus*, another gram-positive bacteria, is another invasive pathogen for immunocompetent hosts. Effective vaccines to protect against infection by these organisms are not currently available.

The biochemical interactions occurring at cell surfaces between bacterial membranes and their surroundings are complex and not well understood. Certain bacterial surface structures and secreted products have been suggested to contribute to tissue invasion. One of these secreted products, streptokinase, is a plasminogen activator and converts the host zymogen plasminogen to the active protease, plasmin (Siefring, G. E., F. J. Castellino [1976] *J. Biol. Chem.* 251:3913-3920). Although classically described as the enzyme responsible for fibrin degradation, plasmin is a serine protease with trypsin-like specificity and has activity for a broad range of substrates. Plasmin can degrade several mammalian extracellular matrix proteins, such as fibronectin and laminin, and can enhance collagenase activity (Liotta, L. A., R. H. Goldfarb, R. Brundage [1981] *Cancer Res.* 41:4629-4636). Therefore, the ability to generate and capture active plasmin may contribute to the invasive propensity of certain streptococcal strains. The interaction of plasmin with group A streptococci has high affinity ( $K_d$ ,  $10^{-10}$ M) and is specific for plasmin, with no significant binding demonstrated for structurally related proteins (Broescker, T. A., M. D. P. Boyle, R. Lottenberg [1988] *Microb. Pathog.* 5:19-27; DesJardin, L. E., M. D. P. Boyle, R. Lottenberg [1989] *Thromb. Res.* 55:187-193).

As described herein, the plasmin receptor of the subject invention has significant similarity to the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH). GAPDH is a key enzyme involved in glucose metabolism and has been the subject of many genetic studies. Multiple copies of GAPDH genes have been identified for mammals, with many described as pseudogenes (Piechaczyk, M., J. M. Blanchard, S. Riaad-El Sabouty, C. Dani, L. Marty, P. Jeanteur [1984] *Nature* 312:469-471). Multiple GAPDH genes have also been identified for *E. coli*, *Trypanosoma brucei*, *Saccharomyces cerevisiae*, and *Drosophila melanogaster* (Alefounder, P. R., R. N. Perham [1989] *Mol. Microbiol.* 3:723-732; Holland, J. P., L. Banieniec, C. Swimmer, M. J. Holland [1983] *J. Biol. Chem.* 258:5291-5299; Michels, P. A. M., M. Marchand, L. Kohl, S. Allert, R. K. Wierenga, F. R. Opperdoes [1991] *Eur. J. Biochem.* 198:421-428; Tso, J. Y., X. -H. Sun, R. Wu [1985] *Nucleic Acids Res.* 13:1251). *E. coli* and *T. brucei* each have two GAPDH genes with significant differences in deduced amino acid sequence (Alefounder et al., supra; Michels et al., supra); however, the translated product of the second *E. coli* GAPDH gene has not been reported. One of the trypanosomal isoenzymes is localized in the glycosome, a specialized metabolic organelle, while the other GAPDH is found in the cytoplasm (Lambier, A. -M., A. M. Loiseau, D. A. Kuntz, F. M. Vellieux, P. A. M. Michels, F. R. Opperdoes [1991] *Eur. J. Biochem.* 198:429-435).

In addition to its usual intracellular location, GAPDH has been identified on the surface of hematopoietic cells and *Schistosoma mansoni*, an invasive parasite (Allen, R. W., K. A. Trach, J. A. Hoch [1987] *J. Biol. Chem.* 262:649-653; Goudot-Crozel, V., D. Cail-

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101, M. Djabali, A. J. Dessein [1989] *J. Exp. Med.* 170:2065-2080). Allen and Hoover ([1985] *Blood* 65:1045-1055) characterized a membrane-associated 37,000-M<sub>r</sub> protein expressed by the erythroleukemic cell line K562. Peptide mapping and molecular cloning studies revealed the protein to be homologous to GAPDH (Allen, Trach, and Hoch, supra). A similar finding has been reported for the blood fluke responsible for abdominal schistosomiasis (Goudet-Crozel et al., supra). A 37,000-M<sub>r</sub> surface immunogen of *S. mansoni* was characterized by isolating the cDNA encoding the protein. The deduced amino acid sequence had significant homology to that of human GAPDH. Like the recombinant plasmin receptor protein (Plr), neither of these surface proteins had domains corresponding to previously described membrane-anchoring structures (Blobel, G. [1980] *Proc. Natl. Acad. Sci. USA* 77:1496-1500; Ferguson, M. A. J., A. F. Williams [1988] *Annu. Rev. Biochem.* 57:285-320). Interestingly, Hekman et al. (Hekman, W. E., D. T. Dennis, J. A. Miernyk [1990] *Mol. Microbiol.* 4:1363-1369), while studying the expression of recombinant plant GAPDH in *E. coli*, were able to target the protein to the outer membrane by genetically fusing the signal sequence of *E. coli* OmpA to *Ricinus communis* GAPDH. Pancholi et al. have recently reported the isolation of a 39 kD surface protein with GAPDH activity from a group A streptococci (Pancholi, V., V. A. Fischetti [1992] "A Novel Multifunctional Surface Protein (MFG) of group A Streptococci," Abstract No. B-252, Abstracts of the General Meeting 1992:68).

GAPDH from streptococci has not been isolated or characterized, and the relationship of the plasmin receptor to the glycolytically active enzyme remains to be seen.

#### BRIEF SUMMARY OF THE INVENTION

The subject application pertains to novel compositions and methods for thrombolytic therapy. A receptor for human plasmin has been identified, purified, characterized, cloned and expressed. This receptor has been found to bind with very high affinity to plasmin. When bound to the receptor, plasmin retains its enzymatic activity but is not regulated by  $\alpha 2$  antiplasmin.

The isolation and purification of the plasmin receptor protein makes it possible to administer novel compositions and treatments to achieve fibrinolytic activity. Specifically, the receptor protein can be used in combination with existing plasminogen activators in ways which enhance the usefulness of the plasminogen activators while minimizing the negative aspects of these agents. For example, by concurrent or sequential administration of plasminogen activators and the receptor protein it is possible to prolong the effects of the plasminogen activator. The prolongation results from the binding of the receptor molecule to free plasmin produced by the activity of the plasminogen activator. Once the receptor binds with high affinity to the plasmin, the plasmin is no longer inhibited by  $\alpha 2$  antiplasmin. In this fashion, the amount of plasminogen activator needed to achieve the desired plasmin activity can be reduced and the disadvantageous effects of the short half life of the plasminogen activator can be minimized.

Immobilized or receptor-bound plasmin can be used in conjunction with the administration of streptokinase, urokinase, tissue plasminogen activator, or other plasminogen activators. The use of our novel plasmin constructs in such a treatment regimen can help to prevent

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re-occlusion of blood vessels, while the reduced concentration of the plasminogen activator lowers the risk of bleeding.

Furthermore, the receptor molecule can be conjugated to a monoclonal antibody specific for fibrin. In this way, the plasmin can be targeted to the fibrin clot where the fibrinolytic activity is needed. Also, this will help to reduce the amount of plasmin circulating freely in the blood system.

In another embodiment of the subject invention, plasmin can be bound to the receptor before administration to the human or animal in need of thrombolytic activity. Plasmin bound to the receptor will supply the fibrinolytic activity without being rapidly inhibited by  $\alpha 2$  antiplasmin.

The subject invention further concerns a process which comprises treating a human or animal in need of fibrinolytic activity with bacteria comprising a selective, high affinity receptor for plasmin, human and otherwise. Exemplified herein is the use of a group A streptococcus receptor which binds human plasmin with, advantageously, a very high affinity and a very slow off-rate. This form of immobilized plasmin has a number of potential applications for thrombolytic therapy, not only in man, but also in animals.

Further aspects of the subject invention pertain to the essentially pure plasmin receptor and the DNA which codes for this protein. The plasmin receptor of the subject invention can be purified from certain bacteria as described below. Also, the receptor can be produced by recombinant techniques using novel clones which express the receptor. The receptor is a M<sub>r</sub> ≈ 41,000 dalton protein that is distinct from the M<sub>r</sub> ≈ 47,000 streptokinase molecule produced by the same bacteria. Unlike the streptokinase, the plasmin receptor protein lacks plasminogen activator activity.

Yet a further aspect of the subject invention pertains to the use of the plasmin receptor as a vaccine to raise an immune response to group A streptococci (GAS) infections and a potentially broad range of infections associated with pathogens expressing GAPDH or GAPDH-like proteins at the cell surfaces.

#### BRIEF DESCRIPTION OF THE DRAWING

FIG. 1 shows the restriction map of the 2.7-kb EcoRI insert of pRL015. Restriction enzyme recognition sites are indicated. The location of plr is denoted by the hatched box. The arrow indicates the orientation of the open reading frame.

#### BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO. 1 shows the composite DNA and translated amino acid sequence of the plasmin receptor protein of the subject invention.

SEQ ID NO. 2 is the translated amino acid sequence of the plasmin receptor protein of the subject invention.

#### DETAILED DESCRIPTION OF THE INVENTION

One of the major problems with plasminogen activator administration is the lytic state that accompanies injection of high concentrations of streptokinase or, to a lesser degree, tissue plasminogen activator. This is due to the generation of plasmin from the plasminogen as the activators circulate throughout the human or animal. Tissue plasminogen activator has a certain affinity for fibrin that allows activation to occur preferentially in the region of the clot.

The purified plasmin receptor of the subject invention can be used in combination with plasminogen activators to produce the desired thrombolytic activity. Advantageously, the use of plasmin receptors can be used to reduce the amount of plasminogen activator needed to achieve the necessary activity. By binding with plasmin generated by plasminogen activators, the receptor is able to greatly prolong the effect of the plasminogen activator. The adverse effects of the short half-life of plasminogen activators are thereby minimized. When the receptor binds to plasmin, the plasmin retains its enzymatic activity but is not inhibited by  $\alpha 2$  antiplasmin. Also, the receptor can be conjugated to antibodies which serve to target the enzymatic activity of the plasmin to the blood clot where activity is needed. The use of this combined therapy of plasminogen activator and plasmin receptor facilitates a much more efficient treatment for blood clots. The combined therapy minimizes the deleterious effects which accompany current thrombolytic therapies.

An alternate form of therapy involves the administration of plasmin already bound to the receptor. The receptor may be purified or still associated with the bacteria surface. Plasmin associated with a bacteria circulates freely in the bloodstream until cleared by the reticuloendothelial system and, by virtue of its particle size, it can accumulate in areas where blood clots have formed. Once there, the bacterium-bound enzyme can degrade the fibrin and clear the clot. The bacteria then continue to circulate until the reticuloendothelial system clears the organisms. Our findings that the plasmin receptor is extremely stable and is present on non-viable bacteria makes this approach particularly attractive. It enables micromolar quantities of plasmin to be immobilized on bacteria by adsorption. The bacterial bound plasmin can then be administered intravenously without significant toxicity. The organisms, being non-viable, do not present any risk to the human or animal patient, and the potential for clearance of the organisms following a reasonable half-life in the plasma, would be anticipated by virtue of the normal reticuloendothelial cell function of the human or animal.

Although a pathogenic group A streptococcus is exemplified herein, plasmin receptors have also been identified on a number of other organisms including isolates that, even when viable, are not human pathogens. Other organisms which can be used to practice the subject invention include other groups of streptococci including B, C and G. Also, bovine *Pasteurella haemolytica* as well as *Staphylococcus aureus* microorganisms can be used. These organisms are well-known in the art and are illustrative, but not exhaustive, of the microorganisms which come within the scope of the subject invention.

The major advantage of using plasmin bound to bacteria is the ability to produce a fibrinolytic agent that has selectivity towards the clot, and would not result in a widespread lytic state, all of which can be achieved at a very moderate cost. In addition, the bacterial plasmin receptor has been shown to bind not only to human but other species of plasmin, thereby enabling this approach to thrombolytic therapy to be extended to domestic and food producing animals.

Of particular interest in this regard is a major economic disease in cattle which involves fibrin pneumonia that occurs in cows infected with *Pasteurella haemolytica*. This is a major killer of animals, particularly those suffering from immunosuppression as a consequence of

viral infection. This is particularly important in feed lots and under conditions where large numbers of cattle are housed together. There is a great need for the ability to treat the fibrin deposits in the lung, which ultimately suffocate the animal, by aerosol treatment with a plasmin-bacterial complex. In addition, situations for which thrombolytic therapy has been used in human medicine could now be applied to similar situations in animals.

Among the advantages of using bacteria coated with plasmin, are (1) the increased selectivity achieved by immobilizing plasmin in a non-regulatable form on a particle that would circulate with a predictable half-life in the serum of an individual animal; and (2) the low cost, which would then enable the approach to be used for animals as well as man.

We have isolated and analyzed plr, the gene encoding a group A streptococcal plasmin receptor. By screening a  $\lambda$  gt11 expression library with antiplasmin receptor antibodies, we identified a plasmin receptor gene within a 2.7-kb streptococcal DNA fragment. This fragment was subcloned into a low-copy-number plasmid, and the receptor protein was stably expressed in *E. coli* under the control of putative streptococcal promoter elements. The recombinant receptor protein demonstrated immunoreactivity and plasmin-binding activity. We determined the nucleotide sequence for plr and upstream elements of the structural gene. An open reading frame of 1,008 bp was identified. The 40.5% G+C content of plr was slightly higher than the 35 to 39% reported for group A streptococcal chromosomal DNA (Hardie, J. M. [1986] "Genus Streptococcus," pp. 1043-1071, In J. G. Holt et al. (eds.), *Bergey's manual of systematic bacteriology*, Williams & Wilkins, Baltimore). The deduced amino acid sequence was identical for 74 amino acid residues at the N terminus as well as three cyanogen bromide fragments obtained from the native streptococcal protein. The amino acid sequence obtained for the streptococcal receptor protein revealed that the initial methionine residue is cleaved.

The deduced amino acid sequence for the recombinant plasmin receptor protein (Plr) was compared with published sequences for other proteins. Plr exhibits significant similarity to the glycolytic enzyme GAPDH, reported for a number of prokaryotic and eukaryotic organisms. The best match was with *B. subtilis* (56% identical and 73% conserved amino acid residues). GAPDH from streptococci has not been isolated or characterized, and the relationship of the plasmin receptor to the glycolytically active enzyme remains to be defined. However, the extensive amino acid homology and similar hydropathy plots for Plr and *B. subtilis* GAPDH strongly suggest that Plr is a member of the GAPDH family of proteins. Furthermore, preliminary analysis of the recombinant protein revealed that Plr has GAPDH enzymatic activity.

The clones have been transformed into and expressed in *E. coli*. An *Escherichia coli* host containing the clones has been deposited into the permanent collection of the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Md. 20852 USA. The culture has been assigned the following accession number:

| Culture                                        | ATCC Designation | Deposit Date     |
|------------------------------------------------|------------------|------------------|
| <i>Escherichia coli</i> ,<br>chi 6060 (pRL015) | 69415            | December 9, 1993 |

The subject cultures have been deposited under conditions that assure that access to the cultures will be available during the pendency of this patent application to one determined by the Commissioner of Patents and Trademarks to be entitled thereto under 37 CFR 1.14 and 35 USC 122. The deposit is available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny, are filed. However, it should be understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

Further, the subject culture deposits will be stored and made available to the public in accord with the provisions of the Budapest Treaty for the Deposit of Microorganisms, i.e., they will be stored with all the care necessary to keep them viable and uncontaminated for a period of at least five years after the most recent request for the furnishing of a sample of the deposit(s), and in any case, for a period of at least 30 (thirty) years after the date of deposit or for the enforceable life of any patent which may issue disclosing the cultures. The depositor acknowledges the duty to replace the deposits should the depository be unable to furnish a sample when requested, due to the condition of the deposits. All restrictions on the availability to the public of the subject culture deposits will be irrevocably removed upon the granting of a patent disclosing them.

In addition to the plasmin receptor amino acid sequence disclosed herein, the subject invention further comprises equivalent plasmin receptor proteins (and nucleotide sequences coding for equivalent proteins) having the same or similar biological activity of the plasmin receptor exemplified herein. These equivalent proteins may have amino acid homology with the protein disclosed and claimed herein. This amino acid homology will typically be greater than 75%, preferably be greater than 90%, and most preferably be greater than 95%. The amino acid homology will be highest in certain critical regions of the protein which account for biological activity or are involved in the determination of three-dimensional configuration which ultimately is responsible for the biological activity. In this regard, certain amino acid substitutions are acceptable and can be expected if these substitutions are in regions which are not critical to activity or are conservative amino acid substitutions which do not affect the three-dimensional configuration of the molecule. For example, amino acids may be placed in the following classes: nonpolar, uncharged polar, basic, and acidic. Conservative substitutions whereby an amino acid of one class is replaced with another amino acid of the same type fall within the scope of the subject invention so long as the substitution does not materially alter the biological activity of the compound. Table 1 provides a listing of examples of amino acids belonging to each class.

TABLE 1

| Class of Amino Acid | Examples of Amino Acids                |
|---------------------|----------------------------------------|
| Nonpolar            | Ala, Val, Leu, Ile, Pro, Met, Phe, Trp |
| Uncharged Polar     | Gly, Ser, Thr, Cys, Tyr, Asn, Gln      |
| Acidic              | Asp, Glu                               |
| Basic               | Lys, Arg, His                          |

In some instances, non-conservative substitutions can also be made. The critical factor is that these substitutions must not significantly detract from the biological activity of the protein. It has been shown that proteins of identified structure and function may be constructed

by changing the amino acid sequence if such changes do not alter the protein secondary structure (Kaiser, E. T. and Kezdy, F. J. [1984] *Science* 223:249-255). Thus, the subject invention includes mutants of the amino acid sequence depicted herein which do not alter the protein secondary structure, or if the structure is altered, the biological activity is substantially retained.

The genes and proteins according to the subject invention include not only the full length sequences disclosed herein but also fragments of these sequences, or fusion proteins, which retain the characteristic receptor-binding or GAPDH activity of the proteins specifically exemplified herein.

It should be apparent to a person skilled in this art that genes coding for receptor-binding proteins can be identified and obtained through several means. The specific genes may be obtained from a culture depository as described herein. Alternatively, these genes, or portions thereof, may be constructed synthetically, for example, by use of a gene machine. Variations of these genes may be readily constructed using standard techniques for making point mutations. Also, fragments of these genes can be made using commercially available exonucleases or endonucleases according to standard procedures. For example, enzymes such as Bal31 or site-directed mutagenesis can be used to systematically cut off nucleotides from the ends of these genes. Also, genes which code for active fragments may be obtained using a variety of other restriction enzymes. Proteases may be used to directly obtain active fragments of these proteins.

#### Materials and Methods

Bacterial strains, plasmids, and media. Group A streptococcal strain 64/14 is a M-untypeable clinical isolate that was passaged in mice 14 times (Reis, K. J., M. Yarnall, E. M. Ayoub, M. D. P. Boyle [1984] *Scand. J. Immunol.* 20:433-439). *Escherichia coli* Y1090 [ $\Delta$ lacU169 proA<sup>+</sup>  $\Delta$ lon araD139 strA supF trpC22::Tn10(Tet<sup>r</sup>)(pMC9)] was used in screening the expression library, and *E. coli*  $\chi$ 6060 [F' (traD36 proAB lac1<sup>+</sup>  $\Delta$ lacZM15)::Tn5 (Km<sup>r</sup>)/araD139  $\Delta$ (ara leu)7967  $\Delta$ lacX74  $\Delta$ phoA20 galE galK recA1 rpsE argE(Am) rpoB thi] was used for transformation and gene expression. A low-copy-number plasmid pYA2204 with a replicon derived from pREG153 (a low-copy-number IncW vector derived from the R plasmid R388) was used (Galan, J. E., J. F. Timoney, R. Curtiss III [1988] "Expression and localization of the *Streptococcus equi* M protein in *Escherichia coli* and *Salmonella typhimurium*," p. 34-40, In D. G. Powell, ed., *Proceeding of the Fifth International Conference of Equine Infectious Diseases*, University Press of Kentucky, Lexington, Ky.). The plr was subcloned into the pUC9 lacZ multiple cloning site of pYA2204.

Streptococci were grown in Todd-Hewitt broth. *E. coli* Y1090 for  $\lambda$  infection was grown in 1% tryptone supplemented with 0.5% yeast extract and 0.4% maltose. *E. coli*  $\chi$ 6060 was grown in L broth supplemented with kanamycin (50  $\mu$ g/ml).

Radioiodination of proteins. Human plasminogen isolated from plasma by chromatography on lysine-Sepharose (Lottenberg, R., F. R. Dolly, C. S. Kitchens [1985] *Am. J. Hematol.* 19:181-193) and streptococcal protein G (Calbiochem, San Diego, Calif.) were labeled with <sup>125</sup>I (Amersham Corp., Arlington Heights, Ill.) by using a mild lactoperoxidase reaction with Enzymo-

beads (Bio-Rad, Richmond, Calif.) (McCoy II, E., C. C. Broder, R. Lottenberg [1991] *J. Infect. Dis.* 164:515-521). Plasmin was generated from radiolabeled plasminogen as previously described (Broder, C. C., R. Lottenberg, G. O. von Mering, K. H. Johnston, M. D. P. Boyle [1991] *J. Biol. Chem.* 266:4922-4928).

DNA manipulations. Streptococcal chromosomal DNA was isolated by a modification of the procedure reported by Hudson and Curtiss (Hudson, M. C., R. Curtiss III [1990] *Infect. Immun.* 58:464-470). Streptococci were grown overnight as standing cultures at 37° C. in Todd-Hewitt broth supplemented with 2.2 mM K<sub>2</sub>HPO<sub>4</sub>. Bacterial pellets were treated with lysozyme, mutanolysin, and pronase. The cells were lysed with sodium dodecyl sulfate (SDS). Genomic DNA was further purified by two successive cesium chloride gradient centrifugations. Plasmid DNA was isolated by the alkaline lysis procedure (Birnboim, H. C. [1983] *Methods Enzymol.* 100:243-255) and purified by cesium chloride gradient centrifugation. Restriction enzyme digestions and restrictions with DNA-modifying enzymes were performed according to the manufacturers' recommendations (Bethesda Research Laboratories, Inc., Gaithersburg, Md., and Promega Corp., Madison, Wis.).  $\lambda$  phage were isolated by the plate lysate method (Silhavy, T. J., M. L. Berman, L. W. Enquist [1984] *Experiments with gene fusions*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) and then precipitated with polyethylene glycol. DNA was purified by using an anion-exchange resin (Qiagen, Studio City, Calif.). DNA fragments of interest were recovered from agarose gels by using GeneClean (Bio 101, La Jolla, Calif.). Other DNA manipulations were performed essentially as described by Maniatis et al. (Maniatis, T., E. F. Fritsch, J. Sambrook [1982] *Molecular cloning: a laboratory manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.).

Preparation of the  $\lambda$  gt11 library. The streptococcal genomic library was constructed as described by Huynh et al. (Huynh, T. V., R. A. Young, R. W. Davis [1985] "Construction and screening cDNA libraries in  $\lambda$  gt10 and  $\lambda$  gt11," pp. 49-78 In D. M. Glover (ed.), *DNA cloning*, IRL Press, Oxford). Chromosomal DNA was mechanically sheared to generate fragments approximately 2 to 7 kb in length. The DNA was treated with EcoRI methylase, treated with Klenow fragment of DNA polymerase to generate blunt ends, and ligated with EcoRI linkers. Excess linkers were removed by digestion with a high concentration of EcoRI. The DNA was ligated into EcoRI-generated  $\lambda$  gt11 arms and packaged into  $\lambda$  phage with Packagene (Promega Corp.).

Screening of the streptococcal library. The resulting non-amplified  $\lambda$  gt11 library was diluted in 10 mM Tris-2.5 mM MgSO<sub>4</sub>-0.01% gelatin-0.1M NaCl, pH 7.5, and used to infect *E. coli* Y1090, yielding a density of 200 to 400 plaques per plate. The infected cells were mixed with 0.45% soft agar, plated on 1.2% L agar supplemented with ampicillin (50  $\mu$ g/ml), incubated at 42° C. for 3 to 4 hours to induce lysis, and overlaid with nitrocellulose filters impregnated with 10 mM isopropylthiogalactoside (IPTG) to induce the lac promoter. After incubation at 37° C. for approximately 16 hours, the filters were removed, washed, and blocked in 100 mM Tris-300 mM NaCl-5 mM EDTA-0.05% Triton X-100-0.25% gelatin, pH 7.4 (NET-gel). The filters were then incubated with murine antiplasmin receptor antibody (Broder et al. [1991], supra) for approximately 18 hours

at room temperature, washed, and then incubated with goat anti-mouse immunoglobulin G (Cappel, Organon Teknika) for 3 to 4 hours. Antigen-antibody complexes on washed filters were detected with <sup>125</sup>I-streptococcal protein G. Autoradiographs were generated by exposing the washed nitrocellulose filters to Kodak XAR-5 film with intensifying screens at -70° C. and then using automated film developing. Immunoreactive plaques were isolated and purified through two additional screenings.

PAGE and protein blotting. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out with a 10% (wt/vol) polyacrylamide separating gel and a 4% (wt/vol) polyacrylamide stacking gel according to the method of Laemmli (Laemmli, U. K. [1970] *Nature* 227:680-685). Gels for Western blotting (immunoblotting) were equilibrated, and separated proteins were transferred by methods described previously (Broder et al. [1991], supra). For studies of plasmin binding, nitrocellulose membranes were blocked for 1 hour at room temperature in NET-gel buffer. The blots were then incubated for 2 hours at room temperature with <sup>125</sup>I-human plasmin to identify plasmin-binding protein bands (Broder et al., supra). The blots were washed in NET-gel and subjected to autoradiography at -70° C.

In vitro transcription-translation. Plasmid-encoded proteins were generated in vitro with a DNA-directed transcription-translation kit (Amersham). Protein products labeled with [<sup>35</sup>S]methionine (specific activity, 1,000 Ci/mmol; Amersham) were subjected to SDS-PAGE and identified by fluorography with sodium salicylate (Chamberlain, J. P. [1979] *Anal. Biochem.* 98:132-135).

Purification of the recombinant streptococcal plasmin receptor protein. *E. coli*  $\chi$ 6060(pRL015) was lysed with a French pressure cell. The resulting material was centrifuged at 10,000 $\times$ g for 30 minutes, and the supernatant fluids were subjected to ammonium sulfate fractionation. Successive additions of ammonium sulfate were performed at room temperature; the precipitates were removed by centrifugation at 10,000 $\times$ g for 30 minutes, dialyzed against phosphate-buffered saline, and analyzed by SDS-PAGE and Western blotting. At 55% ammonium sulfate saturation, the recombinant  $\approx$ 41,000-M<sub>r</sub> plasmin receptor protein remained in the supernatant fluid, whereas the majority of *E. coli* proteins precipitated.

DNA sequencing and analysis. DNA sequencing was performed by dideoxy chain termination by using the Sequenase kit (United States Biochemical Corp., Cleveland, Ohio) with  $\alpha$ -<sup>35</sup>S-ATP (specific activity >1,000 Ci/mmol; Amersham) by following the instructions of the manufacturer. Sequences were determined by using the universal forward primer supplied with the kit, recessed M13 primers, and sequence-specific oligonucleotides synthesized by the University of Florida Interdisciplinary Center for Biotechnology Research DNA Core Laboratory. Sequences were analyzed with the Genetics Computer Group programs (University of Wisconsin-Madison) (Devereux, J., P. Haeberli, O. Smithies [1984] *Nucleic Acids Res.* 12:387-395).

Amino acid sequencing. Mutanolysin-extracted proteins (Broder et al. [1991], supra) from strain 64/14 were subjected to SDS-PAGE. The proteins were electrotransferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corp., Bedford, Mass.) by using 10 mM 2-(N-morpholino)ethanesulfonic acid (pH 6.0)-20% methanol as the transfer buffer. Protein bands

were stained with Coomassie brilliant blue. The  $\approx 41,000$ -M<sub>r</sub> protein band, which had previously been shown to bind plasmin, was excised. Microsequencing by automated Edman chemistry was performed with an Applied Biosystems model 470A gas-phase sequencer with an on-line 12A PTH analyzer (Washington University Protein Chemistry Laboratory). Cyanogen bromide fragmentation of the  $\approx 41,000$ -M<sub>r</sub> protein was performed by immersing the polyvinylidene difluoride membrane-bound protein in 70% formic acid and treating with cyanogen bromide overnight at room temperature. The fragments were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane as described above. Four peptides were identified and sequenced at the University of Florida Interdisciplinary Center for Biotechnology Research Protein Chemistry Core Facility by using an Applied Biosystems model 470 sequencer with an on-line PTH analyzer.

Following are examples which illustrate procedures, including the best mode for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

#### EXAMPLE 1

##### Human Plasminogen

Plasminogen was prepared from human plasma by chromatography on lysine sepharose (Sigma Chem. Co., St. Louis, Mo. U.S.A.) and molecular sieving chromatography on Sephadex G-100 (Lottenberg, R., F. R. Dolly, C. S. Kitchens [1985] *Am. J. Hematol.* 19:181-193). The purified protein appeared as a single band on a silver stain of an SDS-polyacrylamide gel electrophoresis. A given concentration of isolated plasminogen following activation with streptokinase demonstrated the predicted theoretical amidolytic activity, thereby confirming the purity of the isolated human plasminogen.

#### EXAMPLE 2

##### Iodination of Plasminogen

Purified human plasminogen was iodinated by the chloramine T method using Iodobeads (Pierce Chem. Co., Rockford, Ill. U.S.A.) as described in Markwell, M. A. K. (1982) *Anal. Biochem.* 125:427-432. The labeled proteins were separated from free iodine by passage over a G-25 column (PD-10, Pharmacia) and collected in 0.15M Veronal buffered saline, pH 7.4, containing 0.001 M Mg<sup>2+</sup>, 0.00015M Ca<sup>2+</sup>, and 0.1% gelatin(VBS-gel). The labeled proteins were stored in aliquots containing 0.02% sodium azide at -20° C. The specific activity was approximately 25 mCi/mg. The concentration of <sup>125</sup>I-plasminogen was determined using a modification of a previously described enzyme-linked immunosorbent assay (Reis, K. J., G. O. Von Mering, M. A. Karis, E. L. Faulmann, R. Lottenberg, M. D. P. Boyle [1988] *J. Immunol. Methods* 107:273-280). The ELISA could accurately measure 1 ng/ml plasminogen.

#### EXAMPLE 3

##### Generation of Plasmin

Plasmin was generated from radiolabeled plasminogen by incubation with urokinase (20 units/ml, Sigma Chemical Co., St. Louis, Mo., U.S.A.) in VBS-gel that contained 0.02M lysine. Conversion was maximal after 30 min at 37° C. The conversion of the single chain

zymogen molecule to heavy and light chains was monitored, following reduction, on SDS-PAGE using the method of Laemmli as described previously (Lottenberg, R., C. C. Broder, M. D. P. Boyle [1987] *Infect. Immun.* 55: 1914-1918). Greater than 95% of plasminogen was consistently converted to plasmin. The specific activity of labeled plasmin was therefore essentially the same as labeled plasminogen.

#### EXAMPLE 4

##### Bacteria

The group A $\beta$ -hemolytic streptococcal strain 64 had been previously subjected to mouse passage (Reis, K. J., M. Yarnall, E. M. Ayoub, M. D. P. Boyle [1984] *Scand. J. Immunol.* 20: 433-439). The parent strain (64/P), as well as strains isolated after three (64/3) and fourteen (64/14) passages, were grown in Todd-Hewitt broth (DIFCO, Detroit, Mich.) overnight in phosphate-buffered saline (PBS), pH 7.4, containing 0.05% Tween-20 and 0.02% sodium azide. The bacteria were heat killed at 80° C. for 5 min, a treatment that did not alter their plasmin binding potential, but eliminated the production of soluble plasminogen activators which would interfere with these studies. The suspension was centrifuged, the pellet washed twice with PBS 0.02% sodium azide. Samples were stored at -20° C. The concentration of a bacterial suspension was determined by counting bacterial chains in a Neubauer hemacytometer (Fisher Scientific, Orlando, Fla., U.S.A.)

#### EXAMPLE 5

##### Effect of pH and Ionic Strength on Binding

To assess the effect of pH on the bacterium: plasmin (ogen) interaction, 50  $\mu$ m of labeled plasminogen of plasmin (approximately  $2 \times 10^4$  cpm) were added to 1.0 ml of VBS containing 0.05% Tween-20 adjusted to the appropriate pH. After 15 min at room temperature, 50  $\mu$ m of VBS containing approximately  $10^7$  bacteria (strain 64/14) were added and the mixture was incubated at 37° C. for 15 min. The bacterial suspensions were centrifuged at 1000 g for 7 min to separate bacteria from unbound labeled proteins and the pellets were washed twice with 2 ml of VBS at the appropriate pH. The radioactivity associated with the bacterial pellet in duplicate experiments was measured using a Beckman 5500 autogamma counter.

Maximal binding of plasmin to the bacteria was observed between pH 5 and 8 with approximately 60% of counts offered being bound by the group A streptococcus 64/14. In contrast, addition of labeled plasminogen to the bacteria over the entire pH range tested (pH 5-9) resulted in direct binding of less than 10% of offered counts.

To assess the effect of ionic strength on the bacterium:plasmin (ogen) interaction, similar studies were carried out in solutions containing different concentrations of NaCl with 0.05% Tween-20. The bacterial pellets were washed in the appropriate NaCl concentration to remove unbound labeled proteins.

Plasmin binding was dependent on ionic strength and optimal binding occurred between 0.1 and 0.4M NaCl. In this range of salt concentrations, less than 10% of plasminogen bound to bacteria. As the ionic strength was lowered below 0.075M NaCl, significant binding of plasminogen to the bacteria was observed.

EXAMPLE 6

Effect of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  on Binding

5 Bindings of radiolabeled plasmin to group A streptococci strain 64/14 was studied in the following buffers: (1) VBS-gel containing 0.00015M  $\text{Ca}^{2+}$  and 0.001M  $\text{Mg}^{2+}$ , or (2) metal free VBS-gel containing 0.15M ethylenediaminetetraacetic acid (EDTA). In each case 400  $\mu\text{l}$  of buffer was added to 100  $\mu\text{l}$  of VBS-gel containing approximately  $10^7$  bacteria and 100  $\mu\text{l}$  of VBS-gel containing  $3 \times 10^4$  cpm of radiolabeled plasmin. After incubation at  $37^\circ\text{C}$ . for 15 minutes, the mixtures were centrifuged at 1000 g for 7 min to separate bacteria from unbound radiolabel, the pellets were washed twice with 2 ml of the appropriate buffer, and radioactivity associated with the bacterial pellet in duplicate experiments was measured.

The amount of plasmin bound by the bacteria was the same in the presence or absence of divalent cations.

EXAMPLE 7

Inhibition of Binding by Amino Acids

Labeled plasmin (100  $\mu\text{l}$  containing approximately  $1 \times 10^4$  cpm) was added to 200  $\mu\text{l}$  VBS-gel containing varying concentrations of epsilon-aminocaproic acid (EACA), lysine, or arginine, and incubated at  $37^\circ\text{C}$ . for 15 min. The pH of each solution was 7.0. One hundred  $\mu\text{l}$  of VBS-gel containing  $10^7$  bacteria (strain 64/14) were then added and the mixture was incubated at  $37^\circ\text{C}$ . for 15 min. The bacterial suspensions were centrifuged at 1000 g for 7 min and washed twice with 2 ml of VBS-gel containing the same concentration of amino acid present during the incubation period. The percent inhibition of binding was calculated for duplicate experiments by comparison with binding in VBS-gel alone.

The ability of EACA, lysine, or arginine to dissociate bound plasmin from the bacteria was examined in the following manner. Labeled plasmin was incubated with  $10^7$  bacteria in VBS-gel containing no amino acid at  $37^\circ\text{C}$ . for 15 min. The bacteria were pelleted by centrifugation and washed twice with 2 ml on VBS-gel. After determining the radioactivity associated with the bacteria, the pellets were resuspended in solutions of VBS-gel containing varying concentrations of amino acid (pH 7.0) as described above. The mixtures were incubated at  $37^\circ\text{C}$ . for 15 min and washed twice with VBS-gel containing the appropriate amino acid concentration. The radioactivity associated with the bacteria in duplicate experiments was again measured and the percentage dissociated was calculated.

Binding of plasmin to the group A streptococcus 64/14 was inhibited by each amino acid in a concentration dependent fashion. Fifty percent inhibition of binding of plasmin to the bacteria was observed at an EACA concentration of 0.15 mM a lysine concentration of 2.0 mM, and an arginine concentration of 25 mM. In similar studies, plasmin was prebound to the group A streptococcus and a concentration dependent elution of bound radiolabel was observed on incubation with EACA, lysine, or arginine. The concentration of amino acid required to elute 50% of the bound plasmin was approximately equivalent to that required to inhibit plasmin binding by 50%.

EXAMPLE 8

Determination of  $K_D$  and Receptor Density

Labeled plasmin (25,000 to 250,000 cpm) in 100  $\mu\text{l}$  of VBS-gel was added to  $3 \times 10^6$  bacteria in 300  $\mu\text{l}$  of VBS-gel, pH 7.4, and incubated at  $37^\circ\text{C}$ . for 15 min. The bacterial suspensions were centrifuged at 1000 g for 10 min and washed twice with 2 ml of VBS-gel. Experiments were performed in triplicate. Total binding was determined by measuring the radioactivity associated with the bacterial pellet when only labeled plasmin was offered. Non-specific binding was determined by pre-incubation bacteria at  $37^\circ\text{C}$ . for 15 min in VBS-gel, pH 7.4, containing unlabeled plasmin at a 100-fold molar excess of the labeled plasmin. Specific binding was calculated by subtracting non-specific binding from total binding for each amount of labeled plasmin offered. The amount of free labeled plasmin was calculated by subtracting the amount of specifically bound labeled plasmin from the total amount of labeled plasmin offered.

The apparent dissociation constant ( $K_D$ ) was determined by two methods. A non-linear least squares analysis of the total counts offered vs. the counts bound fit to the simple Michaelis-Menten equation was performed (Cleland, W. W., [1967] *Adv. Enzymol.* 29: 1-32). The concentration of plasmin was determined specific activity for the labeled plasminogen. Scatchard analysis (Scatchard, G., [1949] *Ann. N.Y. Acad. Sci.* 51: 660-672) of these data was also performed. Counts bound vs. counts bound/counts free was plotted and the slope (representing  $-1/K_D$ ) was determined by linear regression. The x-intercept (counts bound) was converted to moles of plasmin. Receptor density was calculated using this value and the number of bacterial chains offered (derived by hemacytometer chamber counts).

Plasmin which had been bound to and eluted from strain 64/14 by treatment with lysine was also examined in similar binding studies. Eluted plasmin was obtained by incubating 2 ml of stock 10% wet weight/volume bacterial suspension (strain 64/14) with approximately 20  $\mu\text{g}$  of labeled plasmin at room temperature for 45 min. This suspension was centrifuged at 1000 g for 10 min and washed once with 10 ml of VBS-gel, and the radioactivity associated with the bacterial pellet was measured. The pellet was then resuspended in VBS-gel containing 20 mM lysine and incubated at room temperature for 30 min. The suspension was centrifuged and the supernatant recovered. Approximately 90% of the radioactivity originally associated with the bacterial pellet was dissociated by the lysine treatment. The dissociated plasmin in the supernatant was the subjected to gel filtration of a G-25 column to separate lysine from plasmin. Fractions containing plasmin were collected and stored at  $-20^\circ\text{C}$ .

Non-specific binding demonstrated a linear relationship to counts offered and was less than 5% in all tubes. Analysis of this data by least squares and Scatchard analysis demonstrated a  $K_D$  of approximately  $5 \times 10^{-11}\text{M}$  for the association of plasmin with its receptor on the mouse passaged group A streptococcus strain 64/14. Scatchard analysis of the binding data indicates that there is a single population of plasmin receptors on streptococci, and that strain 64/14 possesses approximately 800 receptors per bacterium.

EXAMPLE 9

Dot-blotting Procedure for the Identification of Plasmin Receptor Activity

Bacterial extracts, chromatography fractions or standards were loaded into the wells of a dot-blotting manifold in 50–200  $\mu$ l aliquots. Commercially available group C streptokinase was used as a positive plasmin binding control in each assay. All wells were washed twice with 200  $\mu$ l aliquots of PBS-azide and vacuum drained. All samples were assayed in duplicate. Dot blots were blocked in 5.0 mM sodium diethylbarbiturate, 0.14M NaCl, 0.5% gelatin, 0.15% Tween 20, 0.004%  $\text{NaN}_3$  pH 7.3. The blots were probed for 3–4 hours at room temperature in the blocking buffer containing 2.0 mM PMSF and  $^{125}\text{I}$ -labeled human plasmin at  $3 \times 10^4$  cpm/ml. The probed blots were then washed in 0.01M EDTA pH 7.3, containing 0.5M NaCl 0.25% gelatin, 0.15% Tween 20, and 0.004%  $\text{NaN}_3$ . Autoradiographs were generated by exposing the nitrocellulose blots to Kodak XAR-5 film with an intensifying screen for 15–24 hours at  $-70^\circ\text{C}$ . followed by automated film developing.

EXAMPLE 10

Polyacrylamide Gel Electrophoresis and Protein Blotting

Gels intended for Western blotting were equilibrated in 25 mM Tris, 0.2M glycine pH 8.0 containing 20% v/v methanol (electroblot buffer) for 25 minutes. Protein blotting, from SDS-PAGE gels, was performed using the 'Trans-Blot SD Semi-Dry' electrophoretic transfer cell (Bio Rad, Richmond, Calif.). Blots were blocked as described for the dot-blot procedure, and probed for 3–4 hours at room temperature with radiolabeled human plasmin in either the presence or absence of 1.0 mM EACA, to identify functionally active protein bands. In studies of antigenic properties of these proteins, blots were probed with rabbit anti-plasmin receptor antibody or anti-group C streptokinase antibody by incubation with 4.3  $\mu$ g IgG per ml of probing solution (approximately a 1:3000 dilution of antisera) for three hours and probed with  $^{125}\text{I}$ -streptococcal protein G containing  $3 \times 10^4$  cpm/ml. For probing with mouse monoclonal antibodies specific for epitopes on group C streptokinase, blots were probed with a 1:100 dilution of the monoclonal antibody stock solution for three hours, followed by probing with goat antibody specific for mouse IgG at 1.0  $\mu$ g/ml, followed by probing with  $^{125}\text{I}$ -streptococcal protein G containing  $3 \times 10^4$  cpm/ml. Blots were then washed with 0.01M EDTA pH 7.3, containing 1.0M NaCl, 0.25% gelatin, 0.15% Tween 20. Autoradiographs were generated by exposing the nitrocellulose blots to Kodak XAR-5 film with an intensifying screen for 15–24 hours at  $-70^\circ\text{C}$ . followed by automated film developing.

EXAMPLE 11

Mutanolysin Extraction

Generation of cell membrane fragments of streptococcal cell strain 64/14 by mutanolysin generates functional plasmin receptor. Additional purification of receptor is accomplished by affinity chromatography with chemically modified plasmin (Broder et al.). The colony blot immunoassay to monitor the expression of Fc receptors on individual bacterial colonies is described in Yarnall, M., K. J. Reis, E. M. Ayoub, M. D.

P. Boyle (1984), "An immunoblotting technique for the detection of bound and secreted bacterial Fc receptors," *J. Microbiol. Meth.* 3:83–93.

This procedure is a modification of the method described by Yarnall, M. and M. D. P. Boyle (1986) "Isolation and partial characterization of a type II Fc receptor from a group A streptococcus," *Mol. Cell. Biochem.* 70:57–66. Approximately 1.0 g wet weight of bacteria was suspended in 5.0 ml of 20 mM  $\text{KH}_2\text{PO}_4$ , 1.0 mM EDTA, 0.02%  $\text{NaN}_3$  pH 7.0 containing 2.0 mM PMSF, 10  $\mu$ g/ml DNase I and 50  $\mu$ g/ml mutanolysin. The suspension was vortexed and placed at  $37^\circ\text{C}$ . for 4 hours with periodic mixing. Supernatants were collected following centrifugation to remove bacteria and debris. For these studies a commercial preparation of mutanolysin was further purified according to the method described by Siegal et al. (Siegal, J. L., S. F. Hurst, E. S. Liberman, S. E. Coleman, A. S. Bleiweis [1981] "Mutanolysin-induced spheroplasts of *Streptococcus mutans* are true protoplasts," *Infect. Immun.* 31:303–815) to remove contaminating protease.

EXAMPLE 12

Preparation of Immobilized Human Plasmin Affinity Support

Human plasminogen at a concentration of approximately  $5.6 \times 10^{-5}\text{M}$  was activated to plasmin by incubating the sample in the presence of an approximately 60 fold lower molar concentration of urokinase. The reaction was carried out with constant agitation for one hour at  $37^\circ\text{C}$ . in a reaction volume of 10 ml of 0.05M Tris, 0.15M NaCl pH 7.4 containing 40 mM lysine. A 50  $\mu$ l aliquot was removed and analyzed by SDS-PAGE under reduced conditions to determine the extent of conversion of the single chain plasminogen molecule to the two chain plasmin form. The remainder of the reaction mixture was flash frozen, and stored at  $-70^\circ\text{C}$ . Preparations in which complete conversion of plasminogen to plasmin was observed were then reacted with constant rotation with a 5 fold molar excess of D-valyl-L-phenylalanyl-L-lysine chloromethyl ketone at ambient temperature with constant rotation. The enzymatically inactive plasmin was then concentrated by ammonium sulfate precipitation (4.0 g/10 ml), dialyzed at  $4^\circ\text{C}$ . against 0.1M MOPS buffer, pH 7.3, containing 0.02% sodium azide. The dialyzed inactive plasmin was chromatographed on a Superose 6 column (Pharmacia, Piscataway, N.J.) in 0.1M MOPS buffer, pH 7.3.

The chloromethyl ketone blocked plasmin recovered from the molecular sieving column, was immobilized to the activated affinity chromatography support, Affi-Prep 10 (Bio Rad, Richmond, Calif.). This matrix couples in aqueous buffers to primary amino groups in the ligand by means of an N-hydroxysuccinimide ester on the end of a 10 carbon spacer arm. Approximately 50 mg of inactivated plasmin in 18 ml of 0.1M MOPS buffer, pH 7.3 was incubated with 6.0 ml of the Affi-Prep 10 bead suspension for 15 hours with rotation at  $4^\circ\text{C}$ . Following ligand coupling, 100  $\mu$ l of 1.0M ethanolamine HCl pH 8 was added and the reaction mixture rotated for 1 hour at  $4^\circ\text{C}$ . to block any remaining active sites on the Affi-Prep 10 matrix.

EXAMPLE 13

Affinity Purification of Plasmin Receptor

The Affi-Prep 10-Plasmin matrix was placed in an HR 10/10 column attached to a Pharmacia FPLC chro-

matography system. The column was equilibrated at room temperature in 0.05M Na<sub>2</sub>HPO<sub>4</sub>, 0.15M NaCl, 1.0 mM benzamidine HCl, 0.02% sodium azide pH 7.2 (equilibration buffer). Approximately 1 or 2 ml of supernatant from the mutanolysin extraction of bacterial strain 64/14 was loaded onto the column. The column was then washed with the equilibration buffer until the OD<sub>280</sub> returned to base line. The column was then eluted with a 50 ml linear gradient of 0.0M-0.1M L-Lysine in equilibration buffer, or eluted in a single step using equilibration buffer containing 0.1M L-Lysine. The absorbance at 280 nm was continuously monitored an 1.0 ml fractions were collected. After each affinity purification procedure the column was washed with 20 ml of 2.0M NaCl, followed by 200 ml of equilibration buffer and stored at 4° C.

#### EXAMPLE 14

##### Production of Polyclonal Antibodies to the Extracted Plasmin Receptor in the Mutanolysin Extract of Strain 64/14

Polyclonal antibodies to the M<sub>r</sub> ≈ 41,000 plasmin receptor protein extracted from strain 64/14 by mutanolysin extraction were prepared in both rabbits and mice. Rabbits were immunized with a gel slice from an SDS-polyacrylamide gel containing approximately 200 μg of the plasmin binding protein emulsified in Freund's complete adjuvant. The rabbit was boosted three times at two week intervals with a gel slice containing approximately 125 μg of the protein emulsified in Freund's incomplete adjuvant. The immunogen was prepared by separating the proteins in a mutanolysin extract of strain 64/14 by electrophoresis on 10% SDS-polyacrylamide gels. The gel was stained to identify protein bands. Plasmin binding proteins were identified by transferring the proteins in one lane of the gel to a nitrocellulose membrane and probing with <sup>125</sup>I-labeled human plasmin, as described above.

Polyclonal antibodies to the M<sub>r</sub> ≈ 41,000 dalton plasmin binding protein were also prepared in mice. For these studies the immunogen was separated on 10% SDS-polyacrylamide gels under reducing conditions using a preparative comb with a single sample well. Separated proteins were transferred to nitrocellulose as described above. Functional plasmin binding proteins were identified by probing with <sup>125</sup>I-labeled human plasmin. The remainder of the nitrocellulose sheet was then stained and the position of the 41,000 dalton band was located and aligned with the autoradiographed strip. The marked band on each nitrocellulose sheet was then carefully cut out to avoid any contamination, and divided into four equal fractions containing approximately 500 μg of protein. The strips were then equilibrated in PBS, sonicated to a fine powder, and subsequently mixed with Ribi Adjuvant. Aliquots of this mixture were used to immunize a group of 6-8 week old out-bred female mice as follows. Initially 10 mice were injected intraperitoneally with approximately 50 μg of antigen immobilized on the nitrocellulose membrane mixed with Ribi adjuvant. Two weeks after the initial injection the mice were boosted intraperitoneally with 10 μg of the nitrocellulose-bound antigen mixed with Ribi adjuvant. Four weeks after the initial injection, the mice were boosted intraperitoneally with 10 μg of nitrocellulose-bound antigen mixed with water. Four days after the final boost the mice were sacrificed and

ascites fluid collected from each mouse, pooled and used as a source of antibody.

#### EXAMPLE 15

##### Solubilization of Plasmin Receptor

A variety of conditions for solubilizing plasmin receptor activity from the group A streptococcal strain 64/14 were compared. These included hot acid, alkaline, and neutral pH extractions, extraction with the detergents TRITON®X-100 with osmotic shock, acetone and TRITON®X-100 extraction, and extractions with the enzymes, lysozyme, trypsin, or mutanolysin. The highest yield of soluble plasmin binding activity was found in mutanolysin extracts.

The size heterogeneity of the soluble plasmin receptor activity in the mutanolysin extract of strain 64/14 was assessed by electrophoresis of a 50 μl aliquot of the extract on a series of parallel reducing or non-reducing SDS-polyacrylamide gels. The majority of the plasmin binding activity was present predominantly in a band with a M<sub>r</sub> of approximately 41,000 daltons. One μg of purified group C streptokinase (M<sub>r</sub> approx. 48,000 daltons) was electrophoresed as a positive control for plasmin binding. An aliquot of a sham mutanolysin digest, containing all the reactants, except the bacteria, was also analyzed by Western blotting techniques and demonstrated no plasmin binding activity (data not shown).

#### EXAMPLE 16

##### Relationship of the Plasmin Receptor to Streptokinase

Although group A streptococcal plasmin receptor is functionally distinct from streptokinase, the release of this secreted plasmin (ogen) activator from strain 64/14, during extraction, would be a possible confusing factor in the isolation and characterization of the surface plasmin receptor.

The binding specificities of the plasmin receptor extracted from strain 64/14 with streptokinase present in the culture fluid of the same culture were compared. There was no plasminogen activator activity associated with any proteins in the extracted plasmin receptor preparation. A plasminogen activator activity at a M<sub>r</sub> ≈ 48,000 was seen in the lane containing the concentrated supernatant from strain 64/14. This protein migrated at a similar molecular weight in this gel system to the reference group C streptokinase protein.

Comparison of the ability of the samples to bind <sup>125</sup>I-labeled human plasmin demonstrate that the M<sub>r</sub> ≈ 48,000 plasminogen activator bands associated with the reference group C streptokinase or the corresponding molecule from the concentrated culture supernatant of strain 64/14 bound the labeled probe. The mutanolysin extracted plasmin receptor displayed a M<sub>r</sub> ≈ 41,000 dalton band capable of binding to human plasmin; however, this molecule lacked any plasminogen activator potential. A minor M<sub>r</sub> ≈ 41,000 dalton band was present in the concentrated culture supernatant of strain 64/14 which also bound <sup>125</sup>I-labeled human plasmin. This band displayed no plasminogen activator activity.

Previous studies have shown that the binding of human plasmin to the bacterial bound plasmin receptor on strain 64/14 could be inhibited in the presence of EACA. The plasmin binding activity of M<sub>r</sub> ≈ 41,000 dalton band present in the mutanolysin extract of strain 64/14 was reduced significantly in the presence of 1.0

mM EACA. Binding of  $^{125}\text{I}$ -labeled human plasmin to the  $M_r \approx 41,000$  dalton plasmin binding protein in the concentrated culture supernatant of strain 64/14 was also inhibited by 1.0 mM EACA. By contrast, addition of EACA had no effect on the binding of plasmin to either the  $M_r \approx 48,000$  plasminogen activator protein from strain 64/14 or to the reference group C streptokinase molecule. Taken together these results indicate that the surface plasmin receptor solubilized by treatment of strain 64/14 with mutanolysin, was unrelated to the streptokinase protein secreted by the same organism. In addition, the results demonstrate that low levels of the plasmin receptor are found in bacterial free culture supernatants in which group A strain 64/14 was grown.

The mutanolysin extracted plasmin binding activity was subjected to further purification by affinity chromatography using an enzymatically inactivated plasmin affinity matrix prepared as described above. One ml of the mutanolysin extract of strain 64/14 was applied to the plasmin affinity column matrix in 0.05M  $\text{Na}_2\text{HPO}_4$ , 0.15M NaCl, 1.0 mM benzamidine HCl, and 0.02%  $\text{NaN}_3$ , pH 7.2. Bound plasmin receptor activity was eluted using a 50 ml linear gradient of 0.0–0.1M L-Lysine in 0.05M  $\text{Na}_2\text{HPO}_4$ , 0.15M NaCl, 1.0 mM benzamidine HCl, and 0.02%  $\text{NaN}_3$ , pH 7.2. The absorbance at 280 nm was monitored continuously and 1.0 ml fractions were collected. Fractions eluted from the affinity column were assayed for functional activity using a dot blotting procedure and  $^{125}\text{I}$ -labeled plasmin as the probe. The functional plasmin binding activity was found to bind to the immobilized plasmin matrix and could be eluted selectively with lysine. The recovered functional activity from the column corresponded to the eluted protein peak as detected by measuring absorbance at 280 nm. Identical results were obtained when a single concentration of 0.1M L-lysine was used to elute the bound plasmin receptor activity from the plasmin affinity column.

The molecular size and number of eluted proteins was determined by SDS-PAGE followed by silver staining. The functional activity eluted from the immobilized plasmin matrix in three 1.0 ml fractions. The greatest activity was found in the second protein containing fraction. Fifty microliters of this fraction was analyzed by Western blotting and probing with  $^{125}\text{I}$ -labeled human plasmin. The results indicated that a single  $M_r \approx 41,000$  dalton protein was affinity purified and that this protein retained fractional plasmin binding activity. This protein corresponded to the activity present in the crude mutanolysin extract indicating that the affinity purification strategy did not result in any modification of the receptor.

Treatment of the affinity purified material with trypsin destroys the ability of the  $M_r \approx 41,000$  dalton molecule to bind plasmin and results in the disappearance of this  $M_r \approx 41,000$  dalton band on a silver stained SDS-polyacrylamide gel. These results indicate that a functionally active plasmin binding protein can be isolated by affinity chromatography for a mutanolysin extract of the group A streptococcal strain 64/14. The affinity purified protein, like the crude extract, lacked any plasminogen activating potential.

The group A streptococcal plasmin receptor and streptokinase are physicochemically and functionally distinct molecules. The possibility that the two proteins arose from a common precursor and shared at least some antigenic determinants was considered. This possibility was examined using a polyclonal antibody to

streptokinase, as well as rabbit and mouse polyclonal antibodies prepared against the plasmin receptor isolated from strain 64/14.

These findings indicate that the plasminogen activator proteins secreted by group A and group C streptococci were antigenically related. By contrast the  $M_r \approx 41,000$  plasmin binding protein extracted from the bacteria or present in low concentrations in culture supernatants displayed no antigenic relatedness with streptokinase as detected by the monospecific polyclonal antibody used.

By contrast, the group A plasmin receptor protein solubilized from the 64/14 bacterial surface was totally devoid of any of the antigenic determinants detected by this the polyclonal anti-streptokinase antibody.

#### EXAMPLE 17

##### Cloning and Expression of the Streptococcal Plasmin Receptor Gene

Strain 64/14 produces at least two distinct proteins that display the ability to bind human plasmin(ogen). In addition to the  $\approx 41,000$ -M<sub>r</sub> plasmin receptor, these bacteria secrete an  $\approx 47,000$ -M<sub>r</sub> plasminogen activator protein, streptokinase. Previous studies from our laboratory have shown that these two proteins are antigenically and functionally distinct (Broder et al., supra). Consequently, a monospecific polyclonal antiplasmin receptor antibody preparation, rather than plasmin, was used to screen a group A streptococcal  $\lambda$  gt11 expression library generated from strain 64/14 chromosomal DNA. Approximately 13,000 recombinant plaques were screened by using a plaque-lift assay as described in Materials and Methods, and three plaques with strong immunoreactivity were identified and designated SPR4, SPR8, and SPR17. After two rounds of plaque purification, protein products from phage lysates of the three clones were collected and analyzed by SDS-PAGE and Western blot analysis. Each positive clone produced an immunoreactive product with an  $M_r$  of  $\approx 41,000$  not present in control lysates, indicating that the protein product was not fused with  $\beta$ -galactosidase.

In order to express the streptococcal plasmin receptor protein (Plr) stably in *E. coli*, subcloning with a plasmid vector was carried out. After digestion of the DNA from SPR4 with EcoRI, agarose gel electrophoresis analysis revealed 2.4- and 2.7-kb DNA fragments in addition to the  $\lambda$  arms. We attempted to subclone each of the EcoRI fragments into the EcoRI site of the low-copy-number plasmid pYA2204. We were unable to isolate recombinant plasmids that carried the 2.4-kb fragment. However, the 2.7-kb fragment was successfully subcloned, yielding pRL015. When this plasmid was transformed into *E. coli*  $\chi$ 6060, an  $\approx 41,000$ -M<sub>r</sub> protein that was recognized by the antiplasmin receptor antibody by Western blotting was observed. This protein was not produced by strain  $\chi$ 6060 alone or by  $\chi$ 6060 transformed with the vector alone.

The  $\approx 41,000$ -M<sub>r</sub> protein expressed from pRL015 was also analyzed for the ability to bind plasmin. Lysates of  $\chi$ 6060 (pRL015) were subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and reacted with  $^{125}\text{I}$ -plasmin. Several proteins contained in lysates of  $\chi$ 6060(pRL015) demonstrated the ability to bind plasmin. Control lysates of  $\chi$ 6060 alone and  $\chi$ 6060(pYA2204) also demonstrated similar plasmin-binding protein bands. Therefore, the streptococcal protein was separated from *E. coli* plasmin-binding pro-

teins in order to determine whether the recombinant protein had plasmin-binding activity. Supernatant fluids from lysates obtained by French pressure cell treatment of  $\chi$ 6060(pYA2204) as a control and  $\chi$ 6060(pRL015) were subjected to ammonium sulfate precipitation as described in Materials and Methods. The supernatant fluids resulting from a 55% ammonium sulfate cut were analyzed by SDS-PAGE and Western blotting. This manipulation resulted in the precipitation of all plasmin-binding proteins in the control lysate, whereas the  $\approx$ 41,000-M<sub>r</sub> protein produced by  $\chi$ 6060(pRL015) remained in the supernatant fluid. This immunoreactive protein demonstrated the ability to bind <sup>125</sup>I-plasmin.

In vitro transcription-translation studies were performed. SDS-PAGE analysis of the protein products revealed that both pYA2204 (the vector) and pRL015 encoded several proteins with M<sub>r</sub> of <30,000; however, pRL015 produced a major protein product with an M<sub>r</sub> of  $\approx$ 41,000 not observed for the control. A minor  $\approx$ 33,000-M<sub>r</sub> protein, which may represent degradation of the  $\approx$ 41,000-M<sub>r</sub> protein or an additional streptococcal encoded product was also identified.

#### EXAMPLE 18

##### DNA Sequence and Characterization of the Plasmin Receptor Gene

The restriction map of the 2.7-kb EcoRI insert of pRL015 is depicted in FIG. 1. DNA sequence analysis of the ends of the 2.7-kb fragment did not reveal evidence of an open reading frame. Therefore, subclones for sequencing were generated to locate the gene. The 1.7-kb PvuII-EcoRI fragment representing the right side of the DNA insert (FIG. 1) was ligated with pYA2204 which had been digested with SmaI and EcoRI. Sequence analysis of each end of the insert DNA was performed, and an open reading frame to the right of the PvuII site was identified. By using pRL015, synthetic oligonucleotides were used to extend the sequence upstream and downstream of the PvuII site. A 1,008-bp open reading frame that began with ATG and terminated with a TAA codon was revealed. The G+C content for the open reading frame was 40.5%.

N-terminal amino acid sequencing of the  $\approx$ 41,000-M<sub>r</sub> plasmin receptor protein from strain 64/14 was performed, and an unambiguous sequence was obtained for 51 residues. Amino acid sequences were obtained from four peptides (M<sub>r</sub>s of 3,000 to 16,000) generated by cyanogen bromide treatment of the  $\approx$ 41,000-M<sub>r</sub> protein. The sequence of one of these peptides had identity with the N-terminal sequence and allowed assignment of an additional 23 residues. The deduced amino acid sequence of the open reading frame exhibited complete identity with 74 amino acid residues of the native protein, indicating that valine following the ATG initiation codon represents the N terminus of the receptor protein. The sequences of two additional peptides (13 and 27 residues) were also determined and found to correspond to residues 160 to 173 and 186 to 216, respectively. Thus, 114 of the predicted 335 amino acid residues encoded by plr have been confirmed by amino acid sequencing of the native streptococcal protein.

Several putative regulatory sequences upstream of the structural gene were identified. A potential ribosomal binding sequence was found at position-9 (AAG-GAGG). Putative-35 (TTCACA) and -10 (TCTAAT) regions were identified upstream of the ribosomal binding site with a spacer of 19 bp. An additional subclone with the HindIII-BamHI fragment of pRL015 placed in

the opposite orientation to the plasmid-encoded promoter expressed the receptor protein in *E. coli*, indicating that the upstream region served as a functional promoter in *E. coli*.

#### EXAMPLE 19

##### Sequence Homologies

The deduced amino acid sequence of Plr was compared with deduced amino acid sequences for genes entered in the EMBL (release 26.0) and GenBank (release 67.0) data bases by using the TFASTA program based on the algorithm of Lipman and Pearson (Lipman, D., W. R. Pearson [1985] *Science* 227: 1435-1441). Glyceraldehyde 3-phosphate dehydrogenases (GAPDHs) of bacterial origins (Branlant, G., C. Branlant [1985] *Eur. J. Biochem.* 150: 61-66; Schlaepfer, B. S., W. Portmann, C. Branlant, G. Branlant, H. Zuber [1990] *Nucleic Acids Res.* 18: 6422; Viaene, A., P. Dhase [1989] *Nucleic Acids Res.* 17: 1251) exhibited the greatest homology with Plr. The gram-positive *Bacillus subtilis* GAPDH demonstrated the highest score. The sequences showed 56% identity and 73% similarity.

Hydropathy plots of Plr and *B. subtilis* GAPDH were determined as described by Kyte and Doolittle (Kyte, J., R. F. Doolittle [1982] *J. Mol. Biol.* 157: 105-132). Plr and *B. subtilis* GAPDH showed similar patterns overall except for differences in the C-terminal portion of the molecules. Common cell wall-spanning and membrane-anchoring motifs have been identified for several gram-positive surface proteins (Fischetti, V. A., V. Pancholi, O. Schneewind [1991] "Common characteristics of the surface proteins from gram-positive cocci, pp. 290-294, In G. M. Dunne et al. (eds.), *Genetics and molecular biology of streptococci, lactococci, and enterococci*, American Society for Microbiology, Washington, D.C.). However, no similar regions were identified for Plr. No significant amino acid sequence homology between Plr and streptokinase, the other well-characterized plasmin(ogen)-binding protein, was identified, supporting our previous biochemical and immunological analyses (Broder et al. [1991], supra).

#### EXAMPLE 20

##### Bacterial Plasmin Receptors as Thrombolytic Agents

The plasmin receptors described above can be either purified from bacteria or produced by recombinant technology. These receptors bind plasmin with high affinity but the plasmin retains enzymatic activity. Although plasmin is normally inhibited rapidly by  $\alpha$ 2 antiplasmin, such inhibition occurs more slowly and to a lesser extent when plasmin is bound to the receptor. These characteristics of the receptor and the plasmin bound to the receptor suggest several novel methods for thrombolytic therapy. First, a composition comprising the purified receptor can be administered in combination with the administration of a plasminogen activator. The receptor may be administered concurrently with the administration of the plasminogen activator. Alternatively, the compositions may be administered sequentially. The receptor should be administered before plasmin produced by the activity of the plasminogen activator has been substantially inhibited by  $\alpha$ 2 antiplasmin. Therefore, for best results the receptor should be administered concurrently with, or shortly before or after, the administration of the plasminogen activator. This procedure has the advantage of decreas-

ing the amount and frequency of administration of the plasminogen activator. Normally, the short half life of TPA necessitates frequent administration of that agent. Also, the administration of TPA or other plasminogen activators can result in substantial amounts of freely circulating plasmin. This situation is most likely to occur with plasminogen activators such as streptokinase, which do not have any particular affinity for fibrin. A reduction in free plasmin is important to minimize the possibility of unwanted bleeding. Also, free plasmin can actually be detrimental to platelet function.

In a further embodiment, the receptor can be bound to plasmin before administration to the human or animal in need of treatment. When the receptor is bound to plasmin the receptor/plasmin complex can then be administered to a human or animal in need of fibrinolytic activity. The plasmin would then supply this activity without being rapidly inhibited by  $\alpha 2$  antiplasmin. This procedure also decreases the amount and frequency of administration of the therapeutic agent.

A further advantage of administering plasmin receptor or plasmin bound to a receptor is that the host's plasminogen is not depleted as much as when plasminogen activators alone are administered. When plasminogen activators alone are administered, much of the plasmin is not specifically directed to the desired location and it simply becomes inactivated through the action of  $\alpha 2$ -antiplasmin. When the plasmin receptor is administered in combination with the plasminogen activator, it is not necessary to use as much activator and, consequently, the host's plasminogen is conserved. Also, administration of the receptor-plasmin complex provides the host with a source of fibrinolytic activity without depleting the host's own plasminogen.

A further embodiment of the subject invention comprises the conjugation of the receptor or a receptor-plasmin complex to an antibody for fibrin. Antibodies to fibrin are known to those skilled in the art and can be easily produced by those skilled in the art. It is a straightforward matter to a person skilled in the art to conjugate the receptor or receptor-plasmin complex to an appropriate anti-fibrin antibody. The resulting conjugate then directs the fibrinolytic activity of the bound plasmin to the specific site where the activity is needed.

An alternative therapeutic procedure comprises the administration of plasmin bound to inactivated bacteria. In this embodiment the receptor remains attached to the bacteria and the plasmin then binds to the essentially immobilized receptor. Heat-treated or otherwise inactivated bacteria can be prepared in a sterile manner. Human plasminogen (pyrogen and microbial organism-free) can be converted to active plasmin using urokinase under conditions previously described (Lottenberg, R., F. R. Dolly, C. S. Kitchens [1985] *Am. J. Hematology* 19: 181-193). Incubation of the bacteria and plasmin to allow complex formation can be followed by washing of the bacteria to remove unbound plasmin. Streptococcal strains non-pathogenic for humans expressing high levels of plasmin receptors provide an example of bacteria which can be used in the novel process disclosed here. Alternative strains of bacteria or other microorganisms, such as fungi, can also be used. Streptococci expressing fibrinogen receptor, in addition to the plasmin receptor, may offer enhanced fibrin specificity in that the fibrinogen receptor binds to fibrin (ogen). For strains of streptococci harboring a fibrinogen binding component, cell membrane fragments containing this region may provide enhanced fibrin specificity. Advan-

tageously, bacteria with associated plasmin may become entrapped by the thrombus. The enzymatic activity of the plasmin would provide for fibrin degradation leading to thrombus dissolution. Clearance of unassociated bacterium-plasmin conjugates by the reticulo-endothelial systems would remove the bacteria from circulation.

Anticipated applications of combination therapies or bacterium-plasmin conjugates as thrombolytic agents in clinical practice include intravenous administration for pulmonary thromboembolism, venous thrombosis, acute myocardial infarction, and local infusion for acute arterial thrombosis, and catheter or other intravascular device occlusion. These treatments essentially target the fibrinolytic activity to the specific location where it is needed. Thus, it is not necessary to provide high level systemic dosages. As described above, one preferred embodiment of the invention involves the combined use of the novel receptor-plasmin conjugates with other available treatments. Such a combination can be used to take advantage of the targeted action of the conjugate as well as the more generalized lytic state resulting from the administration of thrombolytic agents. The deleterious side effects normally associated with the thrombolytic agent can be minimized because lower concentrations are needed when they are administered in conjunction with the immobilized plasmin.

An additional aspect of controlling potential hemorrhage accompanying administration of this agent may be the intravenous administration of epsilon-aminocaproic acid (tradename AMICAR) or tranexamic acid. Typical regimens of these anti-fibrinolytic agents would provide plasma levels of the lysine analogs to dissociate the plasmin from the bacteria (Broeseker, T. A., M. D. P. Boyle, R. Lottenberg [1988] "Characterization of the interaction of human plasmin with its specific receptor on a group A streptococcus," *Microbial Pathogenesis* 5:19-27) and enable rapid inactivation of plasmin by its physiological inhibitors  $\alpha 2$ -antiplasmin and  $\alpha 2$ -macroglobulin. This will also provide an approach to prepare the patient for emergency surgical procedures (e.g., coronary artery bypass grafting or administration of alternative anti-thrombotic agents (e.g., heparin).

#### EXAMPLE 21

##### Vaccines

The novel plasmin receptor described herein can be used advantageously in an immunogenic composition such as a vaccine. Such a composition, when administered to a person or animal, raises antibodies or other immune response which reduces the susceptibility of that human or animal to infection. Specifically, the plasmin receptor of the subject invention can be used to raise an immune response against group A streptococcal infections. Also, the plasmin receptor can be used to raise an immune response against a variety of infective agents which present an immunogenic GAPDH or GAPDH-like protein. Examples of infectious agents for which the plasmin receptor can be used to raise an immune response include, but are not limited to, group A streptococci, group B streptococci, *Schistosoma mansoni*, and *Staphylococcus aureus*.

Immunogenic compositions (such as vaccines) comprising the plasmin receptor disclosed herein, and variants or fragments thereof having immunogenic properties, can be prepared by procedures well known in the art. For example, such compositions can be prepared as

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injectables, e.g., liquid solutions or suspensions. Solid forms for solution in, or suspension in, a liquid prior to injection also can be prepared. Optionally, the preparation also can be emulsified. The active antigenic ingredient or ingredients can be mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Examples of suitable excipients are water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, if desired, the immunogenic composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants such as aluminum hydroxide or muramyl dipeptide or variations thereof. Also, cholera toxin subunit B or other agents which stimulate antibody production at mucosal sites can be used. In the case of peptides, coupling to larger molecules such as KLH or tetanus toxoid sometimes enhances immunogenicity. Vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers include, for example, polyalkylene glycols or triglycerides. Suppositories can be formed from mixtures containing the active ingredient in the range of about 0.5% to about 10%, preferably about 1 to about 2%. Oral formulations can include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions can take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain from about 10% to about 95% of active ingredient, preferably from about 25% to about 70%.

The compounds can be formulated into the immunogenic composition as neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts

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(formed with the free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The immunogenic compositions are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective and immunogenic. The quantity to be administered can depend on the subject to be treated and the degree of protection desired. Advantageously, methods known to promote mucosal immunity can be combined with systemic immunity promoters to maximize protection. Also, the plasmin receptors of the subject invention may be combined with carbohydrate antigenic components to enhance the immunogenic response and provide a broader range of protection. The combination of these antigens may be, for example, through chemical coupling. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and can be peculiar to each individual. However, suitable dosage ranges are of the order of about several hundred micrograms active ingredient per individual. Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed in one or two week intervals by a subsequent injection or other administration.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

# SEQUENCE LISTING

## ( 1 ) GENERAL INFORMATION:

( 1.1 ) NUMBER OF SEQUENCES: 2

## ( 2 ) INFORMATION FOR SEQ ID NO:1:

### ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 1125 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: both
- ( D ) TOPOLOGY: circular

( i.1 ) MOLECULE TYPE: DNA (genomic)

( i.2 ) HYPOTHETICAL: NO

( v ) FRAGMENT TYPE: N-terminal

### ( v.1 ) ORIGINAL SOURCE:

- ( A ) ORGANISM: *Streptococcus pyogenes*
- ( B ) STRAIN: M untypable
- ( C ) INDIVIDUAL ISOLATE: 64/14

### ( v.1.1 ) IMMEDIATE SOURCE:

- ( B ) CLONE: pRL015

### ( i.2 ) FEATURE:

- ( A ) NAME/KEY: CDS
- ( B ) LOCATION: 115..1122

414  
414

27

5,328,996

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-continued

( i x ) FEATURE:

( A ) NAME/KEY: matpeptide  
( B ) LOCATION: 115..1122  
( C ) IDENTIFICATION METHOD: experimental  
( D ) OTHER INFORMATION: /codonstart=115  
/ function="High-affinity binding of  
plasmin(ogen)"  
/ product="Streptococcal plasmin receptor"  
/ evidence=EXPERIMENTAL  
/ gene="plr"  
/ number=1  
/ label=PLR

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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ATAATAGTTC TGTTGAAAGG TTGTTGCAGA TGACTGTAAG TAATCTTTTC ACAATAAGTA      60
GGGAGCATTG CCTCTAATAA TATTCTTTTG ATTTTCATAA GGAGGAAATC ACTA  ATO      117
                                         Met
                                         1
GTA GTT AAA GTT GGT ATT AAC GGT TTC GGT CGT ATC GGA CGT CTT GCA      165
Val Val Lys Val Gly Ile Asn Gly Phe Gly Arg Ile Gly Arg Leu Ala
                    5                      10                      15
TTC CGC CGT ATT CAA AAC ATC GAA GGT GTT GAA GTA ACT CGT ATC AAT      213
Phe Arg Arg Ile Gln Asn Ile Glu Gly Val Glu Val Thr Arg Ile Asn
                    20                      25                      30
GAC CTT ACA GAT CCA AAT ATG CTT GCA CAC TTG TTG AAA TAC GAT ACA      261
Asp Leu Thr Asp Pro Asn Met Leu Ala His Leu Leu Lys Tyr Asp Thr
                    35                      40                      45
ACT CAA GGT CGT TTT GAT GGA ACA GTT GAA GTT AAA GAA GGT GGA TTT      309
Thr Gln Gly Arg Phe Asp Gly Thr Val Glu Val Lys Glu Gly Gly Phe
                    50                      55                      60
GAA GTA AAC GGA AAC TTC ATC AAA GTT TCT GCT GAA COT GAT CCA GAA      357
Glu Val Asn Gly Asn Phe Ile Lys Val Ser Ala Glu Arg Asp Pro Glu
                    65                      70                      75
AAC ATC GAC TGG GCA ACT GAT GGG GTT GAA ATC GTT CTT GAA GCA ACT      405
Asn Ile Asp Trp Ala Thr Asp Gly Val Glu Ile Val Leu Glu Ala Thr
                    80                      85                      90
GGT TTC TTT GCT AAA AAA GAA GCA GCT GAA AAA CAC TTA CAT GCT AAC      453
Gly Phe Phe Ala Lys Lys Glu Ala Ala Glu Lys His Leu His Ala Asn
                    95                      100                      105
GGT GCT AAA AAA GTT GTT ATC ACA GCT CCT GGT GGA AAC GAT GTT AAA      501
Gly Ala Lys Lys Val Val Ile Thr Ala Pro Gly Gly Asn Asp Val Lys
                    110                      115                      120
ACA GTT GTT TTC AAC ACT AAC CAC GAC ATT CTT GAC GGT ACT GAA ACA      549
Thr Val Val Phe Asn Thr Asn His Asp Ile Leu Asp Gly Thr Glu Thr
                    125                      130                      135
GTT ATC TCA GGT GCT TCA TGT ACT ACA AAC TGT TTA GCT CCT ATG GCT      597
Val Ile Ser Gly Ala Ser Cys Thr Thr Asn Cys Leu Ala Pro Met Ala
                    140                      145                      150
AAA GCT CTT CAC GAT GCA TTC GGT ATT CAA AAA GGT CTT ATG ACT ACA      645
Lys Ala Leu His Asp Ala Phe Gly Ile Gln Lys Gly Leu Met Thr Thr
                    155                      160                      165
ATC CAC GCT TAC ACT GGT GAC CAA ATG ATC CTT GAC GGA CCA CAC CGT      693
Ile His Ala Tyr Thr Gly Asp Gln Met Ile Leu Asp Gly Pro His Arg
                    170                      175                      180
GGT GGT GAC CTT CGT CGT GCA CGC GCT GGT GCT GCA AAC ATC GTT CCT      741
Gly Gly Asp Leu Arg Arg Ala Arg Ala Gly Ala Ala Asn Ile Val Pro
                    185                      190                      195
AAC TCA ACT GGT GCT GCT AAA GCT ATC GGT CTT GTT ATC CCA GAA CTT      789
Asn Ser Thr Gly Ala Ala Lys Ala Ile Gly Leu Val Ile Pro Glu Leu
                    200                      205                      210
AAC GGT AAA CTT GAC GGT GCT GCA CAA CGT GTT CCT GTT CCA ACT GGA      837
Asn Gly Lys Leu Asp Gly Ala Ala Gln Arg Val Pro Val Pro Thr Gly
                    215                      220                      225
                                         230                      235                      240
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|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |      |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|
| TCA | GTA | ACT | GAG | TTG | GTT | GTA | ACT | CTT | GAC | AAA | AAC | GTT | TCT | GTT | GAC | 885  |
| Ser | Val | Thr | Glu | Leu | Val | Val | Thr | Leu | Asp | Lys | Asn | Val | Ser | Val | Asp |      |
|     |     |     | 245 |     |     |     |     | 250 |     |     |     |     | 255 |     |     |      |
| GAA | ATC | AAC | TCT | GCT | ATG | AAA | GCT | GCT | TCA | AAC | GAT | AGC | TTC | GGT | TAC | 933  |
| Glu | Ile | Asn | Ser | Ala | Met | Lys | Ala | Ala | Ser | Asn | Asp | Ser | Phe | Gly | Tyr |      |
|     |     | 260 |     |     |     |     | 265 |     |     |     |     | 270 |     |     |     |      |
| ACT | GAA | GAT | CCA | ATC | GTT | TCT | TCA | GAT | ATC | GTA | GGC | GTA | TCA | TAC | GGT | 981  |
| Thr | Glu | Asp | Pro | Ile | Val | Ser | Ser | Asp | Ile | Val | Gly | Val | Ser | Tyr | Gly |      |
|     | 275 |     |     |     |     | 280 |     |     |     |     | 285 |     |     |     |     |      |
| TCA | TTG | TTT | GAC | GCA | ACT | CAA | ACT | AAA | GTA | ATG | GAA | GTT | GAC | GGA | TCA | 1029 |
| Ser | Leu | Phe | Asp | Ala | Thr | Gln | Thr | Lys | Val | Met | Glu | Val | Asp | Gly | Ser |      |
|     |     |     |     |     | 295 |     |     |     | 300 |     |     |     |     |     | 305 |      |
| CAA | TTG | GTT | AAA | GTT | GTA | TCA | TGG | TAT | GAC | AAC | GAA | ATG | TCT | TAC | ACT | 1077 |
| Gln | Leu | Val | Lys | Val | Val | Ser | Trp | Tyr | Asp | Asn | Glu | Met | Ser | Tyr | Thr |      |
|     |     |     | 310 |     |     |     |     | 315 |     |     |     |     | 320 |     |     |      |
| GCT | CAA | CTT | GTA | CGT | ACT | CTT | GAG | TAC | TTC | GCA | AAA | ATT | GCT | AAA |     | 1122 |
| Ala | Gln | Leu | Val | Arg | Thr | Leu | Glu | Tyr | Phe | Ala | Lys | Ile | Ala | Lys |     |      |
|     |     |     | 325 |     |     |     | 330 |     |     |     |     | 335 |     |     |     |      |
| TAA |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 1125 |

( 2 ) INFORMATION FOR SEQ ID NO:2:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 336 amino acids
- ( B ) TYPE: amino acid
- ( D ) TOPOLOGY: linear

( ii ) MOLECULE TYPE: protein

( xi ) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

Met Val Val Lys Val Gly Ile Asn Gly Phe Gly Arg Ile Gly Arg Leu
 1          5          10          15
Ala Phe Arg Arg Ile Gln Asn Ile Glu Gly Val Glu Val Thr Arg Ile
 20          25          30
Asn Asp Leu Thr Asp Pro Asn Met Leu Ala His Leu Leu Lys Tyr Asp
 35          40          45
Thr Thr Gln Gly Arg Phe Asp Gly Thr Val Glu Val Lys Glu Gly Gly
 50          55          60
Phe Glu Val Asn Gly Asn Phe Ile Lys Val Ser Ala Glu Arg Asp Pro
 65          70          75          80
Glu Asn Ile Asp Trp Ala Thr Asp Gly Val Glu Ile Val Leu Glu Ala
 85          90          95
Thr Gly Phe Phe Ala Lys Lys Glu Ala Ala Glu Lys His Leu His Ala
100          105          110
Asn Gly Ala Lys Lys Val Val Ile Thr Ala Pro Gly Gly Asn Asp Val
115          120          125
Lys Thr Val Val Phe Asn Thr Asn His Asp Ile Leu Asp Gly Thr Glu
130          135          140
Thr Val Ile Ser Gly Ala Ser Cys Thr Thr Asn Cys Leu Ala Pro Met
145          150          155          160
Ala Lys Ala Leu His Asp Ala Phe Gly Ile Gln Lys Gly Leu Met Thr
165          170          175
Thr Ile His Ala Tyr Thr Gly Asp Gln Met Ile Leu Asp Gly Pro His
180          185          190
Arg Gly Gly Asp Leu Arg Arg Ala Arg Ala Gly Ala Ala Asn Ile Val
195          200          205
Pro Asn Ser Thr Gly Ala Ala Lys Ala Ile Gly Leu Val Ile Pro Glu
210          215          220
Leu Asn Gly Lys Leu Asp Gly Ala Ala Gln Arg Val Pro Val Pro Thr

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31 5,328,996 32

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| 225                 | 230                 | 235                 | 240             |
|---------------------|---------------------|---------------------|-----------------|
| Gly Ser Val Thr     | Glu Leu Val Val Thr | Leu Asp Lys Asn Val | Ser Val         |
|                     | 245                 | 250                 | 255             |
| Asp Glu Ile Asn     | Ser Ala Met Lys     | Ala Ser Asn Asp     | Ser Phe Gly     |
|                     | 260                 | 265                 | 270             |
| Tyr Thr Glu Asp     | Pro Ile Val Ser     | Ser Asp Ile Val     | Gly Val Ser Tyr |
|                     | 275                 | 280                 | 285             |
| Gly Ser Leu Phe Asp | Ala Thr Gln Thr     | Lys Val Met Glu     | Val Asp Gly     |
|                     | 290                 | 295                 | 300             |
| Ser Gln Leu Val Lys | Val Val Ser Trp     | Tyr Asp Asn Glu     | Met Ser Tyr     |
|                     | 305                 | 310                 | 315             |
| Thr Ala Gln Leu Val | Arg Thr Leu Glu     | Tyr Phe Ala Lys     | Ile Ala Lys     |
|                     | 325                 | 330                 | 335             |

20 tor consisting essentially of the amino acid sequence set forth in SEQ. ID NO. 2.

2. The isolated DNA according to claim 1, said DNA consisting essentially of the sequence set forth in SEQ. ID NO. 1.

We claim:

1. Isolated DNA, encoding a plasmin receptor having a molecular weight of about 41 kD, said plasmin recep- 25

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**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO  
DIVISION DE NUEVAS CREACIONES**

**Radicación No:** 02-112910  
**Clasificación Internacional (IPC):** A23K 1/00  
**Concepto Técnico No.** 1151

|                                 |                                     |                               |                                                                      |
|---------------------------------|-------------------------------------|-------------------------------|----------------------------------------------------------------------|
| Patente de Invención            | <input checked="" type="checkbox"/> | Patente de Modelo de Utilidad | <input type="checkbox"/>                                             |
| Vía Nacional                    | <input type="checkbox"/>            |                               |                                                                      |
| Vía PCT (fase nacional)         | <input checked="" type="checkbox"/> | Cap. I                        | <input type="checkbox"/> Cap. II <input checked="" type="checkbox"/> |
| No. Sol. Internacional          | <u>PCT/CA01/00838</u>               |                               |                                                                      |
| Fecha de Presentación Internal. | <u>11 de Junio de 2001</u>          |                               |                                                                      |

Como resultado del examen definitivo, realizado con fundamento en el artículo 48 de la Decisión 486 de la Comisión de la Comunidad Andina, se le comunica:

**1. Documentos estudiados**

- |                                   |                                                                                          |                                                                  |
|-----------------------------------|------------------------------------------------------------------------------------------|------------------------------------------------------------------|
| 1.1. <u>Descripción:</u>          | Folios: <u>135</u> a <u>263</u>                                                          | como se radicó inicialmente                                      |
| 1.2. <u>Reivindicaciones:</u>     | Folios: <u>264</u> a <u>276</u><br>Folios: <u>393</u> a <u>394</u>                       | como se radicó inicialmente<br>como se modificó el<br>20/03/2009 |
| 1.3. <u>Figuras:</u>              | Folios: <u>82</u> a <u>131</u>                                                           | como se radicó inicialmente                                      |
| 1.4. <u>Rta. del Solicitante:</u> | Folios: <u>388</u> a <u>391</u>                                                          | como se radicó el 17/03/2008                                     |
| 1.5. <u>Prioridad:</u>            | Folio: <u>1</u><br>Fecha: <u>12/06/2000</u><br>País: <u>US</u><br>Número: <u>211.022</u> |                                                                  |

#### 4. Respuesta a los argumentos del solicitante:

- El solicitante argumenta que para la persona con conocimiento medio en la materia, que "la respuesta inmune posterior contra el patógeno invasor al inmunizar un sujeto utilizando un antígeno particular, tal como la proteína GapC, podría en si mismo originar una reacción inflamatoria, incrementándose por lo tanto, a diferencia de reducir, los conteos de células somáticas (ver Anexo 5.2)". Sin embargo, de la lectura de dicho documento se entiende que existe un problema específico para inmunización contra mastitis provocada por Staphylococcus, (no estreptococos) y en ningún momento se menciona las proteínas de unión de la capsula GapC ni ensayos que sustenten la hipótesis que el solicitante trata de probar.
- El solicitante menciona la inclusión en el nuevo capítulo reivindicatorio de la expresión "cantidad terapéutica eficaz" que no presenta validez para ser usada como argumento a favor de la novedad o el nivel inventivo de la solicitud ya que dicha expresión no indica una característica técnica de la composición sino un resultado a alcanzar. No es procedente reivindicar una solicitud por un resultado a alcanzar puesto que no existe posibilidad de realizar su comparación con el Estado de la Técnica. Además, en caso de que los argumentos se limiten a una composición de vacuna para mastitis que incluyan GapC en una cantidad efectiva, existiría una objeción puesto que no es claro cuál es la cantidad de cada compuesto en la composición para combatir la mastitis efectivamente.
- El solicitante argumenta que "ninguno de los documentos citados como anterioridades describen ni sugieren composiciones de vacuna para mastitis que incluyan GapC en una cantidad efectiva, y por lo tanto no describen todas las características técnicas de la invención; sino que adicionalmente, dicha materia objeto de protección no antes divulgada representa un avance inesperado en el Estado de la Técnica".

A este particular, cabe mencionar que los documentos no solo si sugieren composiciones que incluyen GapC, también llamada proteína receptor de plasmina, sino que además presentan la secuencia de dicha proteína, cuyo grado de homología con la reivindicada por la solicitud es alta, como se verá en el análisis de patentabilidad.

Teniendo en cuenta el Estado de la Técnica se concluye que el solicitante no ha superado las objeciones formuladas por este Despacho en el concepto técnico 2155, no se aceptan dichos argumentos.

#### 5. Análisis de patentabilidad (artículo 14 D486)

##### Nivel Inventivo (artículo 18 D486)

- La reivindicación 1 y sus dependientes se refiera a una composición de vacuna que comprende 1) un adyuvante y 2) una proteína de GapC o un polinucleótido que codifica para la proteína GapC que se escoge de: a) proteína GapC de *S. dysgalactiae*, b) proteína GapC de *S. agalactiae*, c) proteína GapC de *S. uberis* d) proteína GapC de *S. parauberis* y e) proteína GapC de *S. iniae*.
- El documento del Estado de la Técnica D1 (folio 365) enseña las secuencias de las proteínas de unión de plasmin(ogeno) de *Streptococcus*

spp. que comprenden 336 aminoácidos, revelando su estructura, su función y su potencial uso en la industria de fármacos como vacunas.

- El documento del Estado de la Técnica D2 enseña la secuencia de ADN y su correspondiente traducción en proteína de unión de plasmina obtenida a partir de estreptococos y su efecto en composiciones inmunogénicas para el tratamiento de infecciones humanas y animales específicamente contra estreptococos del grupo A, y B.

Ambos documentos objetan el nivel inventivo de la presente solicitud como ya se había expuesto en el concepto técnico 2155, en la medida en que presentan las secuencias para la proteína GapC que comprende 366 aminoácidos y es obtenida de diferentes cepas de *Streptococcus* spp. El porcentaje de identidad de la secuencia de ADN de los compuestos reivindicados es muy similar (99%) a la de los documentos del Estado de la Técnica D1 y D2 (ver tabla 1), con una variación en algunos aminoácidos, que no confieren un cambio estructural ni funcional en el compuesto activo que le confiere las características a lo reivindicado por la solicitud.

Como se había presentado en el concepto técnico 2155, aunque las objeciones presentadas por novedad en dicho concepto fueron subsanadas, la altura inventiva de la solicitud no ha sido superada por tanto que las características esenciales que confieren la funcionalidad, la estructura, así como el proceso de aislamiento y el problema planteado, ya habían sido solucionadas por los documentos del estado de la Técnica D1 y D2.

Tabla 1. Secuencia de aminoácidos de la proteína GapC de la solicitud y anterioridades citadas.

|                          | 10         | 20          | 30         | 40         | 50         | 60         |
|--------------------------|------------|-------------|------------|------------|------------|------------|
| S. galactiae (solicitud) | MVVKVGINGF | GRIGRLAFRR  | IQNIEGVEVT | RINDLTDPNM | LAHLLKYDIT | QGRFDGTVEV |
| D1                       | MVVKVGINGF | GRIGRLAFRR  | IQNVEGVEVT | RINDLTDPNM | LAHLLKYDIT | QGRFDGTVEV |
| D2                       | MVVKVGINGF | GRIGRLAFRR  | IQNVEGVEVT | RINDLTDPNM | LAHLLKYDIT | QGRFDGTVEV |
|                          | KEGGFEVNGN | FIK VSAERDP | ENIDWATDGV | EIVLEATGFF | AKKEAAEKHL | HANGAKKVVI |
|                          | KEGGFEVNGN | FIK VSAERDP | ENIDWATDGV | EIVLEATGFF | AKKEAAEKHL | HANGAKKVVI |
|                          | KEGGFEVNGN | FIK VSAERDP | ENIDWATDGV | EIVLEATGFF | AKKEAAEKPL | HANGAKKVVI |
|                          | TAPGGNDVKT | VVFNTNHDIL  | DGTETVISGA | SCTTNCLAPM | AKALHDAFGI | QKGLMTTIHA |
|                          | TAPGGNDVKT | VVFNTNHDIL  | DGTETVISGA | SCTTNCLAPM | AKALHDAFGI | QKGLMTTIHA |
|                          | TAPGGNDVKQ | LFSTLTTSIL  | DGTETVISGA | SCTTNCLAFM | AKALHDAFGI | QKGLMTTIHA |
|                          | YTGDMILDG  | PHRGGDLRRA  | RAGAAIVPN  | STGAAKAIGL | VIPELNGKLD | GAAQRPVPT  |
|                          | YTGDMILDG  | PHRGGDLRRA  | RAGAAIVPN  | STGAAKAIGL | VIPELNGKLD | GAAQRPVPT  |
|                          | YTGDMIDVG  | HRGGDLRRA   | RAGAAIVPN  | STGARKAIGL | VIPELNGKLD | GAAQRPVPT  |
|                          | GSVTELVVTL | DKNVSVDEIN  | SAMKAASNDS | FGYTEDPIVS | SDIVGVSYGS | LFDATQTKVM |
|                          | GSVTELVVTL | DKNVSVDEIN  | AAMKAASNDS | FGYTEDPIVS | SDIVGVSYGS | LFDATQTKVM |
|                          | GSVTELVVTL | DNNVSVDEIN  | AAMKAASNDS | FGYTEDPIVS | SDIVGVSYGS | LFDATQTKVM |
|                          | EVDGSQLVKV | VSWYDNEMSY  | TAQLVRTLEY | FAKIAK*    |            |            |
|                          | EVDGSQLVKV | VSWYDNEMSY  | TAQLVRTLEY | FAKIAK*    |            |            |
|                          | EVDGSQLVKV | VSWYDNEMSY  | TAQLVRTLEY | FAKIAK*    |            |            |

Así mismo, el documento D2 describe el uso de dichas secuencias para su uso en composiciones de vacuna como lo enseña en el ejemplo 21. Explícitamente indica que “el receptor de plasmina descrito puede ser usado ventajosamente en composiciones inmunogénicas como vacunas” (segunda columna, párrafo 4, línea 47). Así mismo determina que estas composiciones inmunogénicas que comprenden dicho receptor de plasmina, y variantes de dichos fragmentos con propiedades antigénicas pueden ser preparados por procedimientos bien conocidos en el Estado de la Técnica, empleando adyuvantes, vehículos y demás excipientes bien conocidos

El aislamiento de la proteína GapC no es novedoso ni inventivo como se evidencia en las anterioridades citadas, en las cuales se emplean las mismas técnicas, con la única diferencia que no se emplean las mismas especies. Sin embargo, dentro de la familia de estreptococos no representa un salto cuantitativo del estado de la técnica aislar dichas proteínas de diferentes especies de estreptococos y formularlas en un compuesto inmunogénico, como ya se ha visto debido a que su homología es obvia para un técnico medio en la materia.

Por tanto se reitera que el problema planteado en la solicitud de vacunas protectoras contra infección de estreptococos a partir de GapC ya se había resuelto por los mismos compuestos y se había demostrando los mismos efectos técnicos con la proteína de la solicitud.

Por tanto para el momento de la solicitud era evidente para un técnico medio en la materia realizar composiciones de vacuna de GapC obtenidos de diferentes cepas o especies de estreptococos como *Streptococcus dysgalactiae*, *S. agalactiae*, *S. uberis*, *S. parauberis* o *S iniae*.

En consecuencia, y como ya se había indicado anteriormente en el concepto técnico 2155, puesto que tanto D1 como D2 divulgaban previamente el componente activo de la solicitud y sugerían su uso en una vacuna, una persona conocedora en la materia lograría una composición de vacuna con la combinación realizada para tratar infecciones por estreptococos. Por lo cual la solicitud en estudio no cumple con el requisito de Nivel Inventivo establecido en el artículo 18 de la Decisión 486.

6. Conclusión:

En consecuencia y por todo lo expuesto anteriormente, se recomienda **Negar** las reivindicaciones: 1 a 3 (folios: 393 - 394)

Bogotá, D.C.,

|                                     |                                                |
|-------------------------------------|------------------------------------------------|
| <b>CONCEPTO TECNICO</b><br>No. 1151 | <b>EXAMINADOR TECNICO</b><br>Código No. 202089 |
|-------------------------------------|------------------------------------------------|