E.E.A.D. (C.S.I.C.) Departamento de Producción Animal, S.I.A. (D.G.A.), Zaragoza, España

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Para Clara con cariño

A Mamen

A mis padres

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RESUMEN

Se ha desarrollado un método para la obtención de células epiteliales de la glándula mamaria ovina de animales vivos o muertos. Se ha observado que la adherencia de bacterias aisladas de mamitis ovinas a dichas células depende de varios factores ambientales: aumenta con el tiempo de incubación (30 min frente a 120 min), la temperatura (37°C frente a 22°C) y el grado de acidez (pH 5,9 frente a 7,2). Se han detectado diferencias entre especies bacterianas y entre cepas dentro de una misma especie en cuanto a su capacidad de adherencia a las células epiteliales.

Se ha encontrado que la interacción de las bacterias entre sí, se altera al adicionar suero lácteo al medio de cultivo. Cuando las bacterias crecieron en Todd Hewitt broth (THB) suplementado con ≥30% de suero lácteo ovino, todas las cepas de *S. aureus* testadas (59), ovinas y bovinas, aglutinaron, pero sólo 4 entre las 22 cepas estudiadas de otras especies de estafilococos lo hicieron. El suero lácteo ovino mostró una capacidad de inducir la aglutinación mayor que la del suero lácteo bovino ($P<0,005$), respecto del número de cepas ovinas y bovinas aglutinadas. Sin embargo, no se encontraron diferencias entre ovejas en cuanto a la capacidad de su suero lácteo para inducir la aglutinación. Las cepas de origen ovino aglutinaron más que las bovinas a altas concentraciones de suero lácteo bovino (≥30% en THB; $P<0,001$) y a las distintas concentraciones estudiadas (≥10%) de suero lácteo ovino ($P<0,001$).

Utilizando un medio apropiado (agar rojo Congo), algunas cepas (21 de las 144 testadas) produjeron mucus. Estas cepas mostraron, en microscopía electrónica, una matriz condensada de exopolisacáridos alrededor de las bacterias, cuya presencia se confirmó mediante inmunofluorescencia. Se obtuvieron 8 variantes no mucosas a partir de cepas mucosas, y dos variantes mucosas a partir de cepas no mucosas.

Tras facilitar que las bacterias crecieran formando microcolonias, no se encontraron diferencias respecto de la concentración mínima inhibitoria entre las cepas mucosas y sus variantes no mucosas, pero sí las hubo en cuanto a la concentración bactericida mínima, mostrando la mayoría de las cepas mucosas una mayor resistencia a determinados antibióticos cuando estos se utilizaron a altas concentraciones.

Entre las 92 cepas bovinas y las 52 cepas ovinas testadas para la presencia de mucus, 25 (26,6%) y 4 (7,5%), respectivamente, pertenecieron al serotipo capsular 5. Sólo una cepa mucosa bovina (V50) perteneció a este serotipo. Los anticuerpos frente al mucus parcialmente purificado y frente a la cápsula de la cepa A, obtenidos por inmunización en conejo, resultaron ser inmunológicamente diferentes, lo cual sugirere que el mucus y la cápsula difieren en su estructura antigénica.

En medios comunes de cultivo (THB, etc.), las bacterias pueden también alterar sus propiedades de superficie durante el crecimiento. Tras determinar la hidrofobicidad de *S. aureus* con xilol, se observó que esta se incrementa durante la fase exponencial de crecimiento. Cuando se estudiaron las bacterias al final de esta fase, las cepas de aislamientos recientes resultaron ser más hidrofóbicas que las cepas viejas (P<0,01). Sin embargo, estas últimas exhibieron una mayor hidrofobicidad después de un pase por la glándula mamaria del ratón (P<0,01). Las cepas bovinas fueron más hidrofóbicas que las ovinas (P<0,01). A excepción de las cepas productoras de mucus, las cepas resultaron ser más hidrofílicas después de crecer en medios inductores de exopolisacáridos (Columbia y m110).

STAPHYLOCOCCUS AUREUS BEHAVIOUR ACCORDING TO BACTERIAL CELL SURFACE PROPERTIES

Studies on *Staphylococcus aureus* adherence to epithelial cells, growth agglutination in whey-enriched media, exopolysaccharide production and bacterial cell hydrophobicity

SUMMARY

A method was developed for obtaining epithelial cells from the mammary gland of life or dead sheep. It was observed that the degree of adherence of bacteria isolated from ovine mastitis to these cells varied with the test conditions applied: it increased with incubation time (30 min vs. 120 min), temperature (37°C vs. 22°C) and acidity (pH 5.9 vs. 7.2). Differences between bacterial species and between strains of a given species were detected with regard to the ability to adhere to epithelial cells.

The bacterial interaction was altered upon addition of whey to the culture medium. When bacteria grew in Todd Hewitt broth (THB) supplemented with ≥30% ovine whey, all *S. aureus* strains tested (59), isolated from sheep and cattle, agglutinated, but only 4 among the 22 strains belonging to other staphylococcal species did so. Ovine whey showed a greater capacity to induce agglutination when compared with bovine whey (P<0.005), with respect to the number of ovine and bovine agglutinating strains. However, no differences between individual sheep were found with regard to the capacity of whey to induce agglutination. Strains of ovine origin agglutinated more than bovine bacterial strains at high concentrations of bovine whey (≥30% in THB; P<0.001) and at different concentrations used (≥10%) of ovine whey (P<0.001).

Using an appropiate medium (Congo red agar), some strains (21 of the 144 tested) produced slime. These strains showed by electron microscopy a condensed exopolysaccharide matrix surrounding the

bacterial cells. The presence of this layer was confirmed by immunofluorescence. Eight non-slime producing variants were obtained from slime producing strains; and two slime producer variants were obtained from non-slime producing strains.

After applying a culture procedure favouring microcolony formation, no differences were found between slime producing strains and their non-slime producing variants with respect to the minimal inhibitory antibiotic concentration, but differences were found with regard to the minimal bactericidal concentration, with the majority of slime producing strains showing a greater resistance to specific highly concentrated antibiotics.

Among the 92 bovine strains and the 52 ovine strains tested for the presence of slime, 25 (26.6%) and 4 (7%), respectively, belonged to the capsular serotype 5. Only one slime producing bovine strain (V50) belonged to this serotype. Antibodies against the partially purified slime and against the capsule of strain A, obtained by immunizations in rabbits, showed immunological differences, which suggests that slime and capsule are different antigenic structures

In regular culture media (THB, etc.), bacteria may also alter their surface properties during growth. After determining the degree of hydrophobicity of *S. aureus* with xylene, an increment of this degree was observed during the exponential growth phase. When bacteria were studied at the end of this phase, recently isolated strains showed a higher hydrophobicity when compared with old strains (P<0.01). However, the latter became more hydrophobic after a passage through the mouse mammary gland (P<0.01). Bovine strains were more hydrophobic than ovine strains (P<0.01). With the exception of slime producing strains, the majority of strains became more hydrophilic after growth in exopolysaccharide inducing media (Columbia and m110).

PREFACIO

Esta tesis está basada en los trabajos que se indican a continuación, según su orden de elaboración. Estos trabajos serán identificados en el texto con los correspondientes números romamos I-VII. El carácter de los trabajos I, II, IV, V y VI es experimental, mientras que el de los trabajos III y VII es conceptual y bibliográfico.

I. Amorena, B., **Baselga, R.**, y Aguilar, B. 1990. Factors influencing the degree of "in vitro" bacterial adhesion to ovine mammary gland epithelial cells. Vet. Microbiol., 24: 43-53.

II. **Baselga, R.** y Amorena, B. 1990. Milk whey induction of agglutination in ovine and bovine mastitis *Staphylococcus aureus*. J. Vet. Med., B., 37: 556-560.

III. Tejedor, T., **Baselga, R.** y Amorena, B. 1990. Aspectos actuales de la lucha contra la mamitis en el ganado vacuno lechero. ITEA, 86A: 73-91.

IV. **Baselga, R.**, Penadés, J.R., Albizu, I., y Amorena, B. 1991. Comparación entre cepas mucosas de *Staphylococcus aureus* y sus variantes no mucosas con respecto a la sensibilidad a antibióticos. ITEA, 11: 754-757.

V. **Baselga, R.**, Albizu, I., Penadés, J.R., Aguilar, B., Iturralde, M. y Amorena, B. 1991. Hydrophobicity of mastitis *Staphylococcus aureus*. Effect of growth phase, exopolysaccharide-inducing media, ageing and source of bacteria. Current Microbiol., (en prensa).

VI. **Baselga, R.**, Albizu, I., Penadés, J.R., Del Cacho., E. y Amorena, B. 1991. Production of ruminant mastitis *Staphylococcus aureus* slime negative variants from slime positive strains. Infect. Immun., (enviado para su publicación).

VII. **Baselga, R.** y Amorena, B. 1991. *Staphylococcus aureus* capsule and slime as virulence factors in mastitis. Review article. Vet. Microbiol., (enviado para su publicación).

ABREVIATURAS

ASS	Agar suave suplementado con suero
ECN	Estafilococos coagulasa-negativos
NB	Caldo nutritivo (Nutrient broth)
THB	Medio Todd Hewitt (Todd Hewitt broth)
MCD	Morfología colonial difusa
m110	Medio 110 modificado para *Staphylococcus*
UFC	Unidades formadoras de colonias

INDICE

INTRODUCCION

En el marco de las mamitis de rumiantes, se han realizado múltiples estudios: epidemiológicos, etiológicos, genéticos, inmunológicos, productivos, etc.; todos ellos quedan enlazados en un sustrato común que comprende los fenómenos de la interacción de las bacterias entre sí y con el hospedador en el plano celular y molecular. Es en este campo donde se han encaminado los esfuerzos de esta Tesis. Por dicho motivo, y en conexión con los trabajos realizados en la misma, se considerarán en este apartado distintos capítulos para poder ubicar cada trabajo en su contexto respectivo.

1.1. Mamitis bovinas y ovinas

La mamitis es, en términos económicos, uno de los problemas sanitarios más importantes en el ganado lechero (Zorraquino, 1986). La enfermedad tiene frecuentemente una etiología bacteriana. Entre las especies bacterianas que intervienen en los procesos mamíticos, merecen destacarse los estafilococos, los estreptococos y los coliformes, además de una amplia variedad de otros microorganismos (Watts, 1988; Marco, 1991). Tanto en el ganado bovino como en el ovino, *Staphylococcus aureus* es el más importante de los patógenos implicados en la mamitis, tanto por su prevalencia, como por la gravedad de las lesiones que origina (Plommet, 1959; Nickerson y Heald, 1981; Albizu y cols., 1991; Amorena y cols., 1991; Marco y cols., 1991).

Para producir la enfermedad, los microorganismos tienen que penetrar en la glándula mamaria a través del canal del pezón, superando el esfínter del mismo (Fig 1; Craven y Williams, 1985, Paape y cols., 1985). En el caso de *S. aureus*, esta penetración puede facilitarse por la lesión del pezón o por el "reflujo" de la leche contaminada dentro del pezón durante el ordeño mecánico (Bramley y Dodd, 1984). Una vez que la bacteria ha conseguido penetrar, para llegar a establecerse en el tejido mamario deberá resistir la acción de los factores bactericidas del canal del pezón y los de la leche, el drenaje propio del ordeño o del amamantamiento, y la fagocitosis mediada por macrófagos y neutrófilos (Fig. 1; Craven y Williams, 1985).

S. aureus es capaz de producir toxinas y enzimas extracelulares (Arvidson, 1983) que dañan el epitelio y alteran la permeabilidad vascular y el sistema circulatorio (Haraldsson y Jonsson, 1984), provocando desde una ligera respuesta inflamatoria, característica de las mamitis subclínicas, hasta lesiones muy graves (mamitis clínicas; Amorena y cols., 1991). Muy frecuentemente, la infección por *S. aureus* tiende a volverse crónica, resultando ineficaz el tratamiento con antibióticos, tanto en el ganado ovino (Watson y Buswell, 1984) como en el bovino (Bramley y Dodd, 1984). Ello ha estimulado el desarrollo de las investigaciones encaminadas al diseño de tests "in vitro" con los que pueda inferirse el grado de eficacia del tratamiento antibiótico "in vivo".

1.2. Adhesinas y proteínas de unión a componentes de la matriz celular

Además de la producción de exotoxinas y exoenzimas, se ha sugerido que ciertos componentes de la superficie de *S. aureus* tienen carácter patogénico (Tabla 1). La pared bacteriana de *S. aureus* está constituída fundamentalmente por peptidoglicano y ácidos teicoicos, conteniendo además proteínas y ácido lipoteicoico (Schleifer, 1983). Asimismo, la bacteria puede producir polisacáridos alrededor de su pared (exopolisacáridos), constituyendo con ello cápsulas, microcápsulas o mucus (Tabla 1).

La adherencia de los microorganismos a las células epiteliales del hospedador y a los tejidos puede ser un factor importante de patogenicidad bacteriana (Ofek y Beachey, 1980). Distintas cepas de *S. aureus* son capaces de adherirse específicamente a las células epiteliales de la glándula mamaria, habiéndose sugerido que ello puede constituir la primera etapa en la patogénesis de la mamitis bovina (Fig. 1; Frost, 1975; Frost y cols., 1977; Wanasinghe, 1981; Bramley y Hogben, 1982; Opdebeeck y cols., 1988a; Lindhal y cols., 1989, 1990). De hecho, Lindhal y cols. (1990) han demostrado que *S. aureus* aislado de mamitis bovina posee dos adhesinas proteicas implicadas en la adherencia a las células epiteliales mamarias bovinas. Una de estas proteínas parece ser el receptor para la fibronectina, mientras que la otra es una hemoaglutinina.

Tabla 1. Componentes de superficie asociados con la virulencia de *S. aureus*

Componentes de la superficie celular	Referencia
Proteína A	Forsgren, 1972; Jonsson y cols., 1985
Acido lipoteicoico	Beachey y Simpson, 1982; Carruthers y Kabat, 1983
Proteína de unión a la fibronectina	Espersen y Clemmensen, 1982; Fröman y cols., 1987; Rydén, 1987
Proteína de unión al fibrinógeno (clumping factor)	Bodén y Flock, 1989; Mamo y cols 1988
Proteína de unión al colágeno	Switalski y cols., 1983; Speziale y cols., 1986; Mamo y cols. 1988
Proteína de unión a la laminina	Lopes y cols. 1985; Vercellotti y cols., 1985
Proteína de unión a la vitronectina	Fuquay y cols., 1986; Chhatwal y cols., 1987
Proteína hemoaglutinante y proteína de adherencia a células epiteliales	Lindhal y cols., 1989; 1990
Exopolisacáridos	Yoshida y Ekstedt, 1968; Willey y Maverakis, 1974; Peterson y cols., 1978; Wilkinson y cols., 1979; Verbrugh y cols., 1982; Wilkinson, 1983; Karakawa y cols 1988; Johne y cols., 1989; Watson, 1989; Jonsson y cols., 1985; 1989; Mamo y cols., 1991

Asimismo, *S. aureus* posee receptores específicos para determinadas proteínas que se encuentran en la membrana basal (Tabla 1). Así, cuando el epitelio resulta dañado como consecuencia de un trauma, o por la acción de las toxinas citolíticas secretadas por la bacteria, los componentes subepiteliales de la matriz celular tales como la fibronectina, el fibrinógeno, la fibrina, la laminina, el colágeno o la vitronectina pueden quedar expuestos (Fig. 1) y facilitar con ello la unión de las bacterias a la membrana basal (Simpson y Beachey 1983; Mamo y cols., 1988; Kuypers y Proctor, 1989; Switalski y cols., 1989).

Tanto las proteínas como los exopolisacáridos de la superficie bacteriana pueden experimentar cambios en su expresión fenotípica en respuesta al ambiente en el que crece la bacteria (Cheung y Fischetti, 1988; Lorian, 1989; Christensen y cols., 1990). Ello se traduce en una alteración de las propiedades de la superficie bacteriana

1.3. Aglutinación

Un importante componente en la defensa de las superficies epiteliales es la prevención de la colonización por la aglutinación bacteriana, facilitándose de este modo la eliminación de los microorganismos (Widders, 1988). Estas observaciones pueden tener implicaciones en la mamitis, donde la posibilidad de eliminación durante el ordeño puede ser grande (Bramley y Dodd, 1984)

1.4. Cápsula y mucus (slime)

Aunque el mucus, al igual que la cápsula, está compuesto por exopolisacáridos (Wilkinson, 1983), algunos autores sugieren que la consistencia de éste parece ser más débil que la de la cápsula (Caputy y Costerton, 1982; Wilkinson, 1983; Watson, 1989). Wilkinson (1983) define a la cápsula de *S. aureus* como una estructura bien definida que rodea a la pared celular, la cual es conservada por la bacteria a través del subcultivo "in vitro", independientemente del medio de crecimiento

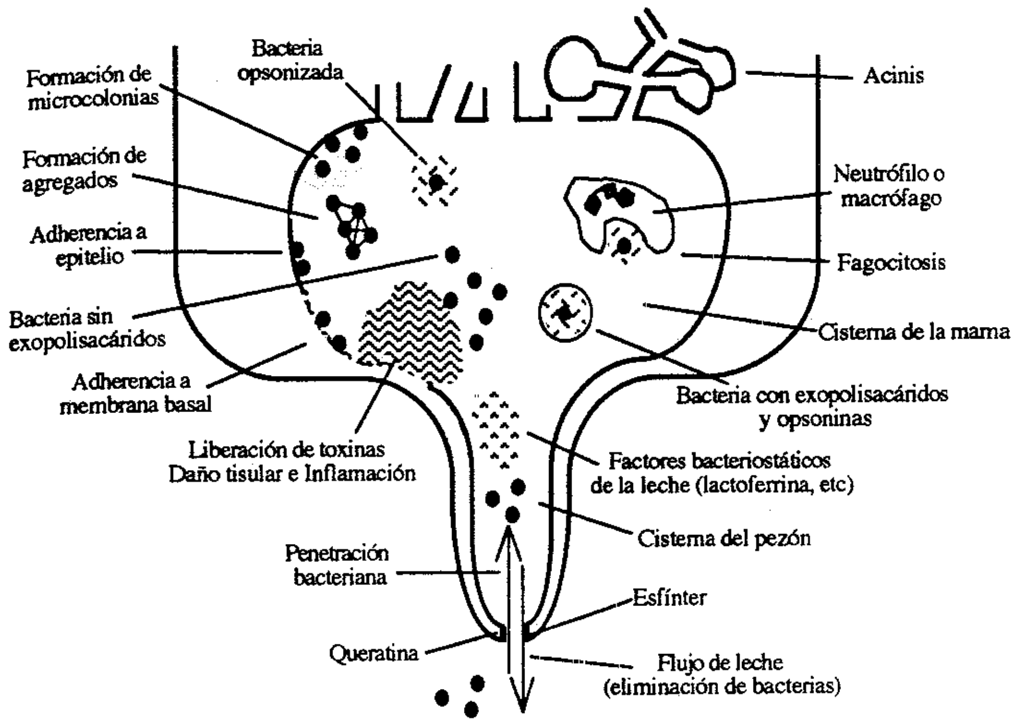

Fig. 1 Fenómenos principales que pueden producirse en un proceso mamítico originado por *S. aureus*. (Por claridad de la ilustración se han plasmado los fenómenos en las cisternas de la mama y del pezón, aunque pueden hacerse extensivos al parénquima).

subcultivo "in vitro", independientemente del medio de crecimiento utilizado. Las cepas de *S. aureus* altamente encapsuladas son capaces de resistir la fagocitosis mediada por neutrófilos "in vitro", aun cuando la bacteria haya sido opsonizada con suero y complemento (Peterson y cols., 1978). "In vivo", se ha demostrado que la virulencia de las cepas de *S. aureus* altamente encapsuladas es mayor que la de sus variantes no encapsuladas. Sin embargo, no parece suceder lo mismo con las cepas que poseen microcápsulas (Lee y cols., 1987a; 1987b; Albus y cols., 1991). Se han descrito varios mecanismos por los que el material capsular puede inhibir la fagocitosis: la incapacidad de la cápsula para activar la vía alternativa del complemento (Verbrugh y cols., 1979), el enmascaramiento de las opsoninas depositadas sobre la pared bacteriana, producido por la cápsula extracelular (inhibiendo así la interacción de la opsonina con el receptor sobre la superficie del neutrófilo; Peterson y cols., 1978) y el decremento de la interacción bacteria-neutrófilo debido a la hidrofilicidad bacteriana (Van Oss, 1978).

En la mamitis, la producción de material capsular debe resultar ventajosa para *S. aureus*, teniendo en cuenta que la fagocitosis mediada por neutrófilos es la principal defensa del hospedador frente a la bacteria, una vez que ésta supera el canal del pezón (Craven y Williams, 1985). De hecho, la presencia de exopolisacáridos capsulares ha sido evidenciada usando microscopía electrónica en cepas de *S. aureus* que fueron estudiadas justamente tras obtenerlas de la leche mamítica bovina (Johne y cols., 1989; Watson, 1989).

Por otro lado, en medicina clínica humana, los estafilococos coagulasa-negativos (ECN) han adquirido una gran importancia, a causa de su capacidad para adherirse a diferentes materiales usados en cirugía (Quie y Belani, 1987; Christensen y cols., 1982). Esta capacidad también ha sido demostrada en algunas cepas de *S. aureus* de infecciones humanas (Mayberry-Carson, 1986; Barth y cols., 1989; Vaudaux y cols., 1989; Trabajos V y VI). Varios estudios sugieren que la producción de mucus (slime) facilita esta unión (Christensen y cols., 1985) y protege a la

bacteria de la fagocitosis y de los agentes antimicrobianos, incluyendo los antibióticos (Quie y Belani, 1987).

La producción de mucus en cepas de ECN puede detectarse estimando la capacidad de las bacterias para colonizar las paredes de un tubo (Christensen y cols., 1985) o estudiando la morfología colonial en agar rojo Congo (Freeman y cols., 1989). Sin embargo, esta metodología no se ha aplicado en *S. aureus*.

1.5. Hidrofobicidad/hidrofilicidad

Las moléculas hidrofóbicas tienden a agregarse expulsando las moléculas de agua y pueden asociarse con solventes insolubles en agua, mientras que las moléculas hidrofílicas (cargadas) forman puentes de hidrógeno con el agua asociándose con sus moléculas (98/1324). Estas dos propiedades permiten determinar la hidrofobicidad en cepas de *S. aureus* aisladas de mamitis bovinas mediante el test de agregación salina "Salt aggregation test" (Jonsson y Wadström, 1983; 1984; Ljungh y cols., 1987., Mamo y cols., 1987a; 1987b; 1988; Jonsson y cols., 1989), o por partición en un sistema de dos fases, una hidrofílica y otra hidrofóbica (Hoght y cols., 1983).

El conocimiento de la naturaleza de la superficie bacteriana es crucial para comprender la interacción de los microorganismos entre sí y con el hospedador (71/1324). Así, se ha sugerido que la hidrofobicidad de la superficie bacteriana interviene en la adherencia de las bacterias a los tejidos del hospedador (Ofek y Beachey 1980; Beck y cols., 1988b; Hazen y cols., 1991). Por otra parte, Van Oss (1978) sugirió que las bacterias altamente hidrofóbicas son más susceptibles a la fagocitosis que las hidrofílicas, ya que la fagocitosis se facilita cuando existen interacciones hidrofóbicas entre las bacterias y los neutrófilos (Van Oss, 1978).

Por último, se ha demostrado que componentes de la superficie bacteriana como la proteína A o la proteína de unión a la fibronectina incrementan la hidrofobicidad bacteriana (Jonsson y Wadström, 1984), mientras que la presencia de un exopolisacárido capsular se asocia a un comportamiento hidrofílico (Hoght y cols., 1983; Jonsson y cols., 1983; Mamo y cols., 1988). De hecho, se ha sugerido que la hidrofilicidad puede ser un criterio para determinar la presencia de exopolisacáridos bacterianos en cepas de *S. aureus* obtenidas de mamitis bovinas (Jonsson y Wadström, 1983).

2. OBJETIVOS DEL TRABAJO EXPERIMENTAL

Se han llevado a cabo una serie de investigaciones encaminadas a elucidar el comportamiento de distintas cepas de *S. aureus* procedentes de mamitis bovinas y ovinas. Especialmente, se han estudiado distintos aspectos de la superficie bacteriana que afectan a la interacción molecular y celular de las bacterias entre sí, con el hospedador y con los antibióticos que se suministran a este último para combatir la infección mamítica.

Los objetivos concretos del trabajo experimental de esta Tesis en las distintas áreas de estudio son los siguientes:

1. **Estudios de adherencia:**
   Determinar los factores que condicionan la adherencia bacteriana "in vitro" a las células epiteliales de la glándula mamaria ovina

2. **Estudios de aglutinación:**
   Investigar la producción de agregados bacterianos tras el crecimiento de cepas de*S. aureus* aisladas de mamitis ovinas y bovinas en presencia de suero lácteo ovino o bovino

3. **Estudios sobre la producción de mucus:**
   a) Estudiar la producción de mucus en cepas de *S. aureus*, aisladas de mamitis ovinas o bovinas; b) obtener variantes no mucosas a partir de cepas mucosas; y c) obtener variantes mucosas a partir de cepas no mucosas.

4. **Estudios sobre la resistencia a antibióticos:**
   Estudiar la resistencia de *S. aureus* a los antibióticos en relación con la producción de mucus.

5. **Estudios sobre la hidrofobicidad:**
   Determinar la hidrofobicidad en cepas de *S. aureus* aisladas de mamitis ovinas y bovinas, en relación a su envejecimiento, su origen (ovino o bovino) y su capacidad de producir mucus.

Aparte de los trabajos experimentales, se han elaborado otros de carácter conceptual y bibliográfico, uno de tipo general sobre la mamitis bovina (Trabajo III) y otro más específico sobre la superficie celular bacteriana; en concreto, sobre la cápsula y el mucus de *S. aureus* (Trabajo VII).

3. MATERIAL Y METODOS

3.1. Cepas bacterianas y condiciones de cultivo

Las cepas bacterianas utilizadas procedían de mamitis ovinas y bovinas, excepto la cepa encapsulada A, suministrada por el Dr K. Yoshida (St. Marianna University, Japon). La información sobre el origen de las distintas cepas, su conservación y las condiciones de cultivo para cada experiencia se detalla en los trabajos I, II, IV, V y VI.

3.2. Ensayos de adherencia

Los ensayos de adherencia se realizaron según la técnica descrita por Krovacek y cols. (1987), con las modificaciones que se especifican en el Trabajo I. Se utilizaron células epiteliales obtenidas de la glándula mamaria ovina directamente, no sometidas a cultivo, y 17 cepas aisladas de mamitis ovinas, pertenecientes a las especies *S. aureus* (9), *S. chromogenes* (1), *S. hyicus* (2), *S. xylosus* (1), *S. intermedius* (1), y *Escherichia coli* (3).

3.3. Formación de agregados durante el crecimiento bacteriano en presencia de suero lácteo

Las bacterias crecieron durante 18-24 h en Todd Hewitt broth (THB), medio modificado para estafilococos (m110), o en THB suplementado con diferentes concentraciones de suero lácteo. La presencia de agregados macroscópicos, fue determinada visualmente después del crecimiento (Trabajo II).

3.4. Producción de mucus

La producción de mucus por parte de la bacteria fue estimada según la capacidad de la bacteria para colonizar la pared del tubo en donde se realiza el cultivo, según describen Christensen y cols. (1985), y según el tipo de morfología colonial en agar rojo Congo (ARC; Freeman y cols., 1989; Trabajos V y VI).

3.5. Morfología colonial difusa

La morfología colonial difusa (MCD) en agar suave suplementado con suero (ASS), fue estudiada siguiendo el método descrito por Opdebeeck y cols (1987), tras crecer las bacterias en medios inductores de exopolisacáridos (m110 y Columbia; Trabajo V).

3.6. Obtención de variantes no mucosas y mucosas a partir de cepas mucosas y no mucosas, respectivamente

Las variantes no mucosas fueron obtenidas a partir de cepas mucosas por subcultivo seriado en ARC, tal y como se indica en el Trabajo VI. Se aplicó un procedimiento similar al descrito por Christensen y cols. (1987) para obtener variantes mucosas de cepas no mucosas (Trabajo VI).

3.7. Detección del serotipo capsular 5 de *Staphylococcus aureus*

Se realizó siguiendo el procedimiento descrito por el Dr. P. Sarradin (comunicación personal), tal y como aparece detallado en el Trabajo VI, utilizando el monoclonal cedido por él mismo y los Dres. P. Rainard y B. Poutrel (INRA, Nouzilly, Francia).

3.8. Purificación del mucus

Se empleó la técnica descrita por Fournier y cols. (1987), con las ligeras modificaciones que se indican en el Trabajo VI.

3.9. Inmunofluorescencia

La presencia del mucus en la superficie de las cepas mucosas de *S. aureus* se determinó utilizando un suero anti-mucus, obtenido en conejo y un anticuerpo secundario marcado con fluoresceina (Trabajo VI).

3.10. Microscopía electrónica

El mucus fue evidenciado mediante microscopía electrónica de transmisión. Para ello, varias cepas mucosas y sus variantes no mucosas, fueron fijadas con glutaraldehído tras crecer en ARC y teñidas con tetraóxido de osmio (Trabajo VI).

3.11. Resistencia a antibióticos

Para determinar la resistencia a los antibióticos de las cepas mucosas con respecto a sus variantes no mucosas, se permitió que las bacterias, una vez adheridas a una superficie (el fondo de un pocillo de una placa microtiter), formaran microcolonias. A continuación se sometieron a distintas concentraciones de antibiótico (Trabajo IV).

3.12. Ensayo de hidrofobicidad

La hidrofobicidad bacteriana fue determinada midiendo la afinidad de las bacterias por el xilol en un sistema de dos fases (agua-xilol), de acuerdo con el método descrito por Hoght y cols. (1983; Trabajo V).

3.13. Infección experimental de la glándula mamaria del ratón

Después de crecer las bacterias durante toda la noche en Nutrient broth (37°C), éstas fueron lavadas una vez en salina tamponada (PBS) para eliminar las exotoxinas bacterianas. Veinticinco microlitros de una suspensión bacteriana en PBS ($5x10^8$-10^9 UFC/ml) fueron inyectados en la cuarta glándula mamaria del lado izquierdo del animal. Tras sacrificar el ratón a las 24 h de la infección, las bacterias fueron recuperadas en condiciones estériles (Trabajo V).

4. RESULTADOS EXPERIMENTALES

A continuación se presenta un extracto de los resultados obtenidos, cuyo detalle figura en los trabajos I, II, IV, V y VI. Las tablas y figuras citadas en este extracto, pueden encontrarse en los trabajos respectivos.

4.1. Factores que influyen sobre el grado de adherencia bacteriana ""in vitro"" a las células epiteliales de la glándula mamaria ovina (Trabajo I)

Previo a la realización de los ensayos de adherencia, se desarrolló un método para la obtención de células epiteliales de la glándula mamaria ovina a partir de animales vivos o muertos. La población celular obtenida

con este procedimiento, estaba compuesta en más de un 65% por células epiteliales, con una viabilidad variable entre el 70 y el 90%.

Bajo las condiciones del ensayo, se observó que la adherencia bacteriana a las células epiteliales se incrementaba con el tiempo de incubación (30-120 min; P<0,05; Tabla 1) y era mayor a 37°C que a 22°C (P<0,001; Tabla 1). Un pH ácido (5,9) también se asociaba con un incremento de la adherencia, en relación a un pH más elevado (7,2; P<0,05; Tabla 2). La presencia de Tween 20, Tween 80 o de albúmina sérica bovina facilitaban la eliminación del fondo bacteriano inespecífico integrado por bacterias no adherentes a células. Se observaron diferencias entre especies bacterianas obtenidas de mamitis ovinas y entre cepas dentro de una misma especie en cuanto a su capacidad de adherencia a las células epiteliales (Tabla 3; Figs. 1, 2 y 3).

4.2. Inducción de la aglutinación de cepas ovinas y bovinas de *Staphylococcus aureus* durante el crecimiento en medios suplementados con suero lácteo ovino o bovino (Trabajo II)

Se utilizaron 59 cepas estafilacócicas aisladas de mamitis. Bajo las condiciones aplicadas, pudo observarse que todas las cepas de *S. aureus* testadas (37), ovinas y bovinas, aglutinaban en THB suplementado con ≥30% de suero lácteo ovino, pero sólo 4 entre las 22 cepas estudiadas de otras especies de estafilococos lo hacían (Tabla 1). Ninguna de las cepas testadas aglutinaba en THB sin suplementar con suero. El suero lácteo ovino tenía una capacidad de inducir la aglutinación mayor que la del suero lácteo bovino (P<0,005; Tabla 2) respecto del número de cepas ovinas y bovinas aglutinadas. Sin embargo, no se encontraron diferencias entre ovejas en cuanto a la capacidad de su suero lácteo para inducir la aglutinación. Las cepas de origen ovino aglutinaban más que las de origen bovino a altas concentraciones de suero lácteo bovino (≥30% en THB; P<0,001; Tabla 2), y a las distintas concentraciones de suero lácteo ovino (≥10%; P<0,001; Tabla 2). La secreción de las vacas secas inducía la aglutinación en todas las cepas ovinas y bovinas testadas (Tabla 2).

4.3. Identificación de cepas mucosas de *Staphylococcus aureus*, procedentes de mamitis ovinas y bovinas, y obtención de variantes no mucosas (Trabajo VI)

Tras el ensayo en ARC se observó que sólo 21 de las 144 cepas testadas eran mucosas. Estas cepas mostraban, en microscopía electrónica (Fig. 1), una matriz condensada de exopolisacáridos alrededor de las bacterias. La presencia de estos exopolisacáridos se confirmó mediante inmunofluorescencia. Se obtuvieron 8 variantes no mucosas a partir de cepas mucosas, y dos variantes mucosas a partir de cepas no mucosas (Tabla 1).

Entre las 92 cepas bovinas y las 52 cepas ovinas testadas, 25 (26,6%) y 4 (7,5%), respectivamente, pertenecían al serotipo capsular 5 (Tabla 1). Sólo una cepa mucosa bovina (V50) pertenecía a este serotipo. Los anticuerpos frente al mucus parcialmente purificado y frente a la cápsula de la cepa A, obtenidos por inmunización en conejo, eran inmunológicamente diferentes (Fig. 2), lo cual sugiere que el mucus y la cápsula son estructuras diferentes a nivel inmunológico.

4.4. Resistencia de *Staphylococcus aureus* a los antibióticos: Diferencias entre las cepas mucosas y sus variantes no mucosas (Trabajo IV)

Utilizando placas microtiter, se observó que, tras 6 horas de crecimiento en Tryptone Soy broth (TSB) suplementado con glucosa, las cepas mucosas crecían formando pequeñas colonias, mientras que las variantes no mucosas tendían a ocupar todo el fondo del pocillo. Al adicionar antibióticos, no se encontraron diferencias respecto de la concentración mínima inhibitoria entre las cepas mucosas y sus variantes no mucosas, pero sí las hubo en cuanto a la concentración bactericida mínima, mostrando la mayoría de las cepas mucosas una mayor resistencia a determinados antibióticos en las altas concentraciones (Tabla 1). La totalidad de las cepas mucosas y de las no mucosas mostró una resistencia a los antibióticos mayor de lo normal (Tabla 1), probablemente debido a que se había permitido que las bacterias formaran una película.

4.5. Hidrofobicidad de *Staphylococcus aureus* aislados de mamitis, en relación con el envejecimiento, el origen (ovino o bovino) y la producción de mucus (Trabajo V)

Tras determinar la hidrofobicidad de *S. aureus* con xilol, se observó que ésta se incrementó durante la fase exponencial de crecimiento (Fig. 3). Considerando a las bacterias al final de esta fase, las cepas de aislamiento reciente resultaban ser más hidrofóbicas que las cepas viejas ($P<0,01$; cepas "rejuvenecidas", Tabla 1). Sin embargo, estas últimas exhibían una mayor hidrofobicidad después de un pase por la glándula mamaria del ratón ($P<0,01$; Tabla 1). Las cepas bovinas fueron más hidrofóbicas que las ovinas ($P<0,01$; Tabla 2). Como era predecible, la mayoría de las cepas eran más hidrofílicas después de crecer en medios inductores de exopolisacáridos (Columbia y m110), a excepción de las cepas capaces de producir mucus en ARC (Tabla 3).

4.6. Algunos aspectos a reseñar entre los resultados obtenidos

En conjunto, las investigaciones realizadas en esta Tesis suponen, por un lado, el estudio por vez primera de adherencia a células epiteliales y de hidrofobicidad en cepas de *S. aureus* aisladas de mamitis ovinas, permitiendo su comparación con las cepas aisladas de mamitis bovinas. Asimismo, en estos estudios, el mucus de *S. aureus*, ha sido identificado por vez primera mediante las técnicas de morfología en ARC y colonización de la pared en tubos de cultivo. Por otra parte, parece haberse confirmado la hipótesis de que el mucus y la cápsula muestran diferencias inmunológicas. Finalmente, se han obtenido variantes mucosas y otras no mucosas de distintas cepas, lo cual ha permitido estudiar por vez primera la resistencia a los antibióticos en cepas de *S. aureus* que sólo difieren en su capacidad de producción de mucus.

5. DISCUSION

5.1. Adherencia a células epiteliales

Durante el ordeño y el amamantamiento, la probabilidad de eliminación de bacterias aisladas no adheridas al epitelio en relación a las adheridas es muy alta (Bramley y Dodd, 1984). De hecho "in vivo", se ha observado que en ocasiones *S. aureus* aparece adherido al epitelio (Gudding y cols., 1984; Amorena y cols., 1991). Análogamente, "in vitro" diversas cepas de *S. aureus* de origen ovino muestran una capacidad de adherencia a las células epiteliales de la glándula mamaria ovina (Trabajo I). Esta adherencia es mayor para *S. aureus* que para el resto de los estafilococos testados, lo cual podría ayudar a explicar la mayor prevalencia y patogenicidad de este microorganismo. Además de estas diferencias entre especies, también se han encontrado diferencias entre cepas dentro de una especie, fenómeno observado anteriormente por otros autores en cepas aisladas de mamitis bovinas (Frost, 1975; Frost y cols., 1977; Wanasinghe, 1981; Bramley y Hogben, 1982; Opdebeeck y cols., 1988a; Lindhal y cols., 1989, 1990). Asimismo, las condiciones ambientales pueden influir en la adherencia (Trabajo I), ello sugiere la extremada precaución que debe adoptarse antes de extrapolar las observaciones sobre adherencia "in vitro" a los fenómenos "in vivo".

5.2. Aglutinación en suero lácteo

Las bacterias no sólo son capaces de interactuar con las células del hospedador presentes en la mama, sino también con los componentes solubles de la leche. Kapral (1966) observó que tras la inoculación intraperitoneal en ratón, las cepas no encapsuladas aglutinaban rápidamente (merced al fibrinógeno, según el autor). Más tarde, los agregados resultantes eran rodeados por PMN. Estas bacterias no podían multiplicarse ni liberar cantidades apreciables de toxinas en el medio, y por lo tanto, el ratón inoculado sobrevivía. En las cepas encapsuladas esta

aglutinación, no tenía lugar, las bacterias resistían la fagocitosis y, tras la liberación de las toxinas, el ratón moría.

Nuestros resultados (Trabajo II) muestran que, durante el crecimiento bacteriano, la agregación de las bacterias puede ser inducida cuando se suplementa el medio con suero lácteo ovino o bovino. En mamitis bovinas, Gudding y cols. (1984) observaron agregados de neutrófilos y de bacterias a las dos horas de la inoculación experimental, los cuales podrían ser fácilmente eliminados en el ordeño. Sin embargo, estos agregados no aparecían a las 18 horas. Es posible que las bacterias, adaptadas a las condiciones "in vivo", produjeran cantidades considerables de exopolisacáridos, lo cual impediría la aglutinación y la fácil eliminación durante el ordeño, favoreciendo así la supervivencia bacteriana dentro de la glándula mamaria.

Comparativamente, con el suero lácteo ovino y las cepas ovinas de *S. aureus* se produjo un mayor grado de aglutinación que con el suero lácteo bovino y las cepas bovinas (Trabajo II). Con respecto a las cepas, parece existir una correlación positiva entre la aglutinación producida durante el crecimiento en presencia de suero lácteo y la patogenicidad de las mismas. De hecho, todas las cepas testadas fueron obtenidas de casos clínicos de mamitis ovinas y subclínicos de mamitis bovinas (Trabajo II). Estas observaciones podrían atribuirse, entre otras causas, a la mayor citotoxicidad observada en los sobrenadantes (toxinas) de las cepas ovinas respecto de las bovinas (Amorena y cols., 1991). Obviamente, el efecto causado "in vivo" por las toxinas bacterianas en ovejas, sólo sería observable en aquellos casos en los que las bacterias no hayan sido eliminadas. Por ello estos resultados no son fácilmente conciliables con una generalización de la hipótesis de que las bacterias sean sistemáticamente eliminadas en el ordeño o en el amamantamiento.

5.3. Cápsula en *Staphylococcus aureus* aislados de mamitis

La producción de exopolisacáridos en forma de cápsula o de mucus por parte de la bacteria puede alterar las interacciones de las bacterias entre sí o con el hospedador. Respecto de la cápsula, Yoshida y Minegishi (1976) propusieron como criterio de encapsulación para *S. aureus* la formación de MCD (colonias en forma de cometa) en ASS, independientemente del medio utilizado previo a la realización del test en ASS (Trabajo VII). Con este criterio, los aislamientos de cepas encapsuladas de *S. aureus* son excepcionales en la clínina humana (Wilkinson, 1983) así como en la mamitis bovina (Yokomizo y cols., 1977). De acuerdo con las observaciones de estos autores, nuestro equipo no ha encontrado ninguna cepa con esta morfología entre las 125 cepas bovinas y las 80 cepas ovinas testadas (datos no presentados).

Karakawa y cols (1985), aplicando criterios exclusivamente inmunológicos para determinar la encapsulación de *S. aureus*, identificaron al menos 11 tipos antigénicos capsulares diferentes. Dos de ellos, los serotipos 5 y 8, representan alrededor del 70% de todos los aislamientos clínicos humanos (Sompolinsky y cols., 1985; Hockeppel y cols., 1987). Análogamente, la mayoría de las cepas aisladas de mamitis bovinas, ovinas y caprinas también pertenecen a estos serotipos (51,4%, 3,0% y 13,0% pertenecen al serotipo 5; y 18,0%, 75,8% y 68,5% al serotipo 8, respectivamente; Poutrel y cols., 1988). En el estudio realizado en esta Tesis (Trabajo VI), se ha observado asimismo que el número de cepas con serotipo 5 es mayor en bovinos que en ovinos (26,6% y 7,5% en bovinos y en ovinos, respectivamente).

5.4. Inducción de la MCD "in vitro"

Cuando las bacterias son inoculadas en ASS directamente de la leche mamítica bovina, la mayoría (85-100%) de las cepas de *S. aureus* muestran una MCD (Norcross y Opdebeeck, 1983; Opdebeeck y Norcross, 1983; Rather y cols., 1986; Opdebeeck y cols., 1988a). Sin

embargo, esta morfología se pierde rápidamente en subcultivo. Varios autores estiman que estas cepas no pueden considerarse como verdaderamente encapsuladas (Anderson, 1984; Jonsson y cols., 1989; Rather y cols., 1986).

Según Opdebeeck y cols. (1987), la MCD en ASS puede ser inducida "in vitro" mediante el subcultivo seriado de las cepas de *S. aureus* en m110 o en otros medios inductores de exopolisacáridos. Asimismo, Sutra y cols. (1990) observaron que un 85% de las cepas de *S. aureus* aisladas de leche bovina, ovina o caprina y pertenecientes a los serotipos 5 y 8 producían la MCD en ASS tras subcultivos en m110. Sin embargo, en ambos casos, la aparición de esta morfología dependía de mantener a las bacterias en bajo número y en la fase logarítmica de crecimiento. Jonsson y cols. (1989) también fueron capaces de inducir la MCD tras crecer las bacterias durante 6-10 horas en suero lácteo, pero no tras crecer las bacterias en m110 (Mamo y cols., 1991a).

Nosotros hemos observado, estudiando cepas jóvenes de *S. aureus* aisladas de mamitis de rumiantes (Trabajo V), que la mayoría de ellas (23 de 26) llegaron a ser hidrofílicas tras crecer en medios inductores de exopolisacáridos (m110 y Columbia). Ello podría sugerir una recuperación de los exopolisacáridos en dichos medios, ya que la hidrofilicidad bacteriana ha sido considerada como un criterio indirecto de capsulación en *S. aureus* (Jonsson y Wadström, 1983). Sin embargo, ninguna de las cepas mostró una MCD tras crecer en estos medios o en suero lácteo.

Tampoco pudo inducirse la MCD mediante el pase de las bacterias por la glándula mamaria de ratón (Trabajo V). Esta glándula ejerció, no obstante, un efecto "rejuvenecedor" sobre las cepas viejas, volviéndolas más hidrofóbicas. Este hecho podría ser debido a una recuperación de proteínas hidrofóbicas "in vivo" por parte de las cepas. Opdebeeck y cols. (1988b) encontraron que el pase a través de la glándula mamaria bovina de las cepas aisladas de mamitis bovinas reinducía la MCD, pero esta reinducción no ocurría cuando estas mismas cepas eran sometidas a pases a través de la glándula mamaria ovina o la cavidad peritoneal ovina. Los autores sugieren que quizá se trate de un fenómeno inherente a la especie.

Nuestros resultados y los de otros autores (Mamo y cols., 1991a) parecen indicar que la formación de MCD no es un criterio fiable para determinar la presencia de exopolisacáridos en *S. aureus* como resultado del cultivo en un medio específico, ya que depende de condiciones muy concretas de crecimiento (fase logarítmica y baja concentración bacteriana). Además, este criterio presenta el inconveniente de que debe ser aplicado tras cultivar una alícuota de la preparación bacteriana en un medio (ASS) diferente al estudiado en cada caso.

5.5. Evaluación de la producción de mucus

De acuerdo con Wilkinson (1983), el mucus es una estructura extracelular producida "in vitro", por la mayoría de las cepas de *S. aureus*, en respuesta a unas condiciones nutritivas concretas; por ejemplo, durante el crecimiento en m110. El mucus está débilmente unido a la pared celular y puede ser fácilmente eliminado durante el lavado de las bacterias (Caputy y Costerton, 1982; Wilkinson, 1983; Watson, 1989).

En esta Tesis, cuando se aplicaron para *S. aureus* los métodos normalmente utilizados para la detección del mucus en ECN (morfología colonial en ARC y colonización de las paredes del tubo de cultivo), sólo 11 de las 92 cepas bovinas (12%) y 10 de las 52 cepas ovinas (19%) testadas fueron capaces de producir mucus (Trabajo VI). Estas cepas mucosas presentaron una matriz de exopolisacáridos condensada alrededor de las células según se evidenció por microscopía electrónica e inmunofluorescencia (Trabajo VI).

Curiosamente, estas cepas, calificadas de mucosas en ARC, no mostraron una superficie hidrofílica tras crecer en otros medios productores de exopolisacáridos (Trabajos VI y VII). Para explicar este

hecho no resulta imprescindible asumir la existencia de dos mecanismos diferentes para la inducción de mucus en ARC y en otros medios, sino que puede explicarse si asumimos que el mucus de *S. aureus*, contrariamente a la cápsula (Dassy y cols. 1991), al ser una estructura débilmente unida a la pared bacteriana, se ha perdido en los lavados, resultando en un comportamiento bacteriano hidrofóbico. Hoght y cols. (1983) obtuvieron resultados similares con cepas mucosas de ECN.

Los resultados presentados en el Trabajo VI sugieren la existencia de diferencias inmunológicas entre el mucus y el antígeno capsular aislado de la cepa A. Estas observaciones coinciden plenamente con las realizadas por Caputy y Costerton (1982, 1984). Así, tras la infección experimental en pulmón de conejo, al igual que tras el crecimiento bacteriano en m110, Caputy y Costerton (1982) encontraron una matriz extensa que rodeaba a la superficie bacteriana tanto en el caso de la cepa Smith, encapsulada, como en el de la cepa Willey, no encapsulada. Este mucus agrupaba a las bacterias en microcolonias y era extracapsular en el caso de la cepa Smith; además, este mucus difería inmunológicamente de la cápsula de la cepa Smith (Caputy y Costerton, 1984). También Yoshida y Ekstedt (1968) concluyeron que determinadas cepas podían producir ciertos exopolisacáridos diferentes a la cápsula en respuesta a condiciones nutritivas específicas (m110).

Según Caputy y Costerton (1982, 1984), la matriz exopolisacarídica de *S. aureus*, también llamada glicocáliz, incluye ambas estructuras (cápsula y mucus). Así, la cepa Smith, encapsulada, muestra estas estructuras "in vivo" y tras crecer en m110, mientras que la cepa Willey, no encapsulada, sólo contiene mucus. De acuerdo con esta hipótesis, se ha descrito en esta Tesis una cepa bovina (V50) capaz de producir mucus en ARC y, simultáneamente, el antígeno capsular 5 (Trabajo VI).

5.6. Obtención de variantes mucosas

Christensen y cols. (1987, 1990) obtuvieron "in vitro" tanto variantes mucosas como no mucosas de ECN y sugirieron un mecanismo de "variación de fase" para explicar el proceso. De acuerdo con estos autores, la expresión de los genes implicados varía rápidamente y en una forma reversible de generación en generación. Nuestros resultados "in vitro" (Trabajo VI) son al menos parcialmente compatibles con esta hipótesis, ya que las variantes (tanto las mucosas y como las no mucosas) fueron fácilmente obtenidas.

Los métodos de obtención de variantes aplicados en esta Tesis son reflejo del tipo de selección aplicada, dependiente del ambiente utilizado en cada caso. Así, para seleccionar las variantes mucosas a partir de una población bacteriana, se han sometido a subcultivos aquellas colonias excepcionales que dentro de una población bacteriana, en conjunto no mucosa según el criterio de Christensen (1982, 1985), eran capaces de adherirse a las paredes del tubo de cultivo (es decir, eran mucosas).

Al contrario, para la obtención de variantes no mucosas, se han seleccionado entre las colonias de cepas típicamente mucosas en ARC, aquellas colonias que se comportaban como no mucosas.

Es probable que este último tipo de selección ocurra en forma de selección natural cuando las bacterias se cultivan en los medios comúnmente utilizados "in vitro" y que en dichos casos las bacterias lleguen a perder el exopolisacárido mucoide (Johne y cols., 1989; Watson, 1989). Este proceso selectivo sería explicable ya que, según indican Lam y cols. (1980), las variantes no mucosas pueden tener en dichos medios un crecimiento más rápido. En dicha situación, un pequeño número de bacterias no mucosas dentro de una población bacteriana, puede dar lugar en definitiva a una población no mucosa.

5.7. Formación de microcolonias y resistencia a antibióticos

En los procesos infecciosos, la mayoría de las bacterias forman microcolonias que se adhieren a los tejidos. Supuestamente, después de la adhesión inicial a los tejidos, las bacterias crecen rodeadas de exopolisacáridos dentro de la microcolonia, uniéndose de forma irreversible a los tejidos en esta segunda fase (Mayberry-Carson y cols., 1986; Costerton y cols., 1987). De acuerdo con esta hipótesis, Chan y cols. (1982) sugieren para *E. coli* un mecanismo de adherencia al epitelio intestinal en el que las fimbrias son las responsables de la adherencia inicial, mientras que los exopolisacáridos "capsulares" son responsables de la formación de microcolonias, dentro de las cuales se multiplican las bacterias. De este modo, la bacteria, inicialmente libre de exopolisacáridos, puede unirse fácilmente a las superficies celulares a través de las fimbrias. Sin embargo, en este estado, las bacterias son altamente susceptibles a la fagocitosis o a la aglutinación mediada por anticuerpos. Posteriormente, tras la adherencia inicial la bacteria se protege de la fagocitosis mediante la elaboración de exopolisacáridos (Mackie y cols., 1979).

Un mecanismo similar podría aplicarse a *S. aureus*. De hecho, Rydén y cols. (1987 1989) identificaron una sialoproteína ósea, que actuaba como receptor específico para las cepas de *S. aureus* aisladas de casos de osteomyelitis. Por otro lado, se ha descrito la implicación del mucus en la adherencia de *S. aureus* al cartílago y al hueso, así como en la formación de microcolonias en afecciones osteomielíticas (Mayberry-Carson y cols., 1984; Speers y Nade, 1985; Power y cols., 1990).

En el caso de la mamitis, *S. aureus* podría adherirse a los conductos glandulares mamarios y alvéolos mediante la unión a las proteínas basales de la membrana (Mamo y cols., 1988) o a través de la unión a las células epiteliales (Frost, 1975; Frost y cols., 1977; Wanasinghe, 1981; Bramley y Hogben, 1982; Opdebeeck y cols., 1988a; Lindhal y cols., 1989, 1990; Trabajo I), para posteriormente formar microcolonias.

En el caso de que el mucus interviniera en la adherencia, no está claro si funcionaría como una adhesina en la adherencia inicial de las bacterias al epitelio, o si sólo se produciría cuando las bacterias, una vez adheridas, experimentan un estrés metabólico (Karakawa y Kane, 1972; Terry et al., 1991). Nuestros resultados sugieren la posibilidad de que el mucus puede actuar como una adhesina para las células epiteliales (Iturralde y cols., 1991).

Muchas infecciones crónicas implican un crecimiento bacteriano en forma de microcolonias adherentes (Brown y cols., 1988), dentro de las cuales las bacterias no son susceptibles a la fagocitosis por macrófagos o por neutrófilos (Lam y cols., 1980). Estas colonias forman focos de inflamación celular y humoral que normalmente son lo suficientemente eficaces como para eliminar las células bacterianas individuales liberadas de la superficie de la microcolonia. Sin embargo, los mecanismos de defensa pueden fallar en algunas ocasiones, especialmente en animales estresados o débiles, donde las bacterias liberadas pueden diseminarse y ejercer un papel patogénico (Costerton y cols., 1987). Este fenómeno podría explicar algunos casos de mamitis crónicas por *S. aureus*.

El crecimiento dentro de estas microcolonias también podría explicar la ineficacia del tratamiento antibiótico en animales con mamitis crónicas, dada la dificultad de los antibióticos en alcanzar concentraciones bactericidas en el interior de las microcolonias (Marrie y cols., 1982; Brown y cols., 1988). De hecho, hay una correlación entre la producción de exopolisacáridos "in vitro" por estreptococos aislados de vegetaciones en conejos con endocarditis y el fallo para erradicar la infección con antibióticos (Dall y Herndon, 1989). En pacientes con prótesis, Davenport y cols. (1986) encontraron que la curación de la infección por ECN mediante el tratamiento antibiótico tuvo lugar en el 100% de los casos asociados a infecciones por cepas no productoras de mucus y tan sólo en el 32% de las infecciones causadas por cepas productoras de mucus. Nuestros resultados en *S. aureus*, obtenidos por el método diseñado en el trabajo IV para medir la sensibilidad a antibióticos, también indican una mayor resistencia a los antibióticos por parte de las cepas mucosas respecto de sus variantes no mucosas, aspecto de aplicación práctica para estudiar la eficacia de nuevos fármacos.

5.8. Hidrofobicidad

A nivel de poblaciones de bacterias indidualizadas, el hecho de que las propiedades hidrofóbicas de la bacteria varíen según el medio de cultivo y la fase de crecimiento bacteriano (Trabajo V) es explicable, ya que ello puede reflejar cambios metabólicos que afecten a la superficie bacteriana. Sin embargo, esta variación debe tenerse en cuenta a la hora de extrapolar las observaciones "in vitro" a una situación "in vivo" y a la hora de cotejar los resultados de los distintos autores en el tema de la hidrofobicidad.

En cualquier caso, nuestros resultados muestran que la mayoría de las cepas bovinas (58%) y una minoría de cepas ovinas (18%) son hidrofóbicas cuando crecen en un medio normal de laboratorio (NB). Se desconoce si estas diferencias entre cepas bovinas y ovinas está relacionada con la capacidad de aglutinación en presencia de suero lácteo (Trabajo II), con las diferencias observadas respecto de la virulencia (Amorena, 1991) o con la cantidad de exopolisacárido capsular. Estos dos últimos aspectos pueden además relacionarse entre sí a nivel fisiológico, ya que diversos trabajos muestran que la presencia de cápsulas (hidrofílicas) incrementa la virulencia bacteriana, lo cual se atribuye a una mayor resistencia a la fagocitosis (Yokomizo y cols., 1977; Yoshida y cols., 1979; Karakawa y cols., 1985).

6. CONCLUSIONES

1. Existen diferencias entre especies bacterianas aisladas de mamitis ovinas y entre cepas dentro de cada especie en cuanto a su capacidad de adherencia a células epiteliales de la glándula mamaria ovina. La temperatura, el pH y el tiempo de incubación pueden influir en el grado de adherencia.

2. El suero lácteo ovino tiene una mayor capacidad de inducir la aglutinación durante el crecimiento bacteriano que el suero lácteo bovino respecto del número de cepas ovinas y bovinas aglutinadas. Las cepas de *S. aureus* de origen ovino estudiadas aglutinan más fuertemente que las de origen bovino.

3. Algunas cepas de *S. aureus* aisladas de mamitis ovinas y bovinas, son capaces de producir mucus en ARC. Este mucus de *S. aureus* ha sido evidenciado por microscopía electrónica y parece ser inmunológicamente distinto a la cápsula.

4. Las cepas de *S. aureus* que se comportan como mucosas en ARC, son capaces de resistir concentraciones más altas de antibióticos que sus variantes no mucosas.

5. La mayoría de las cepas de *S. aureus* testadas se vuelven más hidrofílicas tras crecer en medios inductores de exopolisacáridos (m110 o Columbia). Sin embargo, la totalidad de las cepas mucosas en ARC se comportan de una forma hidrofóbica, después de crecer en medios inductores de exopolisacáridos (m110 o Columbia). Las cepas analizadas de origen ovino son más hidrofílicas que las de origen bovino.

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Factors influencing the degree of "in vitro" bacterial adhesion to ovine mammary gland epithelial cells.

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Factors influencing the degree of in vitro bacterial adhesion to ovine mammary gland epithelial cells

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ABSTRACT

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Bacterial adhesion to mammary gland epithelial cells (EC) may play a role in the pathogenesis of mastitis. In vitro adherence systems have been developed to study mastitis in cattle but little has been done in sheep. In this work, a method is described for obtaining mammary gland cell preparations containing ≥65% EC from live or dead ewes, using a Ficoll–Hypaque flotation method (cell viability=70–90%). An in vitro adhesion assay procedure was also developed to study the interaction between EC and ovine mastitis bacterial strains. It was observed that, under the test conditions, adherence increased as the incubation time was prolonged from 30 to 120 min ($P<0.05$). Adhesion was greater at incubation temperature of 37°C than at 22°C ($P<0.001$). An acidic pH (5.9) was associated with an increase in adhesion, when compared with a higher pH (7.2; $P<0.05$). Tween 20, Tween 80 and bovine serum albumin helped to eliminate a background of unbound bacteria from the test slides, but they also inhibited adhesion to some strains. Strain differences in adhesion and in ability to form a background were also observed. Some of these findings may have in vivo implications.

INTRODUCTION

Bacterial adhesion to mammary gland epithelial cells (EC) appears to play a role in the pathogenesis of mastitis (Frost, 1975; Frost et al., 1977; Wanasinghe, 1981), although this observation has not always been confirmed (Anderson, 1978). Various in vitro assays have been described for evaluating the degree of bacterial adhesion to mammalian cells, such as those of Frost (1975), Bramley and Hogben (1982), Faris (1985) and Opdebeeck et al. (1988). This adhesion may be influenced by pH (Kubín et al., 1983a), incubation time and temperature (Kubín et al., 1983b). The rate of adhesion also depends on the bacterial species used (Wanasinghe, 1981) and on the anatomical source of the epithelium (e.g. buccal vs. vaginal; Kubín et al., 1983a, b), including different regions within the mammary gland epithelia (teat sinus vs. lactiferous sinus; Frost et al., 1977).

EC obtained by gentle brushing of the teat duct (Grier and Paape, 1986) often show aggregates, cellular debris, dead cells and other cell types, which make evaluation of the EC adhesion test difficult. Little work has been done on in vitro adhesion in sheep involving bacteria and EC. This paper describes a method for obtaining preparations of ovine mammary gland epithelial cells from live and dead animals, and reports the effects of pH, temperature, incubation time, detergents and albumin on the degree of adhesion of these cells to ovine mastitis bacteria, using an in vitro assay.

MATERIAL AND METHODS

Animals

Mastitis-infected animals of the Aragonese breed were used as the source of bacteria. Mammary glands from live, healthy, dry ewes of this breed and from slaughtered dry and lactating ewes were used for cell collection.

Bacteria

Seventeen ovine mastitis bacterial strains were studied. Of these, nine were *Staphylococcus aureus* (1A, 2A, 4A, 5A, 16A, 19A, 24A, 25A and 26A), one *S. chromogenes* (3A), two *S. hyicus* (6A and 10B), one *S. xylosus* (12A), one *S. intermedius* (11A) and three *Escherichia coli* (7A, 9A and 10A). Bacteria for adhesion tests were cultured for 18–24 h at 37°C in Todd–Hewitt broth, washed twice and resuspended in PBS (pH 6.65).

Mammary gland cells

Mammary gland cells were obtained from the teat sinus epithelium of live and dead animals, using a modification of the method described by Grier and Paape (1986). In brief, after discarding the gland's secretions, the sinus epithelium was washed twice with 20 ml PBS per wash. Following a third introduction of PBS (20 ml) and gentle brushing of the epithelium using a soft nylon 0.3×3 cm brush introduced with the help of a 40/60 cannula, cells were recovered and incubated for 20 min (22–24°C) in PBS containing carbonyl iron (0.5–0.6 mg ml^{-1}), and layered on a Ficoll–Hypaque gradient (D=1.075). Upon centrifugation (7 min, 700×g) and two washes in PBS (10 min, 700×g), cell viability was tested by eosin exclusion (70–90% live cells), using 1–2 μl cell suspension per sample. The flotation method described eliminated many dead cells, non-cellular artifacts and large clumps of cells. Cells in suspension were submitted to mild fixation (1% paraformaldehyde, 15 min, 4°C), centrifuged and resuspended at a concentration of 10^6 cells ml^{-1} PBS, (ethanol–acetic fixing solution was not used at this stage since clumps would be formed). A drop of this suspension was spread on glass slides, previously cleaned with ethanol–ether. Cells were air-dried, submitted to further fixation in ethanol–acetic acid (99:1) for 15 min at 22–24°C, and stored at 4°C for up to 4 weeks until used. The preparations contained EC as well as three other major types of cells: macrophages, neutrophils and lymphocytes. The ratio of epithelial cells to macrophages among large cells (10–45 μm) was estimated by applying a sideroleukocyte-specific stain procedure (Schalm et al., 1975) to cell spreads on slides; the cytoplasm of epithelial cells was stained red, whereas macrophages had blue cytoplasmic inclusions. Neutrophils and lymphocytes were easily identified by nuclear shape and size. The proportions of the four major cell types varied between samples. Samples with ≥65% EC were commonly found, and were used for adhesion tests. All of the cell preparations used for adhesion tests were free of bacteria, as determined by Giemsa staining and microbiological tests. When cells were obtained from live animals on more than one occasion, samples were obtained once a month.

Adhesion assay

The adhesion assays used the method of Krovacek et al. (1987) with modifications (Amorena et al., 1989). Under slow agitation, cell-carrying slides (8) were incubated with 50 ml bacterial suspension using Hellendahl's staining boxes of 100 ml capacity. The effects of bacterial concentration, incubation time, temperature, pH and detergents were investigated. The slides were subsequently washed ten times under agitation (5 min per wash) in the containers with 50 ml of the medium chosen for the assay, fixed with methanol and stained with a sideroleukocyte-specific stain (Schalm et al., 1975). Tests were done in triplicate. The degree of adhesion was evaluated from the ratio of attached bacteria to adherent cells in the sample, determined from microscopical observations of 100–150 epithelial cells per slide.

Statistical analysis

The data were analysed by paired t tests. Analysis of variance was used to study the effects of pH. Data from different animals were pooled since no significant differences were found between them.

RESULTS

Effect of temperature and incubation time

To determine the effects of temperature and incubation time on adhesion, tests were done in saline (pH 7.2) at a concentration of 5×10^7 bacteria ml^{-1}. As illustrated in Table 1, a higher assay temperature (i.e. 37°C vs. 22°C) was associated with a greater ($P<0.001$) degree of adhesion. Similarly, a longer incubation period (120 vs. 60 or 30 min) favored adhesion ($P<0.05$). Both effects were observed with regard to the percentage of cells showing adherence to bacteria (adherent cells). The average number of bacteria adhering

TABLE 1

Effect of temperature and incubation time on the degree of adherence of *Staphylococcus aureus* (strain 1A)

Time (min)	Temperature (°C)	% Adherent EC ±s.e.
30	22	21±2.12
	37	45±2.82
60	22	26±2.15
	37	50±9.9
90	22	16±3.53
	37	84±6.36
120	22	53±4.24
	37	88±1.41
120	37 (control)[1]	0.0

[1]No bacteria were added.

to individual epithelial cells ranged from 1.5 to 4.8 in this experiment, and did not vary consistently when incubation temperature or time was changed.

Effect of pH

To estimate the effects of the presence of phosphate ions and hydrogen ion concentration of the assay medium on adhesion, tests were done using as media either PBS or physiological saline at three different pH values (5.9, 6.65 and 7.2; Table 2). Tests were carried out at 37°C (120 min; 1×10^8 bacteria ml^{-1}). Adherence appeared to be lower for higher pH values (although P was <0.1 when comparing 7.2 vs. 5.9 in the paired t-test, it was <0.05 in analysis of variance, when separating the effects of different variables). Since the pH of milk is close to 6.65, this pH value was used in the remaining experiments, in order to reproduce physiological conditions as closely as possible in vitro. As a rule, PBS was not detrimental to adhesion when compared with saline and, since it helps maintain a constant pH, it was used throughout the remaining experiments.

Effect of bacterial concentration

Adhesion tests were carried out using different bacterial concentrations (10^6, 10^7, 10^8 and 10^9 bacteria ml^{-1})under otherwise constant test conditions (PBS, pH 6.65, 37°C, 120 min). The experiments were done using cells from sheep Nos. 15 and 20 and bacterial strains 1A and 6A. It was observed that an increase in bacterial concentration in the assay resulted in an increment in the percentage of cells to which bacteria were adhering, number of

TABLE 2

Effect of pH on adherence of two bacterial strains to EC from different ewes

Strain	Medium	pH	Adherence* ± s.e.
1A	Saline	5.9	0.99±0.11
		6.65	2.27±0.07
		7.2	0.33±0.12
1A	PBS	5.9	8.48±3.27
		6.65	3.41±1.60
		7.2	3.39±2.26
6A	Saline	5.9	2.29±0.22
		6.65	1.24±0.03
		7.2	0.89±0.12
6A	PBS	5.9	1.49±0.82
		6.65	1.42±0.49
		7.2	0.84±0.56

*Number of adherent bacteria / total number of celle

bacteria that adhered to a given cell, number of dispersed bacteria in the background, and number of bacterial aggregates in the background.

Effects of detergents and bovine serum albumin (BSA)

Since eliminating the bacterial background helps in the evaluation of the specific adhesion of bacteria to EC, the effects of detergents (Tween 20 and 80) and BSA in the test medium were studied. Several staphylococcus strains were used in the study, at two concentrations (1×10^7, 1×10^8 ml^{-1}) in PBS at pH 6.65. Tests were done at 37°C and 120 min incubation. A bacterial concentration of 1×10^7 yielded results which were difficult to evaluate, because low adherence was found in the presence of a low bacterial background. A difficulty was also found when an exceedingly high number of bacteria (1×10^9 ml^{-1}) was used, due to the presence of a dense background. Hence, a concentration of 1×10^8 yielded the most informative results.

Bacterial background and adherence were reduced by BSA, Tween 20 and Tween 80. Although the experiments were aimed at obtaining high adherence and low background, only intermediate adherence was reached when the background was low (i.e. when using 0.3% BSA during incubation and washing, or when using 0.3% BSA during incubation and 0.05% Tween 20 during washing; this second choice was considered less expensive and was therefore adopted for further tests). Tween 20 and Tween 80 produced similar effects. The efficacy of detergents in eliminating the background differed among strains (e.g. the strain 1A background was more readily removed than that of

TABLE 3

In vitro adherence of *Escherichia coli* and *Staphylococcus aureus* strains to sheep mammary gland epithelial cells

Sheep no.	*Escherichia coli* strain						*Staphylococcus aureus* strain					
	7A		9A		10A		2A		4A		5A	
	% A.C.[1]	Bcgd.[2]	% A.C.	Bcgd	% A.C.	Bcgd.	% A.C.	Bcgd	% A.C.	Bcgd.	% A.C.	Bcgd
50	–	–	50–95	++	40–50	+	10–19	+	10–19	(+)	1–4	(+)
51	–	–	50–95	++	40–50	+	10–19	+	10–19	(+)	1–4	(+)
52	–	–	50–95	++	40–50	+	5–9	(+)	1–4	(+)	–	–
53	–	–	50–95	++	40–50	+	20–39	(+)	10–19	+	–	–
54	–	–	50–95	++	40–50	+	5–9	(+)	1–4	–	–	–
55	–	–	50–95	+	20–39	(+)	1–4	–	5–9	–	–	–
56	1–4	(+)	50–95	+	20–39	+	1–4	–	1–4	(+)	–	–
57	1–4	(+)	N.D.[3]	N.D.	20–39	+	1–4	–	1–4	(+)	N.D.	N.D.

[1]Percentage ranges of cells showing adherence (A.C.) (3–9 slides/test per combination)
[2]Presence of bacterial background (Bcgd.): – = no background; (+) = very weak background; + = intermediate background; ++ = strong background; +++ = very strong background (not observed in these results). Only dispersed bacteria were considered for estimating the degree of background
[3]N.D. = not determined

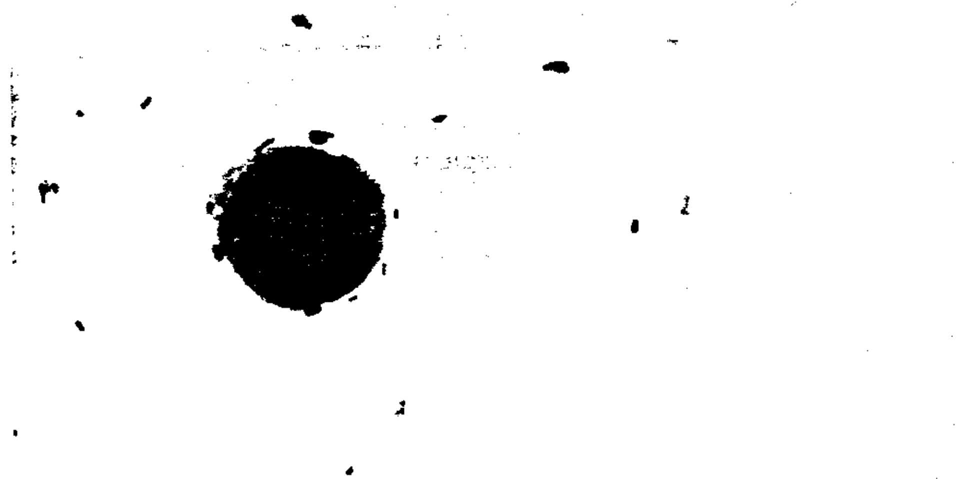

Fig. 1. In vitro adherence of *Escherichia coli* strain 9A to an ovine mammary gland epithelial cell

2A). Efficacy was low for detergents and for BSA when these chemicals were applied only during the washing period of the tests and not during the incubation period.

Strain differences

To further search for differences in adhesion within and between bacterial species, assays were performed involving *E. coli* (strains 7A, 9A and 10A), *S.*

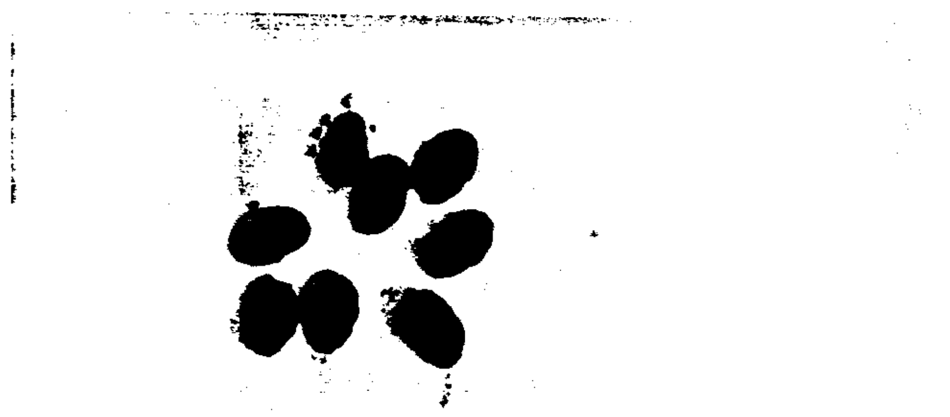

Fig. 2. In vitro adherence of *Escherichia coli* strain 9A to ovine mammary gland epithelial cell clumps (partially disrupted tissue).

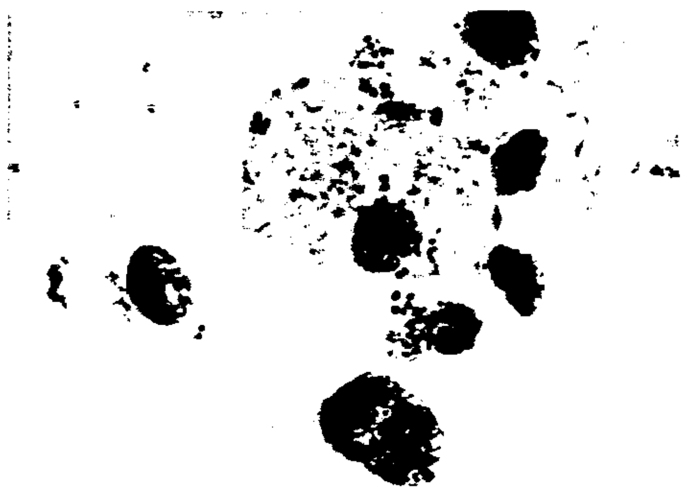

Fig. 3. In vitro adherence of *Staphylococcus aureus* strain 2A to a group of ovine mammary gland epithelial cells

aureus (strains 2A, 4A and 5A), *S. hyicus* (strain 10B) and *S. chromogenes* (strain 3A). Tests were done using 0.3% BSA in PBS as the incubation medium and 0.05% Tween 20 in PBS as the washing medium. Incubation was for 120 min at 37°C and at pH 6.65.

The results obtained with *S. aureus* and *E. coli* strains are illustrated in Table 3. In general, a higher background intensity corresponded to a higher degree of adhesion (percentage of adherent cells). High adhesion (50–95%

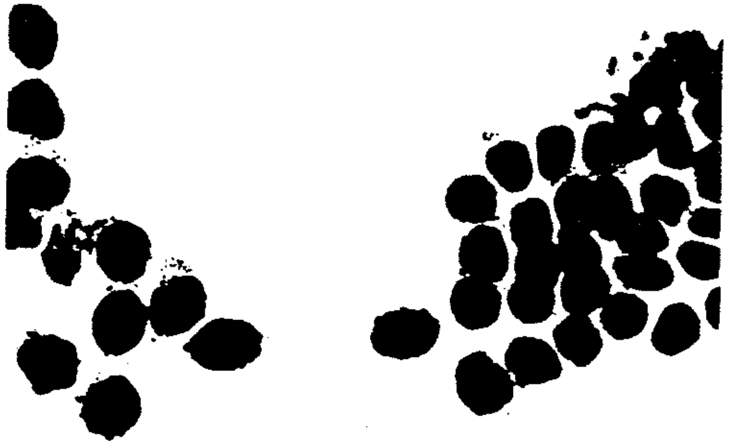

Fig. 4. In vitro adherence of *Staphylococcus aureus* strain 2A to ovine mammary gland epithelial cell clumps (partially disrupted tissue).

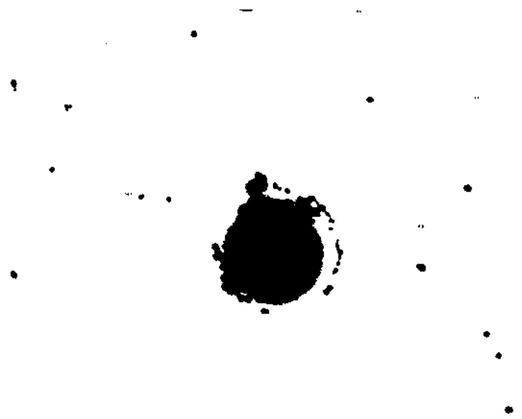

Fig. 5. In vitro adherence of *Staphylococcus aureus* strain 4A to an ovine mammary gland epithelial cell

adherent cells) and background intensity (+ +) was found for *E. coli* strain 9A (Figs 1 and 2), followed by strain 10A of this species and by *S. aureus* strains 2A and 4A (Figs. 3, 4 and 5). *E. coli* strain 7A, *S. aureus* strain 5A, *S. hyicus* strain 10B and *S. chromogenes* strain 3A showed low adhesion (1–9%), detectable only with cells from a proportion of the individuals tested, and with a very weak or undetectable background.

DISCUSSION

A method is described for studying bacterial adhesion to sheep EC. These cells probably have stronger adhesion properties than cultured cells, according to observations in cattle (Opdebeeck et al., 1988). EC donors can be sampled once a month, without causing any harm to the animal. This is advantageous for repeatability and genetic studies. Several methods have been suggested whereby macrophages in the preparations may be distinguished and/or eliminated (electron microscopy, use of antimacrophage serum, etc; McDonald and Anderson, 1981) and to identify EC (antikeratin antibodies; McGrath, 1987). These methods are expensive and laborious for routine work. The method described in this paper is practical and inexpensive, and the epithelial cells are easily recognized.

It may still seem that it would be more convenient to use milk as a source of EC. This procedure has been tried in our laboratory using simple centrifugation techniques as well as the Ficoll–Hypaque flotation method described, after removing the fat. The cells obtained were of various sizes (including leukocytes). EC were often degenerated. This was expected, considering that EC in milk represent an aged cell population that is constantly being renewed. Hence, milk was not considered an ideal source of EC for our studies.

All the factors tested in this work can be regarded as possibly affecting the degree of adhesion. The degree of in vitro adhesion (number of bacteria per adherent cell) increases with increasing incubation period. Whether this reflects the progress of an in vivo situation at the onset of infection is unknown. This increase in adhesion with time has been observed in bacteria–epithelial cell combinations involving several bacterial and mammalian species, although there are cases where maximum adherence is observed within 10 min (Kubín et al., 1983b).

The hydrogen ion concentration also affects adherence, acidic conditions favoring adhesion. A pH value of 5.9 yielded enhanced adhesion compared with pH 7.2. A similar effect has been observed in the adhesion of streptococci to human buccal epithelia, but an opposite situation has been found in the case of vaginal epithelia (Kubín et al., 1983b). Each system therefore appears to have an optimal pH for adhesion. It is known that mastitis is often associated with an increase in pH in milk (0.3 units on average; Walstra and Jennes, 1984), as a result of altered permeability within the udder and the consequent transfer of ions to the milk. We have verified this increase in sheep. This may suggest, in view of our in vitro observation on pH, that during the course of mastitis in vivo, a natural defence mechanism may take place, consisting of a reduction in the adhesion efficiency of bacteria and an increase in pH.

The presence of a bacterial background on the in vitro test slides makes the evaluation of bacterial adhesion to EC difficult. Detergents and BSA have been used in enzyme-linked immunosorbent assays to avoid non-specific binding (Chandler et al., 1986; Faris et al., 1987). Although both agents reduce the bacterial background, they also inhibit adhesion to some extent. The fact that different bacterial strains (1A vs. 2A) show different sensitivity to detergents suggests that strains may differ in the nature of their cell surface.

In cattle, Opdebeeck et al. (1988) succeeded in removing bacterial background and facilitating the interpretation of adhesion tests by labelling bacteria with radioactivity, filtering out unbound bacteria and scoring the radioactive counts of bacteria attached to cells. Whereas in our test the radioactive labelling of bacteria would not allow the scoring of attachment to be restricted to the epithelial cells, elimination of non-specific background by filtration may help to demonstrate the possible specific mechanisms of adherence, which is known to exist in other bacterial infections of epithelium (Faris, 1985; Rapacz and Hasler-Rapacz, 1986). However, the question arises as to whether the tendency of each particular strain to form a background reflects a corresponding in vivo tendency during colonization and subsequent infection. If this were the case, evaluation of the background intensity shown by each bacterial type would yield informative results. Whether this tendency is related to other bacterial properties, such as surface charge, hydrophobicity, encapsulation, the ability to colonize the mammary gland, and pathogenesis, is under study.

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II

Milk whey induction of agglutination in ovine and bovine mastitis *Staphylococcus aureus*.

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Short Communication

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Milk Whey Induction of Agglutination in Ovine and Bovine Mastitis *Staphylococcus aureus*

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With 2 tables

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Summary

A total of 59 mastitis staphylococcic strains were tested for growth agglutination upon supplementation of growth media with ovine and bovine milk whey and mammary secretions from dry cows. Differences were observed when comparing bacterial species or origins (ovine vs. bovine) of bacteria and whey. All of the ovine and bovine *S. aureus* strains tested, but only 4 among 22 other ovine mastitis staphylococcic strains, showed growth agglutination in Todd Hewitt broth (THB) supplemented with ≥ 30 % (v/v) ovine milk whey. None of the strains agglutinated during growth in regular THB medium. Ovine whey had an agglutination induction capacity higher than bovine whey ($P < 0.005$), concerning the number of responsive ovine and bovine *S. aureus* strains. There were no differences between whey samples from different ewes with regard to their capacity to induce agglutination. Ovine *S. aureus* strains were more responsive than bovine strains of this bacterial species, concerning the number of responsive strains ($P < 0.001$) to bovine whey (≥ 30 % in THB), the proportion of responsive strains at low (10 %) ovine whey concentrations ($P < 0.001$), and the strength of reaction (precipitation timing and clump sizes). Secretions from dry cows systematically induced agglutination in all of the bovine and ovine *S. aureus* strains tested.

An important component in the defense of mucosal surfaces is the prevention of colonization by bacterial agglutination, increasing in this manner the ratio of clearance (14). These observations may have implications on mastitis, considering that the development of intramammary infection involves the penetration and attachment of pathogenic organisms to the gland (3, 4, 5, 11), where they have a big chance of removal during milking before they become established (1). SPENCER et al. (10), found agglutinative growth of *S. aureus* M1525 strain in bovine milk whey and suggested that the putative agglutinins are *S. aureus* species specific rather than strain specific and are correlated with the presence of alpha antihemolysin antibodies.

The purpose of this work is to ascertain whether milk whey from ewes and dry cow's mammary secretions, in concentrations high enough to resemble "*in vivo*" conditions (≥ 30 % v/v), have also an agglutinative effect on mastitis staphylococcic bacteria during

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growth and whether there are growth agglutination differences when comparing whey and staphylococcal strains and species from ovines and bovines

Material and Methods

Strains of *Staphylococcus aureus* (21), *S. hyicus hyicus* (6), *S. hyicus chromogenes* (7), and *S. xylosus* (9), were isolated from ewes with clinical mastitis (gangrenous or chronic). Also, 16 *S. aureus* strains, isolated from cows with subclinical mastitis, detected by somatic cell count, were used. Isolation and identification of bacteria were carried out at the Microbiology Lab, Veterinary Faculty, Zaragoza, Spain.

Bacteria were grown at 37 °C for 18–24 hours in Todd-Hewitt broth (THB) or in modified *Staphylococcus* medium No. 110 (m 110), as previously described (7), and in THB supplemented with ovine or bovine milk whey (THB-OW and THB-BW, respectively), sterilized with a 0.22 µm filter. Media were classified as THB-5 %, THB-10 %, and THB-30 %, where percentages indicate whey concentrations. When the strains grown as described above failed to change their agglutinative properties or showed inconsistent results upon retests, they were passaged twice (120 minutes/passage, 37 °C) through the medium used in each case, using 1 ml broth and a transfer inoculum of 10 µl. With these passages, consistent results were obtained.

Milk samples were obtained from various healthy lactating animals of each particular species (ovines or bovines) and used for isolation of whey as a pool, unless otherwise stated. After delipidizing the milk, casein was removed by precipitation (pH 4.5) with acetic acid. Following centrifugation, the pH of the supernatant was restored to 7.2 with sodium hydroxide. Storage was done at −28 °C for up to two months. Floculated material was removed by centrifugation before use. Secretions from dry cows (having been approximately 3 years in the dry period) were pooled and centrifuged to remove floculated material. Supernatants were stored at −28 °C for up to two months.

Reactions of agglutination were macroscopically observed, directly after bacterial growth. Twenty five microlitres of bacterial suspension (5×10^8 bacteria/ml) were mixed with 25 µl of staining solution (2 % crystal violet in 0.002 M PBS) on excavated slides.

Statistical comparisons were made with contingency tests, applying the Yate's correction.

Results

To investigate whether the growth medium by itself or the acidification procedure applied for whey isolation, affected bacterial agglutinability, regular THB medium and THB medium after acidification (pH 4.5) with acetic acid and subsequent reconstitution to pH 7.2, were used for long term (18–24 h) bacterial growth. Neither the ovine -43-, nor the bovine -16- strains agglutinated in these media. Two hour passages of bacteria through these media before the long-term growth incubation did not affect these results. Similarly, m 110 did not induce agglutination in any of the ovine -21- or bovine -16- *S. aureus* strains tested in this medium. When studying the effect of whey, all of the ovine-strains-37 analysed in this test showed growth agglutination in THB-30 % OW (Table 1), but the majority of strains -18/22- from other ovine Staphylococcic species did not show agglutination ($P < 0.001$ for difference between *S. aureus* and other Staphylococcic strains), with

Table 1. Agglutination* of Staphylococcic bacteria during growth in different media: Effect of ovine milk whey

Species	No. of strains	Origin	Growth medium THB	THB-30 % OW
S. aureus	21	ovine	−	+
	16	bovine	−	+
S. hyicus	6	ovine	−	−
S. chromogenes	5	ovine	−	−
	2	ovine	−	+
S. xylosus	7	ovine	−	−
	2	ovine	−	+

* + = Presence of agglutination − = Absence of agglutination

Table 2. Proportion of agglutinative *S. aureus* strains after growth in THB supplemented with ovine whey (OW), bovine whey (BW) or mammary gland secretions from dry cows (SC) at various concentrations

Strain origin	THB supplemented with 5 % OW	10 % OW	> 30 % OW	10 % BW	> 30 % BW	> 30 % SC
Ovine	2/10	16/21	21/21	1/21	12/21	21/21
Bovine	N.D.*	2/14	16/16	0/16	0/16	16/16

* N.D. = Not determined

the exception of two *S. xylosus* and two *S. chromogenes* strains, which agglutinated though weakly, when compared to *S. aureus* strains.

As shown in Table 2, the agglutination reaction observed in *S. aureus* ovine strains, when supplementing THB with bovine whey (BW) at concentrations of ≥ 30 %, instead of ovine whey (OW), was weaker and not so generalized (12/21 vs. 21/21 strains; $P < 0.005$). However, bovine secretions (from dry cows), even when diluted (30 %) in THB, induced a strong agglutination in all of the ovine and bovine *S. aureus* strains analysed. Thus, ovine *S. aureus* strains were more responsive to bovine whey (higher percentage of positive responses) than bovine strains ($P < 0.01$; Tables 1 and 2). These results were not altered when the concentration of whey and/or secretions was increased from 30 % up to 100 % or upon three passages of bacteria through these media.

In general, ovine whey and ovine *S. aureus* strains yielded stronger reactions (considering precipitation time and clump size) than their bovine counterparts. Differences in the strength of reaction were also observed between reactive strains of the same origin (ovine or bovine). As shown in Table 2, concentrations of 10 % and 5 % ovine whey (in THB) were insufficient to induce bacterial agglutination, with the exception of some strains. These showed the strongest positive response to higher (30 %) whey concentrations when compared to other strains. The proportion of agglutinative strains significantly increased ($P < 0.01$) with increasing milk whey concentrations up to ≥ 30 %, except for the combination bovine whey — bovine strains (Table 2). When comparing the proportion of responsive strains at a low concentration of ovine whey (10 %), ovine strains were more responsive ($P < 0.01$) than bovine strains.

When whey from seven ewes, belonging to three different farms, was individually tested against the 37 *S. aureus* strains analysed for agglutination induction capacity, agglutination reactions were in all cases similar in strength, when compared with the whey pool used throughout the experiment.

Discussion

The results presented in this work show that agglutination may be produced during staphylococcus growth, when supplementing a regular medium (THB) with milk whey or mammary gland secretions.

A question arises on whether the agglutination observed in responder strains upon addition of whey to the culture medium, involves wall changes requiring bacterial metabolic activity, or whether adsorption of whey components (milk clumping factor[s]) onto this wall could simply explain this event, independently of the bacterial viability. The latter alternative appears to be the case, since responsive bacteria agglutinated even after inactivation (with 1 % formaldehyde at 4 °C during 24 h). It is known that *S. aureus* form pseudocapsules upon "*in vitro*" culture, in medium supplemented with 10 % ovine or bovine milk they (12, 13) or in m 110 (7). The fact that m 110 does not induce agglutination in any of the strains tested and that metabolic activity is not required for inducing agglutination, suggests that both properties, pseudocapsulation and agglutination are not positively correlated. The bacterial synthesis of an agglutination-mediator wall component, triggered by the presence of whey can thus be ruled out.

We have also found that strains agglutinating in fresh milk whey, do not agglutinate in the presence of autoclaved whey (15 min, 120 °C, 1 atm.) This suggests the presence of a heat-labile substance in whey responsible for agglutination. In any case, bacteria inherent differences in composition or concentration of cell wall components may be responsible for differences in affinity or uptake of specific whey components, affecting the resulting bacterial agglutination. This may explain the observed variation between strains in the strength of reaction and in the whey concentration necessary for induction of agglutination. Also, possible differences in type or concentration of agglutination mediators between whey samples obtained from different animal species may explain the higher capacity of induction of ovine vs. bovine whey.

SPENCER (10) used a growth-agglutination test for detection of antibodies to *S. aureus* in cattle milk. He found growth agglutination antibody titers of up to 1/400 in normal cattle whey and higher in whey from vaccinated or infected animals. Whether these immunoglobulins cause bacterial agglutination during growth was not tested. Fibronectin has been detected in bovine milk (9) in sufficient concentration to induce agglutination of *S. aureus* (8).

Acute gangrenous mastitis is commonly found in ovines as a result of *S. aureus* infection (6), whereas in bovines, gangrenous mastitis is less frequent among clinical *S. aureus* mastitis cases (1). In fact, all of the *S. aureus* strains tested were obtained from clinically and subclinically affected individuals in the case of ewes and cows, respectively. Comparatively, ovine whey and *S. aureus* strains yielded a higher number of responses in the agglutination test ($P < 0.001$), and these were stronger when compared to their bovine counterparts. These observations might suggest the existence of a positive correlation between bacterial agglutination and pathogenicity, considering the differences between both animal species in the pathology of mastitis and in the ability of whey to induce bacterial agglutination. The possible existence of this positive correlation is further supported in this work considering that staphylococcic strains other than *S. aureus*, generally associated with low pathogenicity, show significantly ($P < 0.001$) less agglutination when compared with *S. aureus*.

On the other hand, this interpretation contrasts with that obtained from bacterial clearance studies (14), where a negative correlation between agglutination and pathogenicity has been postulated. A bacterial effective mechanism of eluding phagocytosis may however be the formation of aggregates or filaments sufficiently large to discourage easy ingestion by phagocytes (2). This would be in agreement with our findings.

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III

Aspectos actuales de la lucha contra la mamitis en el ganado vacuno lechero.

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ASPECTOS ACTUALES DE LA LUCHA CONTRA LA MAMITIS EN EL GANADO VACUNO LECHERO

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RESUMEN

La mamitis –inflamación y/o disfunción de la glándula mamaria, asociada con patógenos que la invaden– se halla muy extendida en el ganado vacuno lechero y acarrea graves pérdidas económicas en el sector. En este artículo revisamos la bibliografía reciente sobre su diagnóstico, paso previo para combatir adecuadamente este proceso, así como sobre los factores que influyen en la aparición de la enfermedad, base de nuevos métodos de lucha frente a ella

Palabras clave: Mamitis, Diagnóstico, Prevención, Inmunidad, Marcadores genéticos

SUMMARY

Mastitis –mammary gland inflammation and/or mammary gland problems associated with pathogens invading the gland– is very widespread in dairy cattle and causes significant economical losses In this paper, we review the recent literature about its diagnosis, as a preliminary step for reducing its incidence and about host dependent factors that influence its ocurrence as the basis for developing new methods of fighting against this disease

Key words: Mastitis, Diagnosis, Prevention, Imunity Genetic markers

Introducción

La mamitis o mastitis se ha definido clásicamente como una inflamación de la glándula mamaria, normalmente causada por algún microorganismo, si bien se han descrito mamitis asépticas y mamitis subclínicas, sin evidencia de inflamación. De hecho, ERKSHINE et al (1988) han observado que el 4% de los cuarterones con bajo número de células somáticas (lo cual indica ausencia de inflamación) muestran la presencia de patógenos mayores, especialmente coliformes (43,5%). Puede también ocurrir el fenómeno contrario: al tratar los casos clínicos de mamitis con antibióticos pueden eliminarse los microorganismos, de aquí que algunos cuarterones con elevado número de células somáticas no aparezcan como infectados (mamitis asépticas).

Los problemas mamíticos aparecen con mayor o menor frecuencia en todas las especies de mamíferos domésticos, pero tienen una especial importancia en el ganado vacuno lechero.

Una amplia variedad de organismos y agentes replicativos, incluyendo bacterias, virus, hongos, levaduras, micoplasmas, rickettsias, bedsonias, etc. son capaces de producir mamitis. La incidencia de los distintos patógenos está afectada, entre otros factores, por el tratamiento sanitario en la explotación. Cuando se erradican por antibioterapia y medidas preventivas (teat dipping) algunos patógenos "mayores", los patógenos ambientales pasan a ser responsables de las mamitis (HOGAN et al., 1989). Según POUTREL (1983), en mamitis bovinas son agentes patógenos frecuentes los estafilococos y los estreptococos, entre los que destacan las especies *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* y *Streptococcus uberis*. También se aislan a menudo patógenos como *Escherichia coli*. Asimismo, aparecen otras especies bacterianas de distintos géneros (*Staphylococcus* coagulasa negativos, *Pseudomonas*, *Mycoplasma*, *Pasteurella*, *Klebsiella*, *Corynebacterium*, etc.). Aunque se encuentra *Corynebacterium bovis*, las vacas no parecen ser afectadas negativamente por su presencia según MILLER (1982), hecho que contrasta con las observaciones de PARKER (1977) y RAYNARD y POUTREL (1988). (Para revisión bibliográfica sobre agentes etiológicos principales asociados con mamitis bovinas, véanse WATTS, 1988; MUZAS, 1989).

La mamitis bovina es un proceso ampliamente extendido. Puede decirse que la posibilidad de detectarla depende del tiempo durante el cual se estudian los animales, de modo que si se realiza su seguimiento de por vida, pocas vacas no muestran por lo menos un episodio clínico de mamitis.

Tanto la mamitis clínica como sus formas subclínicas, más frecuentes que la primera, suponen anualmente cuantiosas pérdidas económicas (BARDINAND, 1978). La mamitis subclínica tiene un importante efecto negativo sobre la producción de leche (ALRAWI, 1978; MILLER et al., 1983; DOHOO y MARTIN, 1984a; FOX et al., 1985; SHOOK, 1985). No menos importantes son las consecuentes pérdidas que ocasiona la leche retirada del mercado por vehiculación de antibióticos, el descenso del valor de venta de los animales afectados, el incremento de la tasa de reposición (DOHOO y MARTIN, 1984b), el incremento del trabajo en la explotación por tratamiento de animales enfermos y los costes de dichos tratamientos (MILLER, 1982). El problema se extiende incluso a los países de tecnología avanzada. En Estados Unidos, las pérdidas anuales debidas a la mamitis se han evaluado en más de 2.000 millones de dólares (SEYKORA y MC DANIEL, 1985). En Suecia, según los trabajos de LINDHÉ (1982), una reducción de una quinta parte en la incidencia anual de mamitis equivaldría al beneficio económico de un año de producción lechera.

Ya en 1978, BARDINAND formula la existencia de varios factores a considerar en el estudio de la predisposición a la mamitis bovina: intrínsecos (morfología de la mama, producción lechera, edad, estado de lactación, inmunidad, equilibrio hormonal); extrínsecos (estación del año, desequilibrio

en la alimentación, higiene de la explotación, tipo de manejo y ordeño) y ocasionales (retención de leche, cambios bruscos de alimentación, traumatismo del pezón y bajada brusca de la temperatura). Asimismo, considera que la especificidad, patogenicidad y frecuencia del germen asociado al proceso mamítico son importantes factores a incluir en dicho estudio.

Teniendo en cuenta estas consideraciones, y dada la gran importancia de los procesos mamíticos en las explotaciones lecheras, este artículo intenta resumir bibliografía reciente sobre la lucha contra la mamitis, con especial hincapié en la problemática del diagnóstico, las medidas preventivas frente a la mamitis, las barreras naturales e inducidas frente a dicha enfermedad y los marcadores genéticos de la vaca en relación con la mamitis.

1. Diagnóstico de mamitis

Un problema básico que aparece en cualquier estudio referente a la mamitis es el de escoger un método de diagnóstico adecuado, que permita juzgar si un animal, aparentemente sano, está afectado o no de mamitis subclínica. Los diferentes métodos usados en la actualidad suelen integrarse en el sistema de control lechero y se basan en los análisis de muestras de leche.

El análisis bacteriológico es el método de diagnóstico más deseable entre los existentes (SEARS y HEIDER, 1981; MILLER, 1982), aunque su aplicación en pruebas de campo presenta problemas prácticos (BOUCHOT et al., 1985), considerando la dificultad de recoger las muestras en condiciones estériles, especialmente si dicha recogida ha de ser realizada por el propio ganadero o por el personal encargado del control lechero. Por otro lado, como todos los tests ligados a registros lecheros mensuales, la mayoría de las infecciones quedarían sin registrarse, dada la posibilidad de que una infección aparezca y se resuelva en un plazo de 30 días.

El estudio de la concentración de células somáticas (SCC) en leche es otra prueba diagnóstica de mamitis, muy extendida y de uso creciente. Se realiza sobre las muestras de leche recogidas para el control lechero. Este test se basa en el hecho de que la concentración de células en la leche se eleva marcadamente al comienzo de la enfermedad, particularmente en la fase inflamatoria, debido al paso de leucocitos de la sangre a la glándula mamaria, pudiendo alcanzar, según han informado NATZKE et al. (1972) valores de varios millones por mililitro de leche.

Sin embargo, los valores de SCC presentan una importante variación que hace necesaria una gran cautela en el momento de juzgar como enferma o como sana una vaca en función de dichos valores. Según MILLER y SCHULTZE (1981), el SCC individual está influído por el manejo y el estado fisiológico, así como por el tipo de microorganismo que produce la infección, pudiendo incluso observarse variaciones en los niveles normales de SCC entre vacas no infectadas. Por otro lado, NG-KWAI-HANG et al. (1984) han observado que el SCC está influido por la estación del año (es más elevado en invierno que en verano), el estadío de la lactación (elevado al principio, muestra un mínimo a los 2 meses para elevarse gradualmente después) y la edad del animal (se incrementa a lo largo de su vida, al aumentar el número de lactaciones). VECHT et al. (1989) han obtenido conclusiones similares, aplicando factores de corrección respecto del número de partos y estadío de lactación para comparar valores de SCC indivuales y facilitar la interpretación de los mismos como diagnóstico de mamitis. BROLUND (1985) considera también como factores de variación, la producción lechera diaria, la hora de toma de muestras (mañana/tarde) y la raza.

Así pues, el valor crítico de SCC, a partir del cual un animal se considera mamítico, resulta difícil de estimar. En este sentido, ANDREWS et al. (1983) han comparado las distribuciones del logaritmo de SCC de vacas infectadas y no infectadas, observando que el solapamiento de ambas distribuciones no permite establecer un umbral de discriminación estadísticamente seguro. No obstante, estos autores han definido un umbral de 250 000 células/ml como indicativo de animales afectados. Conviene recordar que existen invididuos con elevado número de neutrófilos (elevado SCC) en la glándula mamaria, que logran eliminar la población bacteriana mediante fagocitosis lo cual se traduce como fallos en los diagnósticos microbiológicos (HILL et al. 1978). Algunos autores (BALL et al., 1989) han fijado el umbral en 400 000 células/ml, lo cual podría evitar éste y otros tipos de falsos positivos, con el riesgo de que algunas mamitis subclínicas queden sin detectar. BROLUND (1985) ha encontrado en su estudio de determinación de umbrales un valor óptimo de 300 000 células/ml, intermedio entre el de ANDREWS et al. (1983) y el de BALL et al. (1989), señalando que distintas especies bacterianas pueden comportarse de distintas formas con respecto a los correspondientes valores umbrales y que el factor población (un mal estado sanitario redundaría en un aumento del umbral) y el número de lactación pueden influir en el cómputo del valor umbral.

Es preciso tener en cuenta que hay muchos tipos de células involucrados en el recuento de células somáticas (LEE et al., 1980). Los macrófagos tienen un papel fisiológico en la eliminación de detritus celulares y materias extrañas en la glándula mamaria, por lo que su presencia puede considerarse como normal. Cuando hay una infección de la ubre, se produce como consecuencia de ello un incremento de los leucocitos polimorfonucleares neutrófilos (PMN) fagocíticos, que migran desde la sangre durante la fase inflamatoria, incrementando con ello el SCC. De aquí que pueda ser de interés la realización de un test para los diferentes tipos celulares, más sensible para la detección de mamitis que la estimación del número total de células somáticas. En este sentido, cabe destacar un trabajo realizado por TOLA y CABELI en 1985, en el que vacas con un SCC de menos de 0,3 millones/ml y un 10-12% de neutrófilos son consideradas como normales, mientras que animales con más de 0,5 millones de células somáticas/ml y más de 20% de neutrófilos se catalogan como mamíticos, juzgándose como sospechosos todos aquellos que presentan valores intermedios. Resultados similares han sido obtenidos por MIELKE (1989).

Por otro lado, el uso del SCC como método de diagnóstico de mamitis presenta un problema adicional de interpretación. Dado que con él se determina la presencia en la glándula mamaria de células activas en la defensa del organismo, parecería deseable que los animales en producción presentaran SCCs no demasiado bajos. De este modo, trabajos realizados en la década de los setenta (SCHALM et al., 1971 y 1976; POUTREL y LERONDELLE, 1978) muestran que mayores recuentos celulares, previos a la infección, hacen disminuir la tasa de ésta. Sin embargo recientes estudios de correlación genética entre los valores de SCC y la frecuencia de mamitis clínica muestran resultados positivos y generalmente altos (MADSEN et al., 1987), de modo que en dichos

estudios un alto recuento celular está ligado a mayores frecuencias de mamitis.

COFFEY et al., (1985) han comparado el diagnóstico microbiano con el recuento de células somáticas, obteniendo valores de correlación genética que oscilan entre 0,36 y 0,67. Asimismo, EMANUELSON et al. (1988), han encontrado una correlación genética de 0,6 entre mamitis y SCC.

Entre los métodos indirectos de determinación del número de células somáticas, destaca el test de California (C.M.T.), basado en la aglutinación del material nucléico de las células presentes en la leche. Debido a su fácil aplicación en pruebas de campo, se halla ampliamente difundido entre los ganaderos. Tomando el recuento de leucocitos como standard, el test de California es el más efectivo de dichos métodos indirectos, según KALOREY y DHOLAKIA (1984).

Otros métodos de diagnóstico de la mamitis se basan en las variaciones en la concentración de ciertos componentes de la leche (KITCHEN, 1981). Entre ellos, podemos citar el estudio de la concentración de lactosa, que disminuye en presencia de mamitis, motivo por el cual ha sido propuesta como alternativa al SCC (RENNER, 1980). Las modificaciones de la concentración iónica de la leche (incremento de cloruros en caso de mamitis; SENDER, 1989) afectan a la conductividad eléctrica de la misma; así, esta característica podría usarse directamente para el diagnóstico de mamitis. Aunque, en principio, según BATRA y MCALLISTER (1984) los errores de diagnóstico por este método son más frecuentes que utilizando SCC, el desarrollo de mejores detectores de la conductividad podría invertir los resultados de esta comparación (FEHER y TAKATSY, 1989).

Diversas proteínas aumentan su concentración en la glándula mamítica, debido a la alteración de la permeabilidad o del epitelio mamario en sí. Por ello, el aumento de las concentraciones de diferentes enzimas de la leche, como catalasas, oxidorreductasas, lactato deshidrogenasa, lipasas, fosfatasas, adenosin trifosfatasa, N-acetil-β-D-glucosaminidasa (NAGasa), antitripsina y glicosidasa, puede usarse también como criterio diagnóstico. En 1981 KITCHEN propuso que la determinación de la concentración del lactato deshidrogenasa podría incluso utilizarse como test diagnóstico de campo. SJAUNJA y FUNKE (1989) han estudiado la capacidad predictiva para la mamitis de adenosin trifosfatasa, NAGasa y antitripsina que resultó ser en porcentaje de resultados correctos de 80,8, 75,8 y 74,3, respectivamente. En este estudio, las pruebas de SCC y C.M.T. detectan, respectivamente, el 86% y 78,1% de las vacas mamíticas. En cuanto a la NAGasa, BALL et al. (1989) han demostrado que la mayoría (91%) de las muestras de leche con un número de células somáticas por mililitro superior a 400 000 tienen una cantidad detectable de este enzima, el cual refleja el deterioro celular del epitelio mamario. Respecto de los inhibidores de proteasa, MATTILA y FROST (1989) han determinado los niveles de antritipsina para medir la permeabilidad vascular de los distintos cuarterones de la mama bovina. Considerando otras proteínas, CONNER y ECKERSALL (1986) han encontrado también que los niveles plasmásticos de haptoglobina, ceruloplasmina y α-1 antritriprina son más elevados en vacas mamíticas que en vacas normales.

No obstante, conviene tener en cuenta en este tipo de diagnósticos que, según ha demostrado HURLEY (1987), los niveles enzimáticos, proteicos, etc., pueden cambiar en la mama según el estado del animal (por ejemplo, durante el período seco). También conviene recordar que algunos de estos indicadores ejercen un efecto directo sobre la infección Este es el caso de proteínas de acción antibacteriana como la lactoferrina (SMITH y SCHANBACHER, 1976), la lisozima (SENFT et al., 1977) y la lactoperoxidasa (KORHONEN, 1980; CRAVEN y WILLIAMS, 1985; NICKERSON, 1985).

Ante los problemas de interpretación que presenta el diagnóstico de mamitis, según se ha descrito en los párrafos anteriores, la International Dairy Federation (IDF) recomienda de forma habitual utilizar, no obstante, para la detección de mamitis subclínica, una combinación de métodos bacteriológicos y una prueba para revelar la respuesta inflamatoria. Puede, por ejemplo, utilizarse el SCC como indicativo. Si en tres recuentos mensuales sucesivos se mantiene un elevado SCC, pueden realizarse las pruebas bacteriológicas para la aplicación de antibioterapia (M.A. ZORRAQUINO, comunicación personal).

Asimismo, PYORALA (1988), para comprobar la curación de la mamitis, propone la NAGasa y el diagnóstico bacteriológico por cuarterón como ensayos para el estudio de la inflamación, diez semanas después del tratamiento de mamitis bovina aguda con antibióticos.

2. Medidas preventivas: manejo y antibioterapia

Los métodos más corrientes para controlar la mamitis son la policía sanitaria y la terapeútica (MILLER, 1982). Un buen manejo es fundamental como medida preventiva. Ejemplos de manejo que contribuyen a la prevalencia de *S. aureus* y *S. agalactiae* son: a) no sumergir el pezón en solución desinfectante preventiva; b) el uso de esponjas o toallas para preparación de la ubre comunes a dos o más animales; y c) mal funcionamiento y poca higiene en la ordeñadora mecánica (DARGENT-MOLINA et al., 1988). Por otra parte, para evitar infecciones y, dado que el esfínter del pezón no se cierra completamente hasta 2 horas después del ordeño, conviene que los animales coman tras el mismo, ya que cuando el animal permanece en pie, el pezón se halla alejado del suelo, disminuyendo con ello los riesgos de infección (NICKERSON, 1985).

En cuanto a la terapia mediante antibióticos, ésta se halla muy extendida, sobre todo en países donde los antibióticos son de fácil acceso para el ganadero. En algunas áreas se aplican antibióticos sistemáticamente a todos los animales al tiempo del secado o al final de la lactación, dado que la incidencia de nuevas infecciones es elevada en el período seco, con especial susceptibilidad inmediatamente tras el momento del secado y después del parto. Dicha práctica es recomendable y constituye un método eficaz de eliminar y prevenir infecciones (EBERHART, 1986). El último resorte del control consiste en eliminar las vacas infectadas, lo cual suele recomendarse cuando los organismos patógenos aislados muestran resistencia a los antibióticos. A pesar de estas medidas sistemáticas, la mamitis no se ha erradicado, aunque su incidencia ha disminuído considerablemente. Dado que *S. aureus* y *S. agalactiae* son patógenos no ambientales (dependientes del hospedador), resolviendo las cuestiones de manejo indicadas y aplicado antibioterapia en el secado, se ha conseguido erradicar *S. agalactiae* y reducir considerablemente *S. aureus* (véase HOGAN et al., 1989).

Quedan por tanto sin controlar por este tratamiento combinando, las mamitis producidas por cepas de *S. aureus* resistentes a los antibióticos aplicados (EBERHART, 1986) o por patógenos ambientales (coliformes y estreptococos ambientales), debido a la utilización de antibióticos no adecuados y/o a la erradicación de *S. aureus* y *S. agalactiae* (SMITH et al., 1985).

El resultado del tratamiento de mamitis con antibióticos depende también en gran parte de posibles interacciones entre éstos y los polimorfonucleares (CRAVEN y WILLIAMS, 1985). Se ha postulado asimismo que uno de los mecanismos por los que *S. aureus* de mamitis bovinas puede resistir al tratamiento con antibióticos consiste en la creación de formas L durante el tratamiento, las cuales pueden a veces revertir a formas normales tras el mismo (OWENS y NICKERSON, 1989a). Por todo ello, se han buscado métodos alternativos que, combinados con la antibioterapia, constituyan una lucha eficaz frente a la mamitis. Entre ellos destaca la reducción (o en su caso eliminación) de factores predisponentes inherentes al hospedador o al patógeno, según se detalla a continuación.

3. Barreras naturales e inducidas frente a la mamitis

3.1 Características del pezón y de la ubre que influyen en la defensa

Existe una serie de mecanismos pasivos de defensa de la ubre frente a la infección, uno de ellos atribuíble a la presencia en el canal del pezón de un esfínter que sirve como válvula y previene la penetración bacteriana (PAAPE et al., 1985). Es bien conocido el hecho de que la distancia entre el extremo del pezón y el suelo está correlacionada negativamente con todos los tipos de mamitis (HAMORI, 1980, JANICKI y BALUKIEWICZ, 1980, JENSEN et al., 1985; MADSEN et al., 1987). Por otra parte, el recubrimiento de queratina y la formación de un tapón de ésta en el conducto del pezón durante el período seco tiene también un efecto protector (NICKERSON, 1985; SCHULTZE et al., 1985; DU PREEZ, 1989). Por ello, cuando dicho tapón no se ha formado todavía al inicio del período seco, existe una mayor susceptibilidad a la infección. Asimismo, STAVIKOVA y LOJDA (1987) han encontrado una variación controlada genéticamente en la composición de la queratina del canal del pezón, que podría ser importante para la explicación de las variaciones individuales en la susceptibilidad a este tipo de infecciones. Se sabe también que los patógenos mayores de la ubre se adhieren preferentemente a las células del epitelio degenerado de la cisterna del pezón, que se forman en menor número durante la involución, con lo cual podría incrementarse la resistencia a la infectividad (NICKERSON, 1985).

Otros aspectos morfológicos como son el diámetro del canal del pezón (que puede aumentar con la edad, provocando una mayor incidencia de mamitis), la forma del extremo del mismo y la profundidad de la ubre (SEYKORA y MC DANIEL, 1985), así como la longitud del pezón, y la distancia entre pezones (JANICKI, 1977; JENSEN et al., 1985) están asociados con la salud de la ubre. Por otro lado, las alteraciones en la morfología del pezón, sobre todo las lesiones graves del canal del mismo, están asociadas con una elevada tasa de prevalencia de mamitis (BINDE y BAKKE, 1984). MADSEN et al. (1987) han observado que el tamaño de la ubre está negativamente correlacionado con la mamitis. La morfología de la ubre y de los pezones tiene también un componente genético moderado o alto (HAMORI, 1980; SEYKORA y MC DANIEL, 1985 y MADSEN et al., 1987), hecho de interés de cara a la lucha frente a mamitis.

3.2 Colonización de la mama

Una vez que la bacteria logra pasar por el canal del pezón, se enfrenta a varios mecanismos de defensa. Uno de ellos es el flujo producido por el ordeño, mediante el cual se eliminan las bacterias. La adherencia a células del epitelio mamario podría evitar dicha eliminación (FROST et al., 1977; WANASINGHE, 1981; AMORENA et al., 1990). Distintas especies y cepas bacterianas presentan distintos grados de adherencia al epitelio, dependiendo ésta también de una serie de factores como pH, temperatura, concentración bacteriana, etc. (WANASINGHE, 1981; AMORENA, et al., 1990). Asimismo, las bacterias pueden adherirse a proteínas extracelulares (fribronectina, colágeno, laminina, vitronectina, fibrinógeno, etc., VERCELLOTTI et al., 1985; CHHATWAL et al., 1987; MAMO et al., 1988).

También la grasa puede contribuir al establecimiento de la infección. Las bacterias pueden adherirse a la superficie de los glóbulos de grasa y ser así transportadas por flotación a la parte superior de la mama, favoreciendo la colonización (SANDHOLM et al., 1989).

Otro mecanismo sustancial de defensa frente a la colonización de la mama es la fagocitosis, según se especifica en el siguiente apartado.

3.3 Defensa por fagocitosis: inmunidad natural e inmunoestimulación

La fagocitosis mediada por neutrófilos y macrófagos es, de hecho, el mecanismo más importante para controlar las infecciones mamarias (POUTREL, 1983; CRAVEN y WILLIAMS, 1985), siendo, según se ha indicado, los macrófagos los más abundantes en leche de animales normales y los neutrófilos en los mamíticos. Estas últimas células son atraídas por factores quimiotácticos que pueden formarse en la leche (algunos componentes del complemento, C5a, son quimiotácticos), en células epiteliales, macrófagos y bacterias (CRAVEN y WILLIAMS, 1985).

Los neutrófilos de rumiantes fagocitan bacterias, especialmente las opsonizadas (unidas a los anticuerpos por su porción Fab, a componentes del complemento, etc.,) con Ig G_2 y/o el componente C_3b de complemento, dado que para ellos presentan receptores de membrana (WATSON, 1987b), aunque también se ha descrito la intervención de moléculas como Ig M (MUKKUR, 1989).

Las bacterias han desarrollado diversos mecanismos antifagocitarios. Así, la proteína A de *S. aureus*, análoga a la proteína M del grupo A de estreptococos (WHITNAK y BEACHEY, 1985), parece actuar de diversas formas: la proteína A libre activa el complemento del medio, posiblemente a través de la vía clásica, tras interactuar con las inmunoglobulinas (FORSGREN et al., 1983). Ello resulta en una disminución del complemento disponible para la opsonización. La proteína A fija en la pared bacteriana tiene una función anti-opsonizante, explicable por dos posibles mecanismos: por una capacidad de fijar anticuerpos, compitiendo con los fagocitos para dicha fijación (GREENBERG et al., 1989), o por impedir la activación de la vía alternativa del complemento iniciada por el peptidoglicano de la pared celular (SPIKA et al., 1981).

Varios estudios han mostrado que el peptidoglicano de la superficie de Gram positivos presenta una gran reactividad inmunológica cruzada, lo que provoca la existencia de una inmunidad natural frente a todos ellos (PETERSON et al., 1778; PETERSON y QUIE, 1981). Sin embargo, la capsulación de las bacterias permite a éstas evadir el

ataque del sistema inmune a través de varios mecanismos: la cápsula es hidrofílica, lo cual dificulta la interacción con los fagocitos (VAN OSS 1978), es poco inmunógena (WIDDERS, 1988) y dificulta la opsonización de bacterias.

Concretamente en *S. aureus*, el caso más estudiado, la cápsula "esconde" los antígenos de la pared celular (ácidos teicoicos, peptidoglicanos, etc.), permitiendo el paso de opsoninas hasta la pared celular, pero impidiendo la interacción de éstas con los receptores en los fagocitos (WILKINSON et al., 1979). Por ello, la correcta opsonización de las cepas capsuladas requiere anticuerpos dirigidos específicamente contra los antígenos capsulares (VERBRUGH et al., 1982). Con independencia de la presencia de cápsula, en mamitis bovinas, *S. aureus* desarrolla una pseudocápsula, la cual le confiere asimismo resistencia a la fagocitosis (WATSON, 1989).

En cuanto a las características de la vaca que influyen en la fagocitosis, los trabajos de PAAPE et al. (1981 y 1985) muestran la influencia de la leche en la capacidad fagocítica de los leucocitos, debido a la fagocitosis de glóbulos de grasa y caseínas. La capacidad defensiva de los leucocitos en la leche es inferior a la de los leucocitos de la sangre, según han demostrado HOLMBERG y CONCHA (1985). Estos autores observaron además que la capacidad fagocítica de las células fagocitarias de la leche va incrementándose desde la fase media de la lactación hasta el secado, alcanzando un máximo al iniciarse este período, para disminuir hasta un mínimo al final del mismo.

En esta misma línea, BURVENICH et al. (1989) han demostrado que las cualidades fisiológicas de los leucocitos polimorfonucleares, de las que depende el grado de mamitis tras el ataque bacteriano, empeoran en algunos animales en el período cercano al parto. Además, es preciso tener en cuenta que la actividad fagocítica depende también del tipo de estímulo que provocan distintas especies bacterianas; en particular, *S. uberis* no parece estimular actividad fagocítica en los polimorfonucleares (ZECCONI y RUFFO, 1989).

Conviene también recordar que el fenómeno de fagocitosis no se halla aislado en el ambiente natural de la ubre. Le acompañan fenómenos naturales de defensa como la secreción en la glándula mamaria de sustancias bactericidas (lisozima, sistema lactoperoxidasa –tiocianato– H_2O_2; PAAPE et al., 1985).

Dado el papel positivo que la fagocitosis desempeña en la lucha frente a la mamitis, se han realizado numerosos ensayos para aumentar el número de fagocitos en la mama (inmunoestimulación).

PAAPE et al. (1988) han observado un 60% de protección frente a *S. uberis* y aumento del número de células somáticas al insertar previo a la infección, como inmunoestimulante, un dispositivo intra-mamario (loop) sometido a abrasión. PAAPE y WEINLAD (1988) han observado que este dispositivo no daña el tejido mamario (como puede medirse por la falta de efecto sobre la actividad NAGasa), ni afecta significativamente a la producción lechera. Algunos dispositivos intramamarios, aunque parecen reducir la infección (apareciendo un menor número de bacterias y de casos de mamitis clínica), pueden ejercer efectos nocivos, como fijar estafilococos coagulasa negativos (NICKERSON et al., 1988). Otros promueven la aparición de eritrocitos en leche y cambios en el epitelio como adelgazamiento, hiperplasia y metaplasia (HUSTON y HEALD, 1983; CRAVEN y HILL, 1986). También acompañando a un aumento de la resistencia a la infección se encuentran dispositivos intramamarios asociados con anomalías en la secreción láctea (BRIGHT et al., 1987). Teniendo en cuenta estas consideraciones y dado que el recuento celular es utilizado por las centrales lecheras para valorar la calidad de la leche y detectar mamitis subclínicas, la aplicación práctica de dispositivos intramamarios resulta dificultosa.

Como método inmunopotenciador alternativo, se han ensayado también sustancias que afectan a la respuesta inflamatoria. El uso de thymosin-α-1 (distribuída comercialmente en forma de producto ID-1), un supuesto agente inmunoterapéutico, aunque aumenta la migración al azar de polimorfonucleares, no parece afectar positivamente a la remisión de infecciones por *S. aureus*, "in vivo" (KEHRLI et al., 1989). Sin embargo, con mayor éxito OWENS y NICKERSON (1989b) han aislado un factor anti-inflamatorio (AIF), a partir de leche de vacas hiperinmunizadas con *S. aureus*, que aumenta "in vivo" la capacidad fagocítica de los macrófagos frente a esta especie bacteriana. Este factor parece así modular la función leucocitaria.

La inmunoestimulación inducida no queda confinada a los procedimientos mencionados. Actualmente, están utilizándose también vacunas "no específicas" que contienen patógenos menores para prevenir infecciones por patógenos mayores. A este respecto, algunos autores recomiendan el uso de *Corynebacterium parvum* para mejorar la respuesta inmune no específica frente a posteriores patógenos mayores (OWENS y NICKERSON, 1989a). También WOODWARD et al. (1988) proponen el uso de *Staphylococcus hominis* 1 para prevenir mamitis bovinas causadas por patógenos Gram-positivos.

HOGAN et al (1988) encuentran sin embargo, que las tasas de infecciones intramamarias estreptocócicas y coliformes aumentan (a más del doble) cuando los cuarterones están infectados bien con *C. bovis* o con estafilococos, por lo que estas últimas especies no parecen jugar un papel protector en algunos casos.

Según OWENS y NICKERSON (1989a), una de las medidas preferentes para la prevención de mamitis, es, además de un manejo adecuado, una mejora de las defensas naturales. En lo referente a inmunomodulación, estos autores señalan que las tendencias actuales consisten en mejorar la defensa inmune no específica aumentando el número y la actividad de fagocitos mediante el uso de dispositivos intramamarios e inyecciones de inmunomodulares (monosacáridos, polisacáridos, glucano de levadura, glicanos de plantas, liposacárido bacteriano o *C. parvum*).

3.4. Implicaciones inmunogenéticas de las sustancias bacterianas extracelulares patogénicas

Las sustancias extracelulares patogénicas producidas por las bacterias merecen analizarse según su variabilidad entre las cepas implicadas y sus interacciones con el sistema inmune, ambas propiedades relacionadas con la diversidad de la patogenicidad en la mamitis. Entre los efectos asociados con toxinas bacterianas, figuran: favorecer la inflamación (MATTILA y FROST, 1989), afectar a la fagocitosis y quimiotaxis, dañar a células sanguíneas y tejidos mamarios y favorecer la coagulación intravascular (EASMON y ADLAM, 1983; LOEFFLER et al. 1988a y 1988b). En suma, las toxinas son altamente responsables de los efectos nocivos observados en la mama infectada (para revisión sobre modos de acción de las toxinas véanse EASMON y ADLAM, 1983 y RUTTER, 1987).

Por ejemplo, la α-toxina, producida por la mayoría de las cepas de *S. aureus*, da

lugar en rumiantes a necrosis de mama que desemboca en mamitis gangrenosa. La inmunización del animal frente a esta toxina elimina la necrosis, aunque no el germen, y desempeña un papel protector para localizar la enfermedad en lugares concretos dentro de la mama (EASMON y ADLAM, 1983), de ahí que sea importante incluir algunos toxoides como parte del inmunógeno en algunas vacunas (WATSON, 1988).

Con respecto a la intervención del complejo mayor de histocompatibilidad de la vaca (BoLA; AMORENA y STONE, 1978) en la respuesta frente a toxinas implicadas en la mamitis, no se han realizado estudios hasta la fecha. Sin embargo, en sistemas análogos, se ha encontrado una intervención directa. Concretamente, en humanos y ratones se ha observado recientemente que la proliferación de clones particulares de células T en respuesta a algunas enterotoxinas de *S. aureus* (SEA), requiere la unión de estas toxinas a células accesorias a través de sus antígenos de clase II del complejo mayor de histocompatibilidad (MOLLICK et al., 1989; FLEISCHER, 1989; VROEGOP y BUXSER, 1989). Además, cada tipo de toxina reacciona con células T que llevan determinadas secuencias de la porción Vβ del receptor αβ propio de estas células (KAPPLER et al., 1989; FRASER, 1989). Algunos clones de células T experimentan respuestas proliferativas y otros citotóxicas frente a estas toxinas (FLEISHER, 1989). No sólo las enterotoxinas presentan este tipo de fenómenos sino también otras toxinas estafilocócicas (KAPPLER et al., 1989). Todo ello, lleva a pensar que la especificidad de estas toxinas para la Vβ las coloca en la clase de superantígenos, descrita recientemente y puede explicar la distinta sensibilidad de diferentes individuos a los efectos tóxicos de estas proteínas (KAPPLER et al., 1989), aspecto cuya investigación resultaría de especial interés en el campo de la mamitis bovina.

3.5. Defensa por vacunación y aplicación de inmunógenos compuestos

La vacunación específica frente a los patógenos causantes de la mamitis ha demostrado ser un difícil problema (MILLER, 1982). Una de las razones para ello es la amplia variedad de microorganismos implicados (en las infecciones consideradas y/o en infecciones subsiguientes), muchos de los cuales presentan mecanismos especiales de patogenicidad. OWENS y NICKERSON (1989b) señalan que se ha alcanzado un éxito limitado, lográndose por ejemplo, mediante vacunación frente a *S. aureus*, una disminución de la severidad de la mamitis y un aumento de curación espontánea. Para casos concretos de cepas de *S. aureus* y *E. coli*, se han conseguido no obstante vacunas bastante eficaces (WATSON, 1984 y 1989; GONZALES et al., 1987). En general, el aumento de anticuerpos por vacunación va acompañado de una disminución de la severidad de la mamitis y de un incremento de la tasa de curación espontánea de mamitis subclínicas (NICKERSON, 1985).

Los estudios realizados por LIE en 1978 muestran que las variaciones individuales en los niveles de inmunoglobulinas frente a distintos antígenos en sangre tienen un componente genético; éste podría interactuar con los mecanismos de la vacunación. Por otra parte, según WATSON (1987a), frente a la relativamente benigna ingestión bacteriana en neutrófilos, mediada por el receptor C3b en ausencia de anticuerpos, las bacterias opsonizadas que penetran vía el receptor Fc en los neutrófilos desencadenan una vía metabólica en dichas células que facilita la muerte bacteriana. Por este doble motivo (facilitación de opsonización y de muerte bacteriana) interesa la presencia de anticuerpos específicos en leche, ya que éstos tienen capacidad de compensar la capacidad antifagocítica de la pseudocápsula. Dado que en rumiantes los neutrófilos poseen receptores abundantes para la IgG_2 (Fc) y que estos anticuerpos son potentes agentes opsonizantes, interesan vacunas que favorezcan la producción de esta subclase de inmunoglobulinas, teniendo en cuenta que interviene de forma primordial en la fagocitosis en lugar de otras (IgG_1, IgA, etc.). Ello parece favorecerse inmunizando con organismos vivos en lugar de muertos, obteniéndose altas proporciones de IgG_2: IgG_1 (Watson, 1987b). El sinergismo IgG_2-neutrófilos ayuda a proteger la mama frente a la mamitis. Por tanto, una combinación de inmunoestimulación idónea y vacunación parece aconsejable (Watson, 1987a).

Hasta la fecha, además de las vacunas mencionadas, se han utilizado muy diversos tipos de inmunógenos, entre otros, vacunas compuestas por bacterinas de dos especies bacterianas como *S. aureus* y *S. agalactiae* (Opdebeeck y Norcross, 1985); vacunas vivas atenuadas de *S. aureus*, con pérdida de la capacidad hemolítica (Watson, 1984): vacunas muertas de *S. aureus* (Brock et al., 1975); extractos de pared celular de *S. aureus* (Frost y Mattila, 1988); cepas capsuladas de *S. aureus* en combinación con polisacárido capsular de *Staphylococcus epidermidis* (Yoshida et al., 1984); y toxoide de leucocidina, para producción de anticuerpos frente a la toxina (Loeffler et al., 1988a y 1988b).

Una tendencia actual para vacunación consiste en la utilización de vacunas con varios componentes. Por ejemplo, recientemente, Watson (1988), trabajando en mamitis ovinas, ha desarrollado una vacuna con tres componentes: 1) células muertas de *S. aureus* que habían sido cultivadas para inducir la síntesis de pseudocápsula; 2) toxoide de β-hemolisina estafilocócica; y 3) el adyuvante dextrano sulfato. Con ello ha logrado una reducción de la incidencia de mamitis grangrenosa y clínica, con el consiguiente aumento de producción lechera.

4. Marcadores genéticos moleculares de la vaca en relación con la mamitis

Además de considerar las características indicadas, que pueden proporcionar alguna información sobre la propensión de los individuos a sufrir una infección a nivel de la ubre, interesaría detectar o medir de algún modo la capacidad global, inherente a los individuos, de resistir la infección antes de que ésta se produzca, es decir, conocer el genotipo para la resistencia y predecir la interacción genotipo-ambiente.

Junto a los estudios genéticos sobre caracteres anatómicos, productivos, fisiológicos, celulares y moleculares relacionados de forma cuantitativa con la mamitis, son numerosos los estudios realizados sobre posibles marcadores de resistencia de tipo genético-cualitativo (distintos alelos, asociados con distintos efectos), aspecto en el que se centra este apartado. En lo referente a las proteínas de la sangre, Malik et al. en 1970 han detectado diferencias significativas entre los genotipos de transferrina (Tf) en cuanto al porcentaje de cuarterones infectados por *S. agalactiae* y por estafilococos hemolíticos. Las vacas con fenotipo Tf D_1 tenían mayor incidencia de infecciones por ambos tipos de agentes microbianos, así como puntuaciones del test de California (CMT) y del recuento de células somáticas (SCC) significativamente superiores. Las vacas con fenotipo Tf E aparecían libres de infecciones por *S. agalactiae* y con un registro medio estreptocócico por vaca significativamente inferior (en el mismo ambiente que las anteriores).

Wijeratne et al. (1976) midiendo la susceptibilidad a la mamitis en función del número de cuarterones infectados por cualquier germen en las diversas etapas de la lactación, también han observado la existencia de una relación, aunque pequeña, entre dicha susceptibilidad y los genotipos, no sólo de Tf, sino también de Amilasa I y II (Am I y II). No obstante, cuando realizaban la estimación de la susceptibilidad a partir de criterios de todo o nada (animal enfermo o animal sano), no había relación o ésta era muy pequeña, entre susceptibilidad y los genotipos de las proteínas consideradas.

En 1983, Dzumkov et al., han observado asimismo diferencias entre los tipos de Tf con respecto a la resistencia a la mamitis, de modo que las vacas Tf AE y Tf DE exhiben mayor resistencia que los animales Tf A. Los trabajos de Makaveev y Angelov (1984), muestran que el mayor porcentaje de las vacas estudiadas que sufren de mamitis presenta los fenotipos Tf AD, Cp (ceruloplasmina) AC, Am-I BC; Ca (anhidrasa carbónica) FS y NP (nucleósido fosforilasa) HL, mientras que la menor frecuencia de mamitis se observa en vacas con los fenotipos Tf DE, Tf AE, Am B, Ca F y NP L.

Por otra parte, también se han estudiado las relaciones de los antígenos de grupos sanguíneos y del complejo mayor de histocompatibilidad (BoLA) con la aparición de mamitis. De todos los antígenos de grupos sanguíneos estudiados, solamente el M' parece ejercer de forma comprobada una influencia sobre la incidencia de mamitis, ya que los animales carentes de M' muestran generalmente menor frecuencia de mamitis (Jensen et al., 1985; Larsen et al., 1985; Madsen et al., 1987). Asimismo, algunos antígenos BoLA de Clase I (w16, w6 y w6.1) se asocian con altos recuentos celulares (Oddgeirsson et al., 1988). Hasta la fecha, se desconoce la base molecular de las asociaciones observadas.

Los diversos tipos de las proteínas lácteas β-lactoglobulina (β-Lg); αs_1-, β y k-caseínas (αs_1-Cn, β-Cn y k-Cn) han sido relacionados con el diagnóstico de la mamitis como tal, así como con la presencia de microorganismos patógenos en las ubres enfermas. Osterhoff et al. (1973) han hallado una correlación significativa entre el fenotipo βLg AB y una baja incidencia de mamitis. Según estos autores, esta menor incidencia de la enfermedad no estaría relacionada con la propia molécula proteica, sino con otras particularidades metabólicas indeterminadas de la vaca y de su ubre. Según Atroshi et al. (1982), las vacas de fenotipo β-Lg A tienden a presentar mayores recuentos de células somáticas en leche (pero con bajas concentraciones de seroalbúmina), que el resto de los genotipos de β-Lg. Por el contrario, Han et al. (1986), estudiando la frecuencia de los alelos de β-Lg en leche mamítica y en leche normal así como los porcentajes de los diversos genotipos para esta proteína en animales sanos y enfermos, han sugerido que las vacas con β-Lg A tienen mayor resistencia a la mamitis que las vacas con β-Lg B. Pero de nuevo Madsen et al. (1987) han observado que los animales portadores del fenotipo β-Lg A eran más frecuentemente afectados por mamitis clínicas que los β-Lg B.

Por otro lado, el nivel de lactoferrina, una proteína de gran actividad bacteriostática, está determinado genéticamente, lo que también podría explicar alguna de las diferencias individuales en cuanto a sensibilidad a la mamitis (Senft et al., 1977).

Como puede deducirse de los estudios indicados, todavía no es posible detectar "a priori" con exactitud la capacidad de resistencia de los animales. Algunos de los resultados presentados son incluso contradictorios y carecen de una evaluación del riesgo de contraer mamitis al que han estado sometidos los diversos animales de la experiencia. Sería más deseable cuantificar los efectos de los marcadores realizando infecciones experimentales. GROOTENHUIS (1976) ha realizado este tipo de investigaciones entre grupos de medias hermanas, pero ha utilizado solamente dos grupos de progenie y sus resultados no son suficientemente concluyentes.

La heredabilidad de la sensibilidad a la mamitis varía ampliamente, dependiendo de la población estudiada y del diagnóstico utilizado para la detección de esta enfermedad. De acuerdo con la revisión realizada por MILLER (1982), la heredabilidad oscila entre 0,10 y 0,20. Algunos de estos estudios, no tienen en cuenta el tipo de patógenos causantes de la mamitis. A nuestro entender, y considerando lo descrito en apartados anteriores, cada estudio de heredabilidad debería implicar infecciones frente a determinados tipos de patógenos, ya que la interacción microorganismo-hospedador no sólo depende de las características del individuo vacuno en cuestión, sino también de las propiedades de la especie bacteriana implicada. En cualquier caso, la existencia ya indicada de diferencias en susceptibilidad a la mamitis entre los individuos, junto con los valores estimados de la heredabilidad, muestran que es posible, al menos teóricamente previsible, conseguir un cambio genético en el nivel de incidencia de estos procesos. Este cambio sería permanente en la población y acumulable de generación en generación, a diferencia del descenso de incidencia causado por métodos terapéuticos o preventivos, a base de medidas higiénicas o de las siempre problemáticas vacunas.

Sin embargo, existe una correlación negativa entre resistencia a la mamitis y la producción de leche (MONARDES et al., 1984; MADSEN et al., 1987), por lo que una mejora en esta resistencia iría acompañada de un descenso de producción. Pese a ello, los resultados podrían ser considerados como positivos puesto que resulta de mayor interés producir leche de mayor calidad (más sana) y a menor coste que incrementar la producción de leche, y el lastre genético siguiendo los sistemas actuales.

Teniendo en cuenta el conjunto de los resultados ilustrados en este trabajo sería aconsejable que, acompañando a dichos programas de selección y estudios de heredabilidad, se contemplasen de cara a la erradicación de la enfermedad, no sólo los factores genéticos inherentes al individuo y al patógeno, sino también los correspondientes a efectos de los inmunoestimulantes, del control ambiental y del manejo, ya que todos ellos colaboran entre sí en la lucha frente a los procesos mamíticos.

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IV

Comparación entre cepas mucosas de *Staphylococcus aureus* y sus variantes no mucosas con respecto a la sensibilidad a antibióticos.

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Comparación entre cepas mucosas de *Staphylococcus aureus* y sus variantes no mucosas con respecto a la sensibilidad a antibióticos

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INTRODUCCION

Se ha demostrado que *Staphylococcus. aureus* crece formando microcolonias en endocarditis y osteomielitis humana (1). Análogamente, *Streptococcus viridans* forma microcolonias rodeadas por mucus de endocarditis humana. En este último caso, se ha encontrado que la cantidad de mucus producida por la bacteria se correlaciona con el tamaño de las vegetaciones cardíacas y con la resistencia a la terapia antibiótica (2).

En bovinos, las mamitis crónicas producidas por *S. aureus* suponen un grave problema económico, ya que la terapia antibiótica no resulta eficaz, haciéndose necesario el sacrificio del animal. Se ha postulado que esta resistencia al tratamiento antibiótico se debe a la permanencia de las bacterias dentro de los macrófagos (3). Paralelamente, podría proponerse para las mamitis crónicas producidas por *S. aureus* un mecanismo similar al descrito para *S. viridans*.

En este trabajo, se ha estudiado la resistencia que presentan las cepas de *S. aureus* mucosas y sus variantes no mucosas frente a los antibióticos, una vez que han crecido las bacterias correspondientes formando un biofilm.

MATERIAL Y METODOS

Cepas bacterianas. Se han utilizado 34 cepas de *S. aureus* aisladas de mamitis; de ellas, 19 eran mucosas (9 de origen bovino y 10 de origen ovino) y 15 eran no mucosas (10 de origen bovino y 5 de origen ovino). La determinación de la presencia de mucus se realizó según las técnicas de Christensen y de agar rojo Congo (ARC; véase Baselga y cols. en este Congreso).

Obtención de variantes. Tras varios subcultivos (10-20) en ARC, se obtuvieron variantes con morfología colonial no mucosa a partir de cepas mucosas. Con el objeto de favorecer el crecimiento de variantes mucosas y, por lo tanto, capaces de formar biofilms en las paredes de un tubo, se cultivaron 15 cepas no mucosas en tryptic soy broth (TSB) suplementado con glucosa al 2% (TSB-G). Diariamente, el medio fue eliminado tras el crecimiento y el interior del tubo fue lavado con PBS estéril, llenándose después dicho tubo con TSB-G. Este ciclo se repitió durante 30 días, a lo largo de los cuales, la aparición de variantes mucosas fue comprobándose sembrando alícuotas en agar rojo Congo.

Periódicamente, y previo al test con antibióticos, todas las cepas originales y las variantes obtenidas fueron analizadas con el test de Christensen y sembradas en agar rojo Congo para comprobar que no hubiera reversión de las últimas a su estado original.

Para confirmar que las variantes obtenidas no fueran producto de una contaminación, todas las cepas y sus variantes fueron biotipadas con el sistema API (BioMérieux) y su espectro de resistencia a antibióticos fue determinado usando un panel de 14 antibióticos. Las variantes que no coincidieron en sus características con las cepas originales en todas los tests fueron descartadas del estudio.

Test de resistencia a antibióticos. Para esta experiencia, se utilizaron 3 cepas mucosas, sus variantes no mucosas, y 3 antibióticos a los cuales eran sensibles. Se usaron placas estériles de cultivo celular con 96 pocillos y fondo plano. Se inocularon en cada pocillo 50 μl de una suspensión bacteriana ($\approx 5\times10^4$ CFU/ml), obtenida a partir de un cultivo bacteriano (18h, 37°C) en TSB. Tras 1h a 37°C, se añadieron 200μl de TSB-G a cada pocillo, incubándose 6h a 37°C. Tras este período de incubación, se comprobó el crecimiento bacteriano al microscopio invertido. El medio se descartó y se añadieron 200μl de TSB suplementado con antibióticos a las siguientes concentraciones: 1000, 250, 63, 16, 4, 1, 0,25 y 0,0. Tras una incubación a 37°C durante 24h, se determinó la concentración mínima inhibitoria. Los pocillos donde no hubo crecimiento se sometieron a 2 lavados con PBS estéril para eliminar los restos de antibiótico. Finalmente, se añadieron 200 μl de TSB a cada pocillo. Tras 24h de incubación, se determinó en qué pocillos hubo crecimiento bacteriano, interpretándose que en estos la concentración de antibiótico no fue capaz de eliminar a todas las bacterias, es decir, no alcanzó la concentración bactericida mínima. Todos los tests se realizaron por triplicado.

RESULTADOS Y DISCUSION

Obtención de variantes. Se obtuvieron variantes no mucosas (-) de las cepas mucosas (+) bovinas (C101+, C104+, C110+ y C171+) y ovinas (72+, 76+, 77+ y 80+) y una variante mucosa de la cepa ovina no mucosa C195-.

Resistencia a antibióticos. A las 6h de crecimiento, previo a la incubación con antibióticos, se observó que las cepas mucosas crecían formando pequeñas microcolonias, mientras que las variantes no mucosas tendían a ocupar todo el fondo del pocillo. Respecto de la concentración mínima inhibitoria, no se encontraron diferencias entre las cepas mucosas y no mucosas, pero sí las hubo en cuanto a la concentración bactericida mínima, mostrando la mayoría de las cepas mucosas una mayor resistencia a determinados antibióticos en las altas concentraciones (Tabla 1). Se desconoce el porqué, en las condiciones de la experiencia, este efecto se observa con distintos antibióticos para distintas cepas. Todas las cepas (mucosas y no mucosas) mostraron una resistencia a los antibióticos mayor de lo normal, probablemente debido a que se había permitido que las bacterias formaran un biofilm.

Tabla 1. Concentración bactericida mínima en cepas mucosas (+) y sus variantes no mucosas (-).

Cepa	Antibiótico		
	Penicilina*	Vancomicina**	Novobiocina**
C104+	250	1000	250
C104-	63	250	63
80+	250	1000	63
80-	16	1000	16
77+	1000	ND†	1000
77-	1000	ND	250

†ND = No determinado
*Unidades Internacionales
**microgramos

En otra comunicación presentada a este congreso (Iturralde y cols.) hemos demostrado que el mucus puede favorecer la adherencia bacteriana a las células epiteliales. Si las bacterias adheridas son capaces de crecer formando microcolonias, tal y como se evidencia "in vitro", éstas podrían permanecer en la mama, resistiendo el flujo de leche producido en el ordeño o en el amamantamiento. Este mecanismo de adherencia/biofilms también podría explicar la ineficacia del tratamiento antibiótico encontrada en las mamitis crónicas originadas por *S. aureus*.

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V

Hydrophobicity of mastitis *Staphylococcus aureus*. Effect of growth phase, exopolysaccharide-inducing media, ageing and source of bacteria.

Baselga, R., Albizu, I., Penadés, J.R., Aguilar, B., Iturralde, M. y Amorena, B.

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Hydrophobicity of Ruminant Mastitis *Staphylococcus aureus* in relation to bacterial ageing and slime production.

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ABSTRACT

Hydrophobicity of 72 bovine and 53 ovine mastitis *Staphylococcus aureus* strains was studied throughout the logarithmic phase using a water-xylene two phase system. Hydrophobicity increased during this growth-phase. Freshly isolated strains were more hydrophobic than old strains ($P<0.01$). Old (bovine) strains became more hydrophobic (i.e., "refreshed") after passage through the mouse mammary gland ($P<0.01$). Bovine strains were more hydrophobic than ovine strains ($P<0.01$). For the majority of strains, bacteria became more hydrophilic ($P<0.001$) after growth in exopolysaccharide inducing media (Columbia broth and modified *Staphylococcus* 110 medium). This could be expected, since exopolysaccharides are hydrophilic. However, in these media, those strains able to produce slime in Congo red agar or in TSB supplemented with 2% glucose (w/v), did not become more hydrophilic.

It is proposed that different mechanisms may be involved in triggering exopolysaccharide production when using different exopolysaccharide inducing media.

INTRODUCTION

Hydrophobicity (Hty) has been proposed as a virulence factor. In humans for example, the frequency of very hydrophobic (Ho) *S. aureus* strains isolated from infectious processes is higher than in strains isolated from the nose of healthy human carriers (21). Furthermore, Ho interaction appears to play a major role in the initial phase of bacterial attachment to inanimate surfaces such as intravascular catheters, sutures, tissue protheses, etc. (10, 14) and to host epithelial cells (27). Specifically, Beck et al. (7) proposed that Hty plays a role in *S. aureus* attachment to human buccal epithelial cells

In bovine mastitis, successful bacteria must be able to overcome the cleansing forces of milking and/or suckling (3). Thus, bacterial attachment to epithelial surfaces (1) and likely Hty, as proposed for human infections, may play a role in invasiveness.

Staphylococus aureus cell surface Hty has been measured by the salt aggregation test (17, 18, 21, 22, 23, 24) and by hydrophobic interaction chromatography (16), showing both methods a good correlation. Also, partition in water-hexadecane two-phase systems hase been used (6, 7).

There are several factors affecting the hydrophobic properties of *S. aureus* strains. Beck et al. (6) have observed that *S. aureus* Hty increases during growth, reaching a constant value in the stationary phase. On the other hand, it is well known that a variety of enviromental factors greatly influence the cell surface components of pathogens (8). Specifically, *S. aureus* Hty may change when altering the composition and/or physical state (solid vs. liquid) of the growth medium used (21).

"In vivo"-grown bovine mastitis *S. aureus* shows an exopolysaccharide matrix (15, 30) which may alter the cell surface Hty. Indeed, highly encapsulated *S. aureus* strains show a hydrophilic (Hi) cell surface (22). This matrix can be indirectly evidenced by the presence of a diffuse colony morphology (DCM) when bacteria are directly transferred from mastitis milk in serum soft agar (SSA; 28). Jonsson (18) found that bacteria became more Hi when a DCM was induced after growth in bovine whey. This may be explained by the formation of an exopolysaccharide matrix. This matrix can also be produced in response to different growth media: Columbia (19), modified *Staphylococcus* medium 110 (15) and Nutrient broth supplemented with whey (30).

In coagulase negative staphylococci, slime producing strains have commonly been identified using the Christensen's method (9), based on the estimation of the capacity to form a biofilm on the walls of a glass tube, or by the colony morphology in Congo red agar (12). However, to our knowledge, these criteria have not been applied so far to *S. aureus*.

The present work was aimed at studying the variation of cell surface Hty of ruminant mastitis *S. aureus*, specially concerning bacterial ageing and slime production.

MATERIAL AND METHODS

Bacterial strains. A total of 125 mastitis *S. aureus* strains (72 of bovine and 53 of ovine origin) were used. Sixty seven of them (22 bovine and 45 ovine) were isolated and identified (API system, BioMèrieux) by Drs. Ducha and Latre (Microbiology Laboratory, Veterinary Faculty, Zaragoza, Spain), and were maintained at -20°C for up to three years, with various (up to six) freeze-thaw cycles. These strains were considered old with respect to the remaining 58 (50 bovine and 8 ovine) strains which were recently isolated in our laboratory and were identified as *S. aureus* by colonial morphology, Gram staining, haemolytic activity, tube coagulase test and catalase activity. These 58 bacterial strains were never subcultured more than twice in blood agar before freezing. The encapsulated strain A kindly supplied by Dr. K. Yoshida, was used as a Hi and DCM positive control. The majority of ovine strains were isolated from severe clinical mastitis ewes, whereas bovine strains were mainly isolated from cows with subclinical or chronic mastitis.

Growth conditions. After thawing, bacteria were cultured in 5 per cent (v/v) sheep blood agar, maintained at 8°C and subcultured fortnightly for up to two months. Thereafter for Hty tests, bacteria were cultured either in Nutrient broth (NB) or in one of the following exopolysaccharide inducing media (EIM): Columbia broth (CB) supplemented with NaCl (4% w/v) as described by Karakawa et al. (19), modified *Staphylococcus* medium 110 (m110) as described by Opdebeeck et al. (28) and NB supplemented with 10 per cent (v/v) filter sterilized bovine whey (NBBW) as described by Watson (30). Bovine whey was obtained by rennin treatment of delipidized milk and stored at -20°C for up to four months; NBBW was maintained at 8°C for up to 48 h.

Cultures were carried out using 10 ml of medium and incubating for 18 to 24 h at 37°C without agitation. Subsequently, 10-100 μl were transferred to 5-300 ml of the same broth and incubated at 37°C, under agitation, until reaching the medium-late exponential growth (MLEG) phase.

The growth curve of *S. aureus* in the different media was determined by measuring the culture optical density (OD; A_{540}) at various time intervals.

Hydrophobicity test. Bacterial Hty was determined by measuring the affinity of bacteria towards xylene in a water-xylene two-phase system, according to Hoght et al. (14). Briefly, bacteria were harvested from culture by centrifugation, washed once with PBS (0.02 M, pH 7.2) and, unless otherwise stated, resuspended to a concentration of 10^9 bacteria per ml PBS, as determined by OD (A_{540}). A volume (0.25 ml, unless otherwise specified) of p-xylene (Merck) was added to the test tubes containing the bacterial aqueous suspensions (4 ml). Tubes were vortexed during 1 min. Ten minutes later, the OD (A_{540}) of the aqueous phase was measured. Bacterial suspensions without xylene were used to measure the initial OD (A_{540}). All strains were tested at least on three different dates and frequently using different subcultures. Hty was estimated according to the following expression:

$$\text{Hty} = 1 - \frac{A_{540}\text{ aqueous phase after addition of xylene}}{A_{540}\text{ aqueous phase before addition of xylene}} \times 100$$

To standardize the test, the effects of xylene:aqueous phase ratio bacterial concentration were determined, according to the following expectations: a) *for effect of bacterial concentration*, 0.25 ml xylene and different concentrations of bacteria, ranging from 5×10^7 to 2×10^9 MLEG bacteria per ml PBS; b) *for effect of xylene:aqueous ratio,* various volumes of p-xylene (0.05, 0.1, 0.25, 0.5, 1.0 and 2.0 ml), and 10^9 MLEG bacteria per ml PBS.

The effect of the growth phase was determined with the following specifications: 0.25 ml xylene, 10^9 bacteria per ml PBS, growth periods of 3.5 to 12 h. The effects of bacterial ageing, passage of bacteria through the mouse mammary gland, EIM-growth and ovine/bovine origin comparisons were studied using the following specifications: 0.25 ml xylene, 10^9 MLEG bacteria per ml PBS. Bacteria were harvested when cultures reached an OD (A_{540}) from 1.3 to 1.5 in NB and from 1.7 to 2.0 in EIM, after a 5 to 7 h incubation period.

Standardization of the test and Hty estimations.

a) Effect of the xylene:aqueous ratio used in Hty assays on Hty estimates. The experiment on the effect of the xylene:aqueous ratio on Hty was carried out using 12 strains. As illustrated in Fig. 1, all strains showed higher Hty estimates when increasing the volume of xylene. However, this effect was very mild for the encapsulated strain A. Only xylene volumes from 0.25 to 1 ml helped classifying the strains into three categories: Hi (Hty<40%; e.g., strains C94 and C143), Ho (Hty>70%; e.g., strains C16, C26, C104, C113, C114, C123, C141) or intermediate (Int; Hty $\geq$40% to $\leq$70%; e.g., strains C139 and C142). The volume of 0.25 ml xylene was selected for the remaining Hty experiments as it was one of the most discriminatory volumes for establishing these categories.

b)Effect of the bacterial concentration used in Hty assays on Hty estimates. The effect of bacterial concentration on Hty estimates was assayed on 12 strains. It is illustrated in Fig. 2, using 5 strains for simplicity of the graphic. Bacterial concentrations increasing from $4x10^8$ to $1.25x10^9$ bacteria per ml, were associated with a decrease in the estimated Hty, Ho strains (C16, C123) showing a more pronounced slope than Hi strains (C94, C143). The reverse situation was found at higher concentrations (>$1.25x10^9$). Lower concentrations (<$4x10^8$) gave rise to unreliable results in several cases (e.g., strain C143). The encapsulated strain A was almost insensitive to changes in bacterial concentration. The concentration of 10^9 bacteria/ml was selected for the remaining experiments since it is one of the most discriminatory concentrations for establishing Hty categories.

Slime production. a) Christensen's method. Bacterial cells (one loopful) were cultured in 5 ml m110, CB or tryptic soy broth (TSB) supplemented with glucose (2% w/v; TSB-G) and incubated for 18 to 24 h at 37°C. Twenty microliters were transferred to 5 ml of the same medium and incubated for 18 h at 37°C. After 24 h, tubes were emptied again. Slime producing strains formed a visible film lining the walls of the tube which was evidenced by adding 0.5 ml cristal violet (0.4% w/v in PBS) and vortexing carefully. A ring formation in the air-liquid interface was not considered indicative of slime production. To clarify some of the results, further empty-refill cycles were neccesary; **b) Congo red method.** Bacteria were grown (18 h, 37°C) in NB, m110, CB or TSB-G. Subsequently, they were cultured in Congo red agar as described by Freeman et al. (13). Also, m110 and CB agar (1% w/v) media supplemented with Congo red (0.8 gr/l) were assayed. In all cases, a positive result (slime production) was indicated by the presence of black or pink colonies with a dry cristalline surface.

Diffuse colony morphology. The presence of exopolysaccharide was determined by the production of DCM in SSA, according to the method described by Opdebeeck et al. (28), with the addition of 1% rabbit normal serum to 0.15% agar in m110.

To induce a DCM, bacteria were passaged 10 times through bovine whey every 24 h. The DCM formation was evaluated in each passage after a 6 to 10 h incubation period at 37°C under agitation, as described by Jonsson et al. (18).

Passage of bacteria through the mouse mammary gland. After growth overnight (16-20 h) in NB (37°C), bacteria were washed once and resuspended in PBS; 0.025 ml of a bacterial suspension ($5x10^8$-10^9 CFU per ml) were inoculated using 28G needles into the left fourth mammary gland of a 4 to 6 day post-parturition female mouse Offspring were removed at the time of inoculation Twenty four hours later, the mouse was killed with chloroform The mammary gland was washed with ethanol (70%), sectioned under sterile conditions, immersed in NB and disrupted by strong vortexing with glass beads. The resulting bacterial suspension, considered at this moment as "refreshed" was directly cultured in SSA and Congo red agar to determine the DCM and the slime production

Statistical analysis. Data were analysed by Chi-square for ovine/bovine and fresh/old comparisons and paired t-tests for effect of growth media, and old/refreshed comparisons

RESULTS

Effect of the growth phase selected for Hty assays on Hty estimates. The effect of growth on Hty estimates was assayed using 6 strains. The growth curve was studied for each of them in relation to Hty. Figure 3 illustrates the Hty curves of all six strains and, for simplicity, the growth curve of one strain (C143) Hty increased during the logarithmic phase, until reaching the stationary growth-phase. Thereafter, Hi strains (C94, C143) became less hydrophilic, simultaneously decreasing the culture OD. This decrease was not related to aggregate formation (none of the six strains tested formed aggregates). The encapsulated strain A did not suffer any change in Hty during the growth-phase period studied

Effect of bacterial ageing on Hty. Forty-five fresh and eighteen old bovine strains were used to compare the effect of bacterial ageing on Hty. Fresh bovine strains were more Ho than old strains ($P<0.01$; Table 1). After a passage, through the mouse mammary gland, of 7 of the old bovine strains, showing Hi and Int Hty, they became more Ho ($P<0.01$). No effect on Hty was observed when the passage was done with fresh bovine strains (10). However, when the strains were of ovine origin, the passage of old strains through the mouse mammary gland had no significant effect on their cell surface Hty.

Ovine-bovine comparisons on bacterial Hty. Since no statistically significant difference was found on the proportion of Ho, Hi and Int between fresh (8) and "refreshed" (14) ovine strains, both categories were grouped to compare ovine strains with bovine fresh strains (45). Under the conditions of this experience, bovine strains showed a higher Hty than ovine strains ($P<0.01$).

Slime production in Congo red agar and in TSB-2% glucose. With regard to classification into slime-producing and non slime-producing strains, there was a total agreement between the results obtained from the Congo red and from the Christensen's tests for slime production However, the Congo red method yielded reliable results upon retests whereas the Christensen's method required several passages for some strains for clear evaluation. Only 15 among 125 strains showed a capacity to produce slime according to these methods. Studying 31 fresh bovine strains, it was observed that the growth medium used (NB, m110 or CB) before culturing in Congo red agar or in TSB-G had no effect on slime production. None of the bacterial strains (33) passaged through the mouse mammary gland became slime producer, according to both methods

Effect of exopolysaccharide inducing media (m110 and Columbia broth) on Hty. Thirty-three strains were used to compare the effect of the growth media on Hty (Table 3). Bacterial Hty was lower after growth in CB or m110 rather than in NB (P<0.001). No significant differences for this decrease were found between CB or m110, the decrease was frequently less patent among the Ho strains (P<0.001) with some exceptions (strains C114, C141 and C193). Standard deviations corresponding to CB or m110 culture data were frequently higher when compared with those of NB culture data. Hty estimates obtained after growth in NBBW were unreliable upon retest, ranging the means from Ho to Hi for some strains. The encapsulated strain A was Hi in all media.

All Congo red positive strains were Ho (Hty= 90.6±6.3) after growth in NB. This Hty was not significantly altered when growing bacteria in CB or m110 (Table 3).

Diffuse colony morphology. The encapsulated control strain A always showed a DCM after growth in all media, including NB, but none of the 31 fresh strains showed a DCM after growth in any of the media used (three EIM and NB), or after one passage through the mouse mammary gland. Similarly, a DCM was not detected in 15 strains upon 10 passages through bovine whey.

DISCUSSION

Our results indicate that using the water-xylene procedure for *S. aureus* Hty estimation requires an adequate selection of the xylene:aqueous phase ratio and a careful monitoring of the bacterial concentration. Also, ageing of bacteria must be considered. In fact, repeated bacterial subcultures in laboratory media may alter the *S. aureus* cell wall proteins (8), the exopolysaccharide envelopes (31) and consequently, the cell surface Hty. Aged bacteria may however be "refreshed" by an "in vivo" passage through the mouse mammary gland, as also shown by Anderson (4).

On the other hand, our results show that bacterial growth phase should also be carefully monitored and selected for Hty estimations, since *S. aureus* Hty increases during the logarithmic growth-phase until reaching the stationary phase. Beck et al. (6), using one strain, obtained similar results. Furthermore, they found that at the stationary phase, Hty remained constant. We have observed a similar phenomenon in Ho and Int strains, in contrast with Hi strains, which show a definite Hty decrease after bacteria reach this phase.

In any case, and considering that ruminant milk is a very rich natural broth (13), which is frequently renewed under natural conditions and may allow an exponential type of bacterial growth, a medium-late logarithmic growth phase was selected for the remaining Hty estimations of this study using broth media. Ljungh et al. (21) using 12 *S. aureus* strains did not find any difference in Hty after 6, 18, 24 and 48 h of bacterial incubation at 37°C under agitation. Likely, under these conditions bacteria reach the stationary growth-phase by 6 h.

The growth phase-Hty relationship may be related to the fact that surface proteins, specifically protein A and fibronectin binding protein, are apparently related with Hty (16, 17). The amount of bacterial cell surface protein A and fibronectin binding protein increases along the growth curve during the logarithmic phase (11, 20), most likely resulting in an increase of Hty. The stable values observed for both proteins at the bacterial stationary growth phase (11, 20) may explain the constant Hty values observed by different authors in this phase (6, 21).

Another factor to consider when estimating Hty is the medium used for bacterial growth. Although several workers (18, 22, 23) have found that the majority of *S. aureus* bovine mastitis bacteria are highly Ho (autoaggregating) after growth (18 h, 37°C) in blood agar, patent differences in Hty have been found in relation to the type of growth medium used and to its physical state (solid vs. liquid; 21). Specifically, Cheung and Fischetti (8) showed that the cell wall proteins of *S. aureus* varied, qualitatively and quantitatively according to the type of medium used and its physical state (solid vs. liquid). This may help to explain the strong Hty variation found in this study, whereby most bacterial strains were more Hi after growth in EIM (CB or m110) rather than in NB, a regular broth medium (P<0.001; this would be expected given that bacterial capsules imply a Hi surface in *S. aureus* and other staphylococci; 14, 16, 24). These observations are similar to those of Mamo et al. (22,24) who found that the proportion of Hi bacteria after growth in m110 was higher than after growth in regular solid media (blood agar). However, in their experiment the effect due to the different physical state of media (broth vs. solid) was not separated from the effect caused by the composition of the medium.

On the other hand, as suggested by Karakawa et al. (19), the majority of *S. aureus* strains present capsular antigens ("capsule") after growth in particular media (Columbia). It is therefore likely that in our experiments a decrease in Hty is also associated with an increase in the amount of capsular antigens. In fact, Hty has been suggested as a method to determine *S. aureus* exopolysaccharide production (16).

Concerning ovine-bovine comparisons, we have found that a majority (58%) of bovine, and a minority of ovine (18%) strains are Ho in NB (P<0.001), but, we have not observed any autoaggregation (data not shown), a phenomenon which would occur in highly Ho strains, as described by other authors (21, 22, 23). This lack of autoaggregation was also observed by Ljungh et al. (21) after growing human strains in NB.

Whether the differences between bovine and ovine strains are related to differences in bacterial growth-aggregating capacity in the presence of ruminant whey (5) or to pathogenicity (more severe mastitis was found in the ovine hosts involved; 2) is unknown.

With regard to the method for exopolysaccharide detection, the presence of a DCM has been linked to the production of this extracapsular layer in *S. aureus* (15, 26, 28). Opdebeeck et al. (28) succeeded in inducing the DCM in "old" strains after growth in m110 and bovine milk, but this morphology required keeping bacteria in a logarithmic growth phase and in very low numbers. "In vivo", Opdebeeck et al. (29) reinduced this morphology after a passage through the bovine mammary gland, but not through the sheep mammary gland or peritoneal cavity. This species specific phenomenon may explain our failure to induce the DCM in the mouse mammary gland.

Jonsson (18) showed that bacteria with DCM after growth in bovine whey were more Hi than before this growth (DCM-negative). Heat labile factors in whey are responsible for the "in vitro" induction of this morphology (28). However, Mamo et al. (23) found that bovine *S. aureus* became more Hi after growth in autoclaved milk containing-medium rather than after growth in blood agar or even in m110 broth. We have used filter-sterilized bovine whey and fresh strains, but we neither succeeded in inducing a DCM, (even after 10 passages through bovine whey), nor in obtaining a Hi response after two o three passages in NBBW under the conditions indicated by Watson for induction of pseudocapsule (30). This response was not consistent upon retests. Furthermore, in our hands, the Hi response of bacteria to CB or m110 was independent from the induction of a DCM.

Costerton et al. (10) proposed that the attachment of coagulase negative staphylococci to inanimate surfaces involves two steps: firstly, bacteria attach to the surface in a weak and inespecific manner, including hydrophobic interaction, and later, bacteria bind strongly to this surface after producing a fibrous exopolysaccharide matrix (slime; 11) within which bacteria grow. The formation of *S. aureus* microcolonies surrounded by an exopolysaccharide matrix has been described "in vivo" by Mayberry-Carson et al. (25).

With this in mind, it is tempting to hypothesize that Congo red positive *S. aureus* strains also develop this strategy, requiring a Ho surface for the first attachment step. The fact that these Congo red positive strains (also forming biofilms on glass tube walls, as detected by the Christensen's method) did not show a Hi response after growth in CB or m110 may have two alternative explanations, both suggesting that the nature of the slime production mechanism in Congo red would be different from that in CB or m110: a) these strains did not produce slime in CB or m110; and b) these strains lost the slime produced in CB or m110 more easily than other strains which behave as Hi in these media. The latter explanation is in agreement with findings in coagulase negative staphylococci, where slime, detected by the Christensen's method, is a very loose structure which is lost during washing (14).

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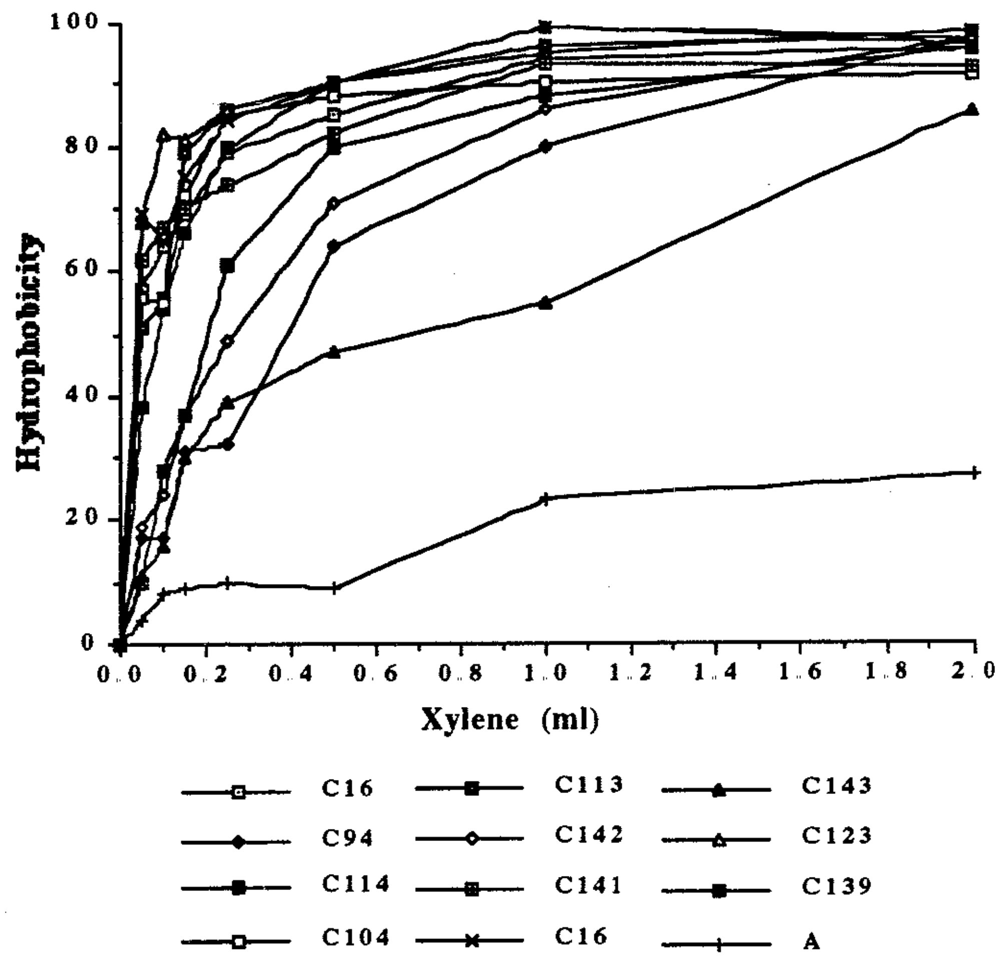

Fig. 1. Illustration of the effect of the xylene:aqueous phase ratio on hydrophobicity

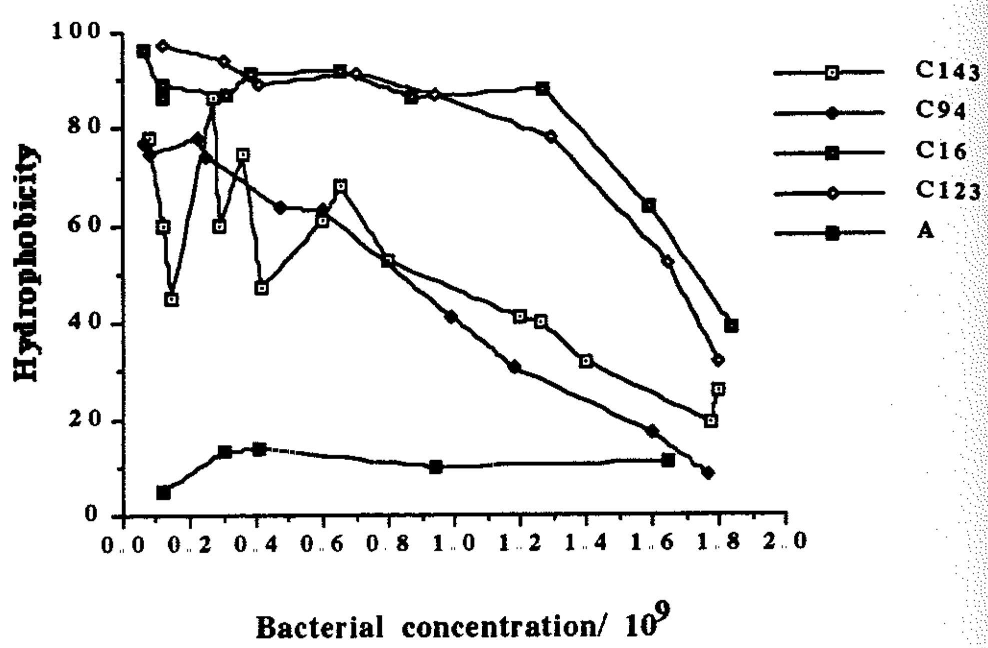

Fig. 2. Illustration of the effect of bacterial concentration on hydrophobicity using 5 strains.

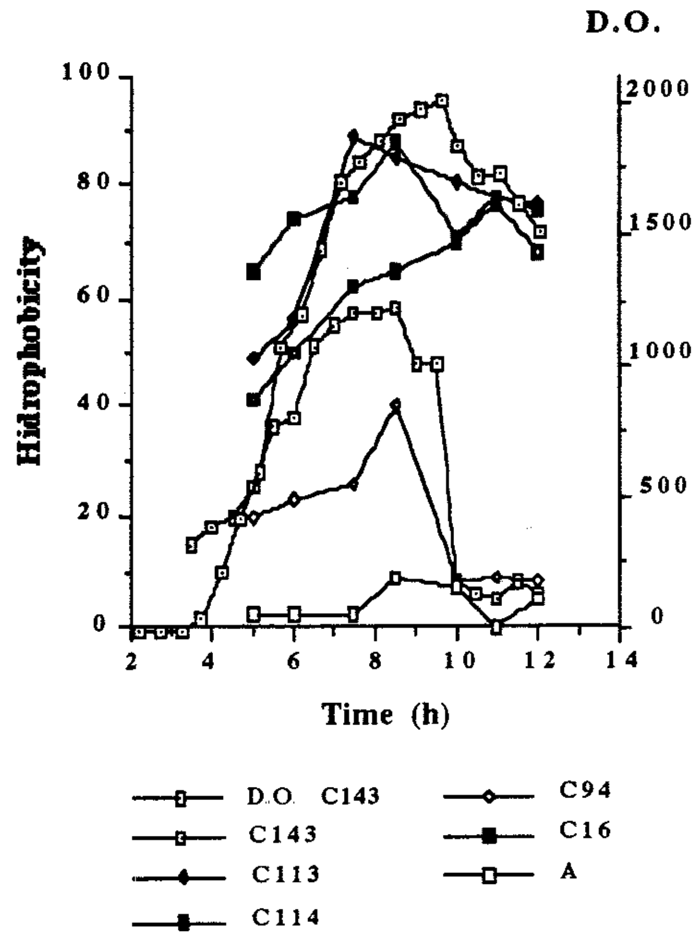

Fig. 3. Effect of bacterial growth phase on hydrophobicity

Table 1. Effect of bacterial ageing of bovine strains. Strain proportions indicating the hydrophobicity types

Strain age	Hydrophobic type		
	Hydrophobic (Hty>70%)	Intermediate (Hty>40%;<70%)	Hydrophylic (Hty<40%)
Fresh	26/45	14/45	5/45
Old	0/18	16/18	2/18
Refreshed	5/7	2/7	0/7

*Old strains after passage through the mouse mammary gland

Table 2. Effect of the host (bovine/ovine) used as bacterial source on the strain proportion obtained with regard to hydrophobicity types

Strain source and age	Hydrophobic type		
	Hydrophobic (Hty>70%)	Intermediate (Hty>40%;<70%)	Hydrophylic (Hty<40%)
Bovine Fresh	26/45	14/45	5/45
Ovine Fresh	2/8	5/8	1/8
Refreshed	3/14	9/14	2/14
Total	5/22	14/22	3/22

Table 3. Effect of the growth medium on Hty and in fresh strains and the encapsulated strain A and slime production

Strain	Hty in different growth media			Slime production
	NB	CB	m110	
C108	96,7±3,4	95,3±3,7	84,0±5,7	+
C113	94,5±2,7	96,4±2,6	85,5±2,5	+
C171	92,3±1,3	85,2±5,6	89,4±4,3	+
C172	92,0±1,3	93,2±3,3	91,7±1,5	+
C16	90,7±4,0	88,7±2,4	82,7±3,7	-
C104	88,7±4,9	83,2±9,9	85,4±8,5	+
C193*	85,0±2,9	54,9±5,8	21,5±4,8	-
C26	86,6±5,3	63,7±10,8	67,7±8,0	-
C167	83,7±2,6	82,2±5,6	70,9±8,6	-
C164	83,7±4,9	67,0±7,8	78,0±5,4	-
C163	83,0±6,2	74,0±1,6	84,2±9,7	-
C141	83,0±4,3	0,0±0,0	28,0±12,4	-
C114	81,7±2,5	16,7±21,3	15,2±16,0	-
C123	80,0±6,0	67,2±8,8	63,7±4,6	-
C175	79,7±6,1	58,0±5,3	72,3±5,0	-
C144*	78,5±5,2	37,7±6,9	26,5±9,7	-
C160	72,0±4,7	10,8±16,3	1,6±10,2	-
C162	67,7±7,0	21,7±7,4	29,7±14,8	-
C137	65,5±2,0	22,0±9,0	34,0±10,9	-
C159	64,7±2,1	48,6±14,5	32,2±10,8	-
C158	61,5±5,9	64,7±14,7	39,1±18,8	-
C225*	57,5±3,2	19,0±7,0	27,0±4,6	-
C139	57,2±5,2	59,0±9,1	37,3±7,6	-
C195*	47,3±6,0	60,2±9,9	49,3±7,3	-
C142	46,5±5,6	23,0±11,9	19,7±14,2	-
C161	46,2±7,2	4,4±6,1	28,0±11,4	-
C224*	45,2±5,3	4,7±4,0	31,7±10,8	-
C143	39,5±3,4	0,0±0,0	0,0±0,0	-
C176	35,4±2,9	0,0±0,0	0,0±0,0	-
C94	30,0±5,4	14,5±14,5	7,6±12,7	-
C194*	30,0±1,6	19,0±8,7	29,7±2,9	-
A	3,0±3,1	0,0±0,0	0,0±0,0	-

* Ovine strains (the remaining strains are of bovine origin, with the exception of the human strain A).

VI

Production of ruminant mastitis *Staphylococcus aureus* slime negative variants from slime positive strains.

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Production of Ruminant Mastitis *Staphylococcus aureus* Slime Negative Variants from Slime Positive Strains.

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ABSTRACT

Slime was detected in 21 out of 144 ruminant mastitis *Staphylococcus aureus* strains applying the methods commonly used for coagulase negative staphylococci (i.e., tube biofilm formation and Congo red agar). The 21 slime producing (SP) strains showed an exopolysaccharide matrix condensed around the bacterial cells, as observed by electron microscopy and immunofluorescence. Upon repeated subcultures in Congo red agar, eight non slime producing variants (4 ovine and 4 bovine) were obtained from the SP strains, and two SP variants (ovine) were obtained from their corresponding non slime producing strains.

Among the 92 bovine and 52 ovine strains tested, 26.6% and 7.5%, respectively, had the capsular serotype 5. Only one SP strain had this exopolysaccharide type. Partially purified slime from SP strains and capsular material from a heavily encapsulated strain were used for immunizations. The antibodies produced were immunologically distintc, suggesting that slime and capsule are different structures.

INTRODUCTION

Bacterial capsules are present in a low percentage of mastitis *Staphylococcus aureus* strains, according to the classical negative staining capsulation criterion (31). However, the majority of *S. aureus* strains produce an exopolysaccharide layer when immunological detection methods are applied. Serotypes 5 and 8 are the most frequent among the majority of strains, including ruminant mastitis strains (18, 27). The production of this layer (slime) by coagulase negative staphylococci (CNS) has been related to "in vivo" colonization of surgery materials (28) and has been detected "in vitro" either by the type of colony morphology produced in Congo red agar (CRA; 13) or by the capacity of bacteria to form biofilms on the walls of a tube (9). Furthermore, slime producing (SP) and non slime producing (NSP) CNS variants have been obtained "in vitro" (9). In this work, the tube biofilm formation and the CRA techniques were used to detect slime in ruminant mastitis *S. aureus* strains. *S. aureus* SP and NSP variants were also obtained in vitro. Immunological differences were found between *S. aureus* slime and capsules exopolysaccharides layers.

MATERIAL AND METHODS

Bacteria. Ninety-two bovine and 52 ovine *S. aureus* mastitis strains were isolated and identified as *S. aureus* by their colony morphology, Gram staining, haemolytic activity, DNase, and coagulase activity. SP strains were further identified as *S. aureus* by the API-Staph system (Biomérieux, France).

Slime production. Slime production was determined by the capacity of bacteria to form biofilms on tube culture walls, as described by Christensen et al. (9), and by the colonial morphology in CRA, as described by Freeman et al. (13). Both techniques showed a complete agreement in *S. aureus* strains. Although in the tube biofilm formation assay, reproducibility was only obtained after several refill cycles.

Production of variants. Nineteen bovine and 20 ovine *S. aureus* strains were used to obtain variants. Nineteen (9 bovine and 10 ovine) of these strains were SP and 20 (10 bovine and 10 ovine) were NSP.

After several passages (10-20) of the 19 SP strains through CRA, variants with a colony morphology characteristic of NSP strains were obtained. A similar procedure to that described by Christensen et al. (8) was used to obtain SP from NSP strains. Briefly, to encourage the growth of SP strains (able to grow in biofilms on tube walls), the 20 NSP strains were seeded in TSB containing 2% glucose (TSB-G) and incubated without agitation at 37°C. Daily, for one month, tubes were washed once with sterile PBS after discarding culture medium to eliminate non-adherent bacteria, and refilled with TSB-G. SP variants were detected by seeding adherent bacteria in CRA.

To insure that variants were not contaminant bacterial strains, all strains and their variants were biotyped with the API-Staph system and API 20E (BioMérieux) and with a panel of 14 antibiotics. The intensity of color and inhibition diameter were scored. All variants which did not resemble the original strain in all tests were discarded.

Serotyping of type 5 capsular polysaccharide. Before testing, bacteria were grown overnight in m110 agar and prepared as described by Opdebeeck et al. (26). Type 5 capsular polysaccharide was serotyped using the anti-type 5 monoclonal antibodies, kindly donated by Drs P. Rainard, P. Sarradin and B. Poutrel (INRA, Nouzilly, France) and according to the enzyme linked immunosorbent assay (ELISA) described by P. Sarradin (personal communication). Briefly, a flat-bottom microplate (Nunc MaxiSorp, Roskilde, Denmark) was coated (100 μl per well) with a bacterial suspension in PBS (A_{600}=0.2). After overnight incubation at 4°C, plates were washed with PBS-0.1% Tween 20, blocked with 0.5% gelatin (150 μl) at 37°C for 45 min, and washed four times with PBS-0.1% Tween 20. The anti-serotype 5 monoclonal antibody was diluted 1:50 in PBS-0.1% Tween 20 plus gelatin (0.5%)

and 100μl aliquotes were added to the wells. Following an incubation of 60 min at 37°C, wells were washed with PBS-0.1% Tween 20 four times and 100 μl of a sheep anti-mouse peroxidase conjugated immunoglobulin G (Pel-Freez; USA) were added to the wells and incubated at 37°C for 60 min. One hundred μl of enzyme substrate [2,2'-azinobis (3-ethylbenzthiazoline sulfonic acid), 0.11 mg/ml; Sigma, USA] were added in citrate buffer (pH 4.2) with 0.001% hydrogen peroxide. After 20 min under agitation at room temperature, the reaction was stopped by the addition of 1N HF (100 μl per well). The optical density was read at A414. All strains were tested at least twice on different dates.

Slime isolation. The technique described by Fournier et al. (12) with modifications was used. Briefly, strain SP C104+ was grown in TSB-G overnight at 37°C. Two mililiter aliquotes were inoculated into 1.5 liter volumes of TSB-G and the suspensions were incubated for 7.5 h at 37°C with agitation (120 rpm) using two liter bottles, until bacteria reached the end of the exponential growth phase (a total of 16.5 TSB-G liters were used). Bacteria were sedimented by two centrifugations of 30 min (5500 x g) and discarded. High-weight soluble components were recovered in approximately 500 ml using a 10,000 dalton Pellicon cassette (Millipore Corp., Bedford, Mass.). The supernatant was supplemented with sodium azide, to prevent bacterial growth, and incubated overnight at 37°C with 50 mg of DNase (Boehringer), and 10 mg of RNase (Boehringer). Both enzymes were added again, in the same proportions, the following morning. After 6 h, an overnight treatment with 50 mg of pronase (Boehringer) digestion at 37°C was applied. Pronase was added again the following morning. After a 6 hour incubation at 37°C, the remaining proteins were removed by phenol extraction (phenol:chloroform:iso-amilic alcohol; 25:25:1). The sample was exahustively washed with distilled water, using a 10,000 dalton Pellicon cassette, and subsequently lyophilized. Slime was purified by this procedure to give a final yield of 1.258 g of polysaccharide containing less than 1% protein, as determined by Bradford's method (4) and less than 0.5% nucleic acid, as determined by absorbance at 260 nm.

Obtainment of capsule-enriched suspensions. The encapsulated strain A (kindly supplied by Dr. K. Yoshida) was grown overnight at 37°C in Columbia broth supplemented with 4% sodium chloride. After seeding 200 μl of this culture in 40 ml of the same medium, bacteria were incubated with agitation at 37°C for 6h, until reaching the end of the exponential growth phase. Subsequently, bacteria were washed twice in PBS, resuspended to a final concentration of 3.5 x 10^9 bacteria/ml. To obtain capsular antigens, bacteria were autoclaved for 60 min at 121°C (12). After centrifugation, supernatants containing capsular material were stored at -20°C.

Immunizations. Two rabbits were subcutaneously immunized with 10 mg of slime (from strains SP C104+), which had been resuspended in 0.75 ml of PBS and emulsified with 0.25 ml of Freund's incomplete adjuvant. Injections were administered 5 times at two weeks intervals. Serum samples were tested 12 days after each injection and the presence of antibodies against slime was determined by double immunodiffusion against a slime suspension (15 mg/ml PBS). Only the strongest of the rabbit antisera obtained was used for further experiments.

The strain variant NSP C104- was used to absorb the anti-slime sera obtained according to the procedure applied by Karakawa et al. (19). Briefly, strain C104- was grown in TSB for 18 h at 37°C. Two ml of this suspension were seeded in 1 liter of TSB and incubated with agitation (120 rpm) during 7 h at 37°C, until bacteria reached the end of the exponential growth phase. Subsequently, bacteria were heat-inactivated at 80°C for 4 h and washed twice with PBS. Rabbit antisera were absorbed adding 8 volumes of serum to 1 volume of packed cells, stirring and incubating overnight at 4°C. Cells were removed by centrifugation and the supernatant (absorbed serum) was stored at -20°C.

Anti-capsule sera were obtained according to the procedure of Norcross and Opdebeeck (26) by inoculating strain A in two rabbits. The strongest of these sera was used for further experiments.

Immunofluorescence. After growth in TSB-G or CRA, bacteria were resuspended to $5x10^8$ bacteria/ml in PBS and a drop of this suspension was allowed to dry on a slide at 37°C. After fixation with acetone (20 min at -20°C), slides were washed and dried. A 15 μl amount of the undiluted rabbit anti-slime serum absorbed with strain C104- was added. After a 45 min incubation in a humid chamber, the slide was gently rinsed with PBS-0.1% Tween 20 (10 min, with agitation) and dried; 20 μl of FITC sheep anti-rabbit serum (kindly supplied by Dr. J.M. Blasco, Dept. of Animal Production, SIA, Zaragoza, Spain) were added. Incubation and washing were carried out as described for the primary antibody. After drying at 37°C the samples were observed under a fluorescence microscope (Nikon Optiphot, Japan).

Immunodiffusion. Double diffusion tests were done using 0.8% agar. Reactions were evaluated at 24h. Rabbit anti-slime and rabbit anti-capsule sera were tested against the partially purified slime and capsule-enriched suspensions obtained, as described, from strains SP C104+ and A respectively. Capsule-negative and slime-negative preparations were obtained from a non encapsulated NSP strain (C16), according to the procedures applied to obtain capsule and slime preparations, respectively.

Electron microscopy. After an overnight growth on CRA, bacteria were scraped off the plates and placed in 2.5% buffered glutaraldehyde for 90 min, washed twice by centrifugation (180 x g for 10 min), resuspended in Milloning buffer and centrifuged at (180 x g for 10 min) to obtain a solid pellet. The pellet was fixed for 30 min in osmium tetroxide. After a final wash in buffer and dehydration in ethanol, the fixed pellet was cleared in propylene oxide and embedded in Epan-Araldite (1:1). Ultrathin (400 to 600 Å) sections were made, using an LKB-8800 ultramicrotome, and stained with uranyl acetate and lead citrate. Ultrathin sections were studied by transmission electron microscopy.

RESULTS

Slime production. A total agreement was observed when the results obtained with the two techniques used (tube biofilm formation and CRA) were compared. Only 11 of the 92 bovine strains (12%) and 10 of the 52 ovine strains tested (19%) were SP (Table 1).

Production of variants. Eight NSP variants (4 bovine and 4 ovine) were obtained from the SP strains, and 2 SP variants from ovine NSP strains. Three other putative variants were discarded because some of the properties analysed were different from those of the original strain.

Electron microscopy. A condensed extracellular layer was observed in the 5 SP strains which were examined by electron microscopy (Fig. 1a), but this layer was not present in any of their NSP variants or in any other NSP strains (Fig. 1b).

Immunofluorescence. In the immunofluorescence assays with slime-specific antibodies, fluorescence was observed in SP strains after growth in TSB-G or in CRA, but not in NSP strains.

Immunodiffusion. As shown in Fig. 2, the capsule-enriched antigen preparation (well I, Fig. 2a; wells I and II, Fig 2b) reacts specifically with one of the antibody populations of the "anticapsular" antiserum (line 1, Figs 2a and 2b). Similarly, the slime antigen preparation (well II, Fig 2a; wells III, IV and V, Fig. 2b) reacts with one of the antibody populations of either the "anti-slime" or the "anti-capsular" antiserum in a specific manner (line 2, Fig 2a; lines 2 and 3, Fig. 2b). Other precipitation lines are also observed, indicating that the "anti-slime" and "anti-capsular" antiserum are polyspecific and that the "slime" and "capsular" preparations have various molecular species.

Capsular serotype 5. Twenty five among 94 bovine strains (26.6%) and only 4 among 52 ovine strains (7.5%) were of serotype 5.

Among the 21 SP strains present, only one bovine SP strain belonged to serotype 5.

DISCUSSION

In this work, *S. aureus* slime production has been shown "in vitro" using the tube biofilm production (9) and CRA (13) methods, commonly applied for slime detection in CNS species. The SP *S. aureus* strains formed a condensed matrix surrounding the bacterial cells, as observed by electron microscopy and immunofluorescence. NSP variants were obtained from SP strains and viceversa. The NSP variants and other NSP strains did not show a matrix after growth in CRA or in TSB-G. As observed in these bacteria, the condensed morphology of the slime in SP strains was likely due to the fact that fixation was carried out in the absence of fixative antibodies (5). This slime was most likely a polysaccharide since it was extracted according to the exopolysaccharide isolation methods applied for other bacteria (12), contained less than 1% protein and 0.5% nucleic acids and had a low immunopotency. The colonies of SP strains in CRA were compact and required an intense sonication to separate bacteria. This compacity was likely attributable to the strong binding of bacteria by the exopolysaccharide matrix.

Bacterial hydrophilicity has also been proposed as a criterion to detect capsular exopolysaccharide production (17). We have shown that strains classified as SP (according to the described tube film production and CRA colony morphology criteria) remain hydrophobic when grown in exopolysaccharide-inducing liquid media (e.g. m110) rather than in regular media (e.g. TSB). In these special media, however, encapsulated strains maintained their hydrophilicity (unpublished data). These results indicate that SP strains behaviour is different from that of encapsulated strains, suggesting that SP strains *S. aureus* slime is different from capsule. In line with this observation is the fact that at least in CNS (15) or in human *S. aureus* strains (32), slime is a loosely bound structure, easily removable by washing and centrifugation. This removal renders

bacterial surface more hydrophobic. In contrast, encapsulated CNS keep their hydrophilicity after washing (15).

Differences between capsule and slime were also evidenced by immunodiffusion. Furthermore, Caputy and Costerton (5,6), demonstrated by immunological methods and by electron microscopy that *S. aureus* may produce slime in addition to capsule "in vivo" and after growth in a exopolysaccharide-inducing media (m110). In this work, two strains were able to produce slime in addition to capsule: one strain (V50) had capsular type 5 after growth in m110 and was SP in CRA; also, the encapsulated strain A was able to trigger the production of anti-slime antibodies after growth in m110, although it was NSP in CRA. The fact that strain A produced exopolysaccharide in m110 but was NSP in CRA could be explained if different mechanisms affecting either the quality or quantity of slime are involved when comparing the exopolysaccharide induction capacity of m110 and CRA.

Christensen et al. (9) obtained "in vitro" SP and NSP variants in CNS and suggested a mechanism of "phase variation" to explain the biological processes involved. According to these authors, the expression of the genes under concern varies in a reversible manner from generation to generation at a relatively rapid rate. Our results are compatible with this hypothesis since variants were easily obtained. This observation suggests that small numbers of NSP may arise out of a given SP bacterial population, giving rise thereafter to a large NSP population by fixing the NSP bacterial cells in place. Likely, "in vitro" NSP variants may have a faster growth-rate. In fact, it is known that although, the exopolysaccharide layer is produced by most *S. aureus* strains isolated from bovine mastitis milk (16, 26, 30), bacteria rapidly loose this layer by "in vitro" subculture. The opposite events would likely occur when changing from the "in vitro" to the "in vivo" situation.

It has been observed that in infectious processes the majority of bacteria form microcolonies. Hence, bacterial adherence to epithelial surfaces appears to proceed in two phases: in the first, microorganisms adhere losely to tissues (or other surfaces) and in the second, adherence becomes irreversible by the formation of microcolonies embedded in an exopolysaccharide matrix (10). However, it is not yet clear whether extracellular slime functions as a "glue" in the initial adhesion phase, or whether it is produced only after bacteria have adhered to other cells or surfaces and have been subjected to metabolic stress (29). Furthermore, it is known that antibody against slime inhibits the "in vitro" and "in vivo" the adherence of SP CNS to silicon elastomer catheters (20). This observation suggests the possibility that "slime" may act as an adhesin to epithelial cells (16).

An "in vivo" mechanism of adherence to the intestinal epithelium for *Escherichia coli* has been proposed whereby adhesins (fimbriae) are responsible for the initial adhesion and "capsular" exopolysaccharides are responsible for the formation of microcolonies in which bacteria multiply thereafter (7). A similar mechanism could be proposed for *S. aureus*, considering the facts that *S. aureus* may adhere to the mammary gland ducts and alveoli via basal membrane proteins (23) or to epithelial cells (1) via specific receptors (22) and that this bacterial species may grow not only "in vitro" but also "in vivo" forming microcolonies enclosed within a fibrous exopolysaccharide matrix (24).

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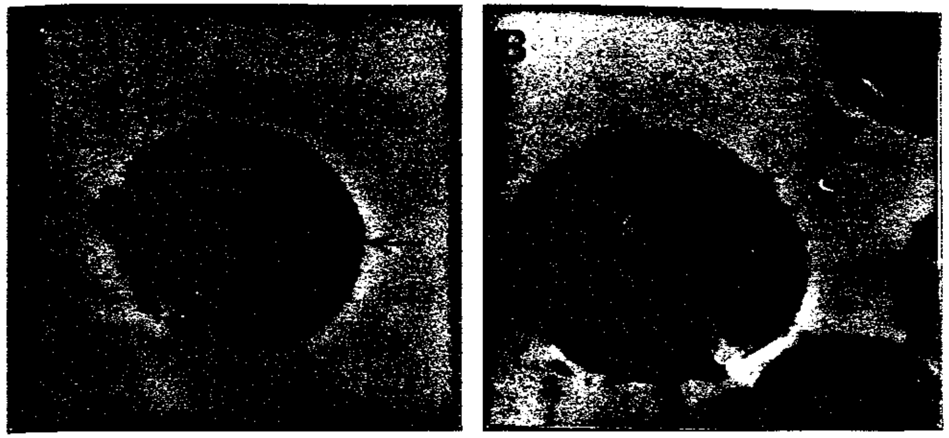

Fig. 1. Electron micrographs of thin sections of *S.aureus* cells grown in CRA. (a) Slime producing strain C104+; and (b) slime non producing strain C104-. Arrows show slime.

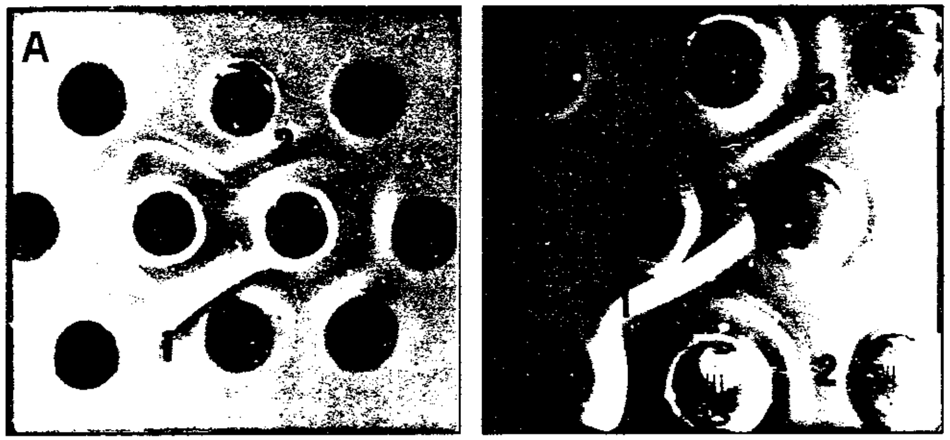

Fig. 2. Two-dimensional immunodifusion. Capsule enriched antigenic preparation of strain A (well I, Fig. 2a and wells I, II and III, Fig. 2b). Slime purified material (well II; Fig. 2a and wells IV, V and VI, Fig. 2b). Slime negative material (wells III, IV, V and VI, Fig. 2a). Capsule negative material (wells VII and VIII). Antiserum against complete cells of the encapsulated strain A (well IX, Fig 2a and well VII, Fig. 2b). Slime purified antiserum (well X, Fig. 2a and well VIII, Fig. 2b).

Tabla 1. Slime and serotype 5 detection among the ruminant strains and the variants produced.

			SP		Obtainment of variants			
					NSP		SP	
Source of strains	No of strains tested	Total No , of serovar 5	No of strains	Serovar 5	No of original SP strains used	No. of NSP variants obtained	No. of original NSP strains used	No. of SP variants obtained
Bovine	92	25	11	1	9	4	10	0
Ovine	52	4	10	0	10	4	10	2

VII

Staphylococcus aureus capsule and slime as virulence factors in mastitis. Review article.

Baselga, R. y Amorena, B.

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Staphylococcus aureus capsule and slime as virulence factors in mastitis

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Review article

ABSTRACT

Baselga, R. and Amorena, B. 1991. *Staphylococcus aureus* capsule and slime as virulence factors in mastitis. *Vet. Microbiol.*,

Staphylococcus aureus is one of the most prevalent bacterial species in bovine mastitis. The interaction of this microorganism with the host is strongly dependent on its cell surface properties, specially concerning the presence of exopolysaccharide-containing outer layers (glycocalyx). Although there is controversial literature on the denomination (capsule, microcapsule, pseudocapsule, slime), nature and role of these outer layers, they appear to play an important virulence role against host defence mechanisms. In this article, literature is reviewed in an attempt to clarify discrepancies and to underline the multiple effects of these layers on various phenomena involved in mastitis, concerning the interaction between host and bacteria (phagocytosis, adhesion to cells, bacterial aggregation, complement fixation, microcolony formation, etc.).

1. INTRODUCTION

Mastitis, a mammary gland disease commonly attributed to infections by microorganisms, constitutes a serious problem, affecting milk production in ruminants (quantity and quality of products). One of the most prevalent and pathogenic bacterial agents involved in this disease is *Staphylococcus aureus*, the species under concern in this study. To elicit an infection, *S. aureus* must reach the gland cistern, after overcoming the physical barrier of the teat, generated by the action of the teat sphincter and by the presence of keratin on the epithelium, covering or occluding the teat duct. It must thereafter avoid or overcome the different host defence mechanisms (phagocytosis, elimination by milking, etc.), in order to reach and colonize the ductular and alveolar mammary gland regions.

Many *S. aureus* strains are able to produce an extracellular layer(s) (capsule, slime, etc.), surrounding the cell wall. This structure has been associated with virulence against host defence mechanisms. In this article, the methods of identification of *S. aureus* extracellular envelopes, the biological role of these envelopes and their possible implication in the development of mastitis are reviewed.

2. PROPERTIES OF TRULY ENCAPSULATED (E) AND SLIME PRODUCING STRAINS

According to Wilkinson (1983), *S. aureus* capsule is a well defined structure that surrounds the cell wall and is maintained through cell subcultures "in vitro", independently of the medium used. It is evidenced by negative staining (for example, with Indian ink), because the stain does not penetrate into the capsular gel. Yoshida and Minegishi (1976), besides carrying out immunizations and subsequent serological absorptions to distinguish encapsulated from non-encapsulated strains, postulated that the truly encapsulated (E) *S. aureus* strains cannot be phagotyped, do not react in the slide coagulase test and produce a diffuse colony morphology (DCM), with the shape of a comet in serum-soft agar (SSA; Fig. 1). On the contrary, non truly encapsulated (NE) strains can be phagotyped, react positively in the slide coagulase test and produce a compact colony morphology (CCM) in SSA (Fig. 1).

Let us consider now the slime of *S. aureus*. According to Wilkinson (1983), this slime is a mucus type of extracellular structure produced "in vitro" by the majority of strains (E and NE strains; Yoshida and Ekstedt, 1968; Caputy and Costerton, 1982) in response to specific nutritional conditions; for example, during growth in modified *Staphylococcus* 110 medium (m110). It is loosely bound to the cell wall and can easily be eliminated during washing (Caputy and Costerton, 1982; Wilkinson, 1983; Watson, 1989a). However, in some strains with great capacity to produce mucoid material in media such as m110-agar, the presence of slime can be evidenced using the criteria already mentioned for the detection of capsule in E strains (Yoshida and Ekstedt, 1968), provided that they are carefully handled in order to avoid the loss of this layer.

According to Caputy and Costerton (1982; 1984), *S. aureus* exopolysaccharide matrix, also called glycocalyx, may include both the true capsule and/or the slime layer. Thus, the freshly isolated and the m110 cultured Smith strain (E) contains both layers, whereas the Willey strain (NE) only contains slime

3. INTERPRETATION OF E STRAINS BEHAVIOUR

3.a. E strains in slide coagulase and phagotyping assays

Boden and FlocK (1989) have corroborated the identity between free extracellular coagulase and coagulase bound to *S. aureus* cell surface, and have shown that this enzyme is able to bind fibrin (a fibrinogen degradation product), exerting with this the function of bacterial aggregation factor or clumping factor. These authors, have suggested that bound coagulase causes first fibrinogen degradation and later bacterial aggregation. For this reason, the resulting fibrin fibers trap staphylococci

forming large aggregates. This hypotesis is consolidated by the results of Umeda et al. (1980), showing by electron microscopy that staphylococci are bound togheter by fibrin fibers in these aggregates.

Wilkinson (1983) proposed that degradation products produced by the action of clumping factor remain immersed within the capsular gel, thus impeding the formation of a net between cells and of the consequent aggregates (Table 1; Fig. 1). This would explain why the E strains behave as clumping factor-negative strains, whereas NE strains of *S. aureus* form big aggregates in the presence of plasma or fibrinogen (Smith et al., 1971).

An analogous explanation could be applied to the fact that, according to some authors, E strains are not phagotypable. Considering that phage receptors are located on the bacterial cell surface (Umeda et al., 1980), phages may be too large to traverse capsules, thus remaining excluded from bacteria (Wilkinson, 1983).

3.b. Formation of diffuse/compact colonies

All *S. aureus* strains form DCM colonies in soft agar (semi-solid medium). However, only NE strains may show a CCM if this medium is supplemented with regular serum (SSA), fribrinogen or fibronectin (Usui et al., 1984). E strains show a DCM under similar conditions, but they may however show a CCM if SSA is supplemented with antisera against capsular antigens (Yoshida, 1971; Chomarat et al., 1989).

Forsum et al. (see Wilkinson, 1983) showed that due to the reaction between the cell wall protein A and the Fc region of IgG, bacterial daugther cells bind to each other during the development of the colony, giving rise to CCM in SSA. This agglutination reaction can easily be understood when the IgG involved is directed against *S. aureus*, or when protein A interacts with immunoglobulins via the Fab region (Forsgren et al., 1983). Wilkinson (1983) proposes that the inagglutinability (i.e., the formation of DCM) of E strains can be attributed to the fact that immunoglobulins traverse the capsule and react with protein A of the bacterial cell wall, but they remain trapped within this capsule and are therefore unable to promote adherence between daughter cells (Fig. 1, Table 1).

As it occurs with fibrinogen, fibronectin is able to agglutinate NE *S. aureus* strains given its dimeric nature (Proctor et al., 1984). For this reason, both proteins can induce the formation of CCM. The presence of a capsule will abrogate agglutination, resulting in the formation of a DCM, as it occurred in the case of immunoglobulins. Other agglutinins of *S. aureus* (type IV collagen, laminin, etc.) can also induce aggregation (Vercellotti et al., 1985) and presumably CCM formation in NE strains.

4. CAPSULAR ANTIGENS FIRST DESCRIBED BY YOSHIDA

4.a. Detection of capsular antigens using antibodies

As we have already mentioned, the addition of anticapsular serum to SA induces in E strains the formation of CCM (Yoshida 1971; Chomarat et al., 1989). With this technique, Yoshida and his group have detected four capsular serotypes (A, B, C and D), being antigens A (rich in teichoic acids), and B (lacking them) the most frequent types (Yoshida et al., 1979). They also found that the majority of human strains only belong to one antigenic type, that is, they are univalent (Yoshida and Minegishi, 1976; Yoshida et al., 1979). Also in bovines, E strains, A and B antigens are the most frequent, but the proportion of polivalent strains is much higher than in humans (50%; Yokomizo et al., 1977).

After observing the bacterial surface at the electron microscope, Norcross and Opdebeeck (1983) concluded that the presence of specific antibodies against capsular antigens in SSA, inhibits the formation of capsules. However, it is not clear whether the bacteria used in this study were E or simply slime producers.

4.b. Presence of capsular antigens in non-truly encapsulated (NE) strains.

Immunization of mice with human NE strains may result in resistance to E strains (Yoshida and Minegishi, 1976), and specific antibodies against capsular antigens may protect mice against infection with human NE strains (Yoshida et al., 1987). These findings are easily understood keeping in mind that the majority (77.6%) of human *S. aureus* NE strains show detectable quantities of capsular antigens on their cell surfaces, as can be shown using fluorescein labelled antibodies (Yoshida et al., 1979). Furthermore, according to the suggestions of Chomarat et al. (1989), the majority of *S. aureus* strains do not produce large capsules, but microcapsules or small quantities of capsular substance. Yoshida and Minegishi (1976) have estimated that these NE strains possess capsular antigens in proportions ranging from 1/7 to 1/20 of the amount produced by the Smith-diffuse E prototype strain, revealing by electron microscopy a partial capsule surrounding the cells (Table 2). These findings may suggest that the possibility to determine the presence of capsule following the criteria described by Yoshida depends on the amount of capsular substance (exopolysaccharide) produced. Yoshida and co-workers (1979) proposed that "in vivo" the majority of bacteria are partially encapsulated, that is, they possess these microcapsules. In agreement with this hypothesis is the fact that the majority of human sera present anticapsular antibodies, suggesting that a broad distribution of staphylococci is able to trigger the production of capsular antigens "in vivo" (Yoshida and Ichiman, 1984b; Yoshida et al., 1987).

It is possible that a hostile environment favours the expression of the genes responsible for the production of "capsules", an effect which is not produced by the majority of culture media commonly used in the laboratory. In fact, Lee et al. (1985) were able to obtain from an E strain (SA1) an NE variant and re-induce "in vivo" the presence of a capsule in this variant.

The regulation of *S. aureus* exopolysaccharide production has been well established (Hamill et al., 1986, Recsei et al., 1986) and extensive work has been done in Gram negative bacteria and Streptococci (Foster, 1991) on the genetic control of exopolysaccharide expression. However, there is only one piece of evidence for the existence of exopolysacccharide expression control in *S. aureus*, whereby a transposon insertion mutation is shown to affect the biosynthesis of capsules (Lee et al., 1987).

5. FREQUENCY OF E STRAINS

With the capsulation criteria established by Yoshida, truly encapsulated strains (Table 2) have only exceptionally been found (Wilkinson, 1983). In bovine mastitis, Yoshida and co-workers (1977) isolated only 19 E strains among 1598 strains (0.92%), maintained through "in vitro" subcultures during several months, and 5 among 79 fresh strains (6.32%); the exact meaning of the term "fresh" was not described in this study. When bacteria are inoculated directly from the mastitic bovine milk in SSA, 85 to 100 per cent of the *S. aureus* strains may show DCM (Norcross and Opdebeeck, 1983; Opdebeeck and Norcross, 1983; Rather et al., 1986; Opdebeeck et al., 1988a), but this morphology is rapidly lost during "in vitro" culture. In human strains, Yoshida and his group (102) obtained similar percentages (83% E strains). However, Opdebeeck and co-workers (1985a) found that only 31 per cent of the fresh human *S. aureus* strains show DCM. In any case, it is clear that for many strains, the ability to form a DCM can be lost through subcultures. These strains cannot be considered as E and consequently, as pointed out by several authors (Anderson, 1984; Jonsson et al., 1989; Rather et al., 1986), a DCM is not necessarily associated with true encapsulation (Fig. 1; Table 2).

The formation of DCM appears to be advantageous for bacteria in staphylococcal mastitis (Table 1), but it is reasonable to expect that once bacteria have been isolated, the selective pressures that induce the DCM formation disappear in the majority of "in vitro" culture media and thus the strains that produce DCM revert rapidly to CCM producers. Lam et al. (1980) suggested that the reversion of bacteria to non slime producers implies the raise and selection of non-mucoid variants with a faster growth rate. According to these authors, the opposite phenomenon could be applied for "in vivo" conditions. In fact, significantly increased *S. aureus* glycoclayx production is observed "in vivo" as compared with the "in vitro" situation in streptococci (Dall and Herndon, 1989).

6. OTHER CAPSULATION CRITERIA: FROM MICROCAPSULES TO LARGE CAPSULES

6.a. Serologic criteria for capsulation first applied by Karakawa

With respect to the concept of capsule and determination of antigenic types, different criteria from those of Yoshida and co-workers have been postulated. Whereas Yoshida and his group only classify as truly encapsulated (E) those bacteria that produce capsules independently from the culture media used, Karakawa and his group determine as encapsulated those strains that exhibit capsular antigens within either a large or a small "capsule" (microcapsule) in some culture media (Columbia). These antigenic structures are constituted by exopolysaccharides, their appearance varies according to the culture conditions and they can be visualized by electron microscopy (Karakawa et al., 1985; Hockeppel et al., 1987; Table 2).

The presence of teichoic acids is considered by Karakawa and co-workers as a characteristic of the bacterial wall and not of the capsular antigens. Hence, these authors propose that teichoic acids remain "hidden" by the "capsule" or the microcapsule. For this reason, and using highly diluted anticapsular antibodies of high titers, these authors established two "capsulation" criteria: the bacterial inagglutinability in the presence of antibodies against teichoic acids and the detectability of the capsular antigen(s) (serotype) implied in each case. The reverse criteria were therefore used to determine the absence of "capsule" (Karakawa et al., 1985). The existence of these capsular antigens "in vivo" has been shown in natural diseases (Albus et al., 1988; Boutonnier et al., 1989) and in experimental infections (Arbeit and Dunn,1987; Arbeit and Nelles,1987).

Karakawa and Young (1979) have suggested that in the strains with microcapsule, the antibodies directed against the bacterial wall can opsonize bacteria. These data suggest that microcapsules may not hide completely the bacterial cell wall, and thus they may allow the interaction of the different agglutinogens to produce CCM in SSA.

Huycke and collaborators (1983) have identified an exopolysaccharide in *S. aureus* which mediates the binding to fibronectin. Some epitopes of this protein present cross-reactivity with the capsular type 8 antigen described by Karakawa's group. Yoshida et al., (1980) also detected the presence of an exopolysaccharide that, as the clumping factor, has coagulase activity and binds fibrin. If these exopolysaccharide belonged to the microcapsule, they could, as described by Huycke and his group (1983), facilitate bacterial aggregation and, consequently, the formation of CCM. Furthermore, this microcapsule would not impede the adhesin (fibronectin, fibrin, etc.) mediated adherence of bacteria to epithelia.

6.b. "Capsular" antigen types

Following the mentioned "capsulation" criteria described by Karakawa and co-workers, 11 different antigenic types have been serotyped. So far, none of the strains has been found polyvalent. Two of the described antigens, serotypes 5 and 8, represent around 70% of all human clinical isolates (Sompolinsky et al., 1985; Hockeppel et al., 1987). No relationship has been found between the distribution of these 2 capsular types and the origin of the isolates, clinical or not, (Sompolinsky et al., 1985; Albus et al., 1988) the intensity of the disease (Arbeit et al., 1984) and the geografic origin (Hockeppel et al., 1987). On the other

hand, the isolates of different provenance within a human being, frequently belong to more than one capsular type (Albus et al., 1988).

When directly serotyping only for types 5 and 8, the majority of strains isolated from mastitis-affected cattle, sheep and goats are found "encapsulated" (69.4%, 71,5% and 78.8%, respectively; Poutrel et al., 1988). In bovine strains, in contrast to human, sheep and goat strains, type 5 antigen is more frequent than type 8 antigen. However, the existence of encapsulated strains non-agglutinating with antibodies against teichoic acids, and at the same time non-typable (lacking known serotypes), suggests that there are "capsular" antigens yet unidentified in these strains.

6.c. Discrepancies between findings using different capsulation criteria

With respect to the capsulation criteria of Yoshida and Minegishi (1976; criteria Y) and Karakawa and co-workers (1985; criteria K), there are obvious discrepancies (Table 2). As already mentioned, the frequency of E strains applying criteria Y is low, but "encapsulated" strains are highly frequent according to criteria K. Furthermore, E strains are non phagotypable using criteria Y, but many of the strains considered as "encapsulated" when using criteria K are phagotypable. These facts could be interpreted if we assumed that a) strains that Yoshida and co-workers classified as E, are a minority whereas strains with a microcapsule seem to be a majority "in vivo" and contain, as the E strains, capsular antigens although in smaller amounts; b) strains that Karakawa and co-workers classified as "encapsulated", correspond to both, E strains and microcapsule containing strains (these microcapsules are maintained or enlarged through "in vitro" subcultures when growing bacteria in especial media like Columbia); and c) the capsule of E strains does not allow the interaction of the phage with the bacteria, whereas the microcapsule does allow this interaction.

Concerning the capsular serotypes identified according K and Y criteria there are also some discrepancies. For example, the majoritary serotype (A) according to criteria Y (Yoshida and Minegishi, 1976), corresponds immunologically to serotype 2, according to criteria K, but the latter is rare (Karakawa and Vann, 1982; Arbeit et al., 1984). Also, Yoshida and Ohtomo (1984a) detected antigenic changes produced during storage of bacteria and found strains with three or even the four antigenic types that they were able to identify (Yoshida et al., 1976). On the contrary, Karakawa and collaborators (1985) did not find changes in the antigenic types and only found cross-reactivity between serotypes 4 and 5.

With respect to the fact that E strains are not phagotypable, Yoshida and Minegishi (1976) did not find any relationship between Yoshida's capsular antigens and NE strain phagotypes. On the contrary, Sompolinsky and his group (1985) and Arbeit and co-workers (1984) found in human strains a close association between the Karakawa's capsular antigens and the phagotype. In ruminant mastitis *S. aureus* strains, this type of correlation (though only partial) was observed (Sutra et al., 1988), but the proportion of strains susceptible to the human set of phages was much lower when compared to human isolates.

Some of these discrepancies between both research groups (Yoshida/Karakawa) referring to capsular serotyping might have an immunological explanation, likely attributable to the criteria applied to prepare antisera and to identify antigens. While Karakawa and his group (1985) in order to purify polyclonal antisera against every serotype systematically absorb these sera with "non-encapsulated" strains, which expose teichoic acids on the cell wall, Yoshida and Mineguishi (1976) do not do this type of absorption and in fact they observe that at least one of the antigens which they describe (A) is very rich in teichoic acids. Whether cell wall teichoic acids were present in the A antigenic preparation is unknown. Furthermore, it is possible that the absorptions carried out by Karakawas' group in order to purify antisera may be responsible for the elimination of antibody populations. This may explain the fact that the

strains which he studies are majoritarily univalent. On the other hand, some of the discrepancies could be attributed to additional differences with regard to the chemical composition of these outer layers (some of these differences are illustrated in Table 3).

7. INDUCTION OF DCM

According to Opdebeeck and collaborators, passage through the bovine mammary gland of strains isolated from bovine mastitis that have lost their DCM, reinduces in bacteria the ability to form DCM. However, this reinduction is not possible when passaging these same strains through the ovine mammary gland (or peritoneal cavity). Hence, a species specific phenomenon appears to be involved (Opdebeeck et al., 1988b). In any case, these authors observed that the DCM was not maintained through "in vitro" subcultures. Using strains maintained in the laboratory for over 3 years, Opdebeeck and his group (1987) succeeded in inducing the DCM in 75 per cent of the strains, after three passages through m110 or BHI supplemented with 30 per cent bovine blood serum, but only in 7 among 192 strains grown in BHI supplemented with 30 per cent bovine milk. Similarly, and using the same method, Sutra et al. (1990) found that about 85 per cent of type 5 and 8 *S. aureus* isolates from cow, goat and ewe milk produced DCM in SSA after growth in m110. This induction required maintaining bacteria in very low numbers and in the logarithmic phase of growth. Jonsson and collaborators (1989) were also able to induce the DCM, after growing the strains in bovine milk whey for six to ten hours, but not in m110 broth or agar (Mamo et al., 1991a). Similarly, Yoshida and co-workers (1969) were able to recover the DCM (in an originally E strain which had lost the ability to form a DCM in SSA) upon prolonged cultures in SSA. On the other hand, Anderson (1984) found that the majority (75 out of 104) of bovine mastitis strains maintained in the laboratory from 18 months to three years, showed a diffuse or intermediate (compact + diffuse) morphology in SSA after growth in Mueller Hinton broth, while the remaining CCM strains, recovered their DCM after passage through the mouse mammary gland. Bacterial hydrophilicity has been considered as an indirect encapsulation criteria (Jonsson and Wadström, 1983). In our labortatory, we have observed that most strains (23 out of 26 ruminant mastitis strains) become hydrophilic after growth in an exopolysaccharide inducing media; however, none of them showed a DCM at that stage, or after the passage through mouse mammary gland (Baselga et al. 1990b).

8. PSEUDOCAPSULE AND SLIME

In bovines, Watson (1989a) named pseudocapsule a structure that appears in about 80 per cent of bacteria, either freshly isolated from bovine mastitis milk or after growth in media supplemented with ruminants' milk whey. This structure only appeared in 10 to 20 per cent of the bacteria after growth in conventional regular media (Watson, 1989a). To visualize it by electron microscopy, an previous fixation with specific antibodies is required.

Watson (1989a) considers that pseudocapsules are not true capsules, since they do not show a negative staining with Indian ink. He also proposes that this structure is different from slime, because the latter presents the following properties: a) it appears abundantly after bacterial growth in m110 (an observation not illustrated in the mentioned study), but not in medium supplemented with milk whey nor "in vivo"; b) it does not react with antibodies directed against the pseudocapsular antigens in immunodiffusion tests; and c) after being produced during growth in m110, the corresponding bacteria do not react in an agglutination test; on the contrary, bacteria do agglutinate in this test after culture in media supplemented with milk whey, which induces pseudocapsulation (Watson, 1989a). Furthermore, slime seems to be a more unstable structure when compared with pseudocapsules, since the latter appears to be maintained during washings (Watson, 1982; 1989a) whereas the former is lost (Wilkinson, 1983). Whether the differences between slime and pseudocapsule are quantitative or qualitative is not clear. In fact, other authors (Johne et al., 1984) also using electron microscopy and carefully

isolating bacteria from bovine mastitis milk, found that these bacteria formed DCM in SSA and detected an extracellular material, surrounding the *S. aureus* cell surface, which they named slime. A similar picture was obtained after growing bacteria in m110 (Table 2).

Some of the differences between the findings of Watson and those of Johne's group could be attributed to the procedure applied for bacterial isolation, since Johne and co-workers used a very mild method to isolate bacteria from milk whey (immunomagnetism), whereas Watson carried out repeated washings, which may have led to the elimination of slime (Wilkinson, 1983).

In any case, it can be concluded that the formation of slime and not of a true capsule after growth in m110 has been widely shown. In experimental infections of the rabbit lung and after growing bacteria in m110 (Caputy and Costerton, 1982) , found an extensive matrix surrounding the Smith-diffuse (E) and the Willey (NE) strains, using electron microscopy. This slime grouped bacteria into microcolonies. It was extracapsular in the case of the Smith-diffuse strain and could not be identified by negative staining. Furthermore, this slime differed immunologically from the true capsule but not from the slime produced by the Willey strain (Caputy and Costerton, 1984). Also Yoshida and Ekstedt (1968) concluded that certain exopolysaccharides different from those of the E capsule can be produced by some *S. aureus* strains in response to specific nutritional conditions (m110; Table 2). Whether the slime and the pseudocapsules besides presentig different aspects at the electron microscopy are in fact immunologically different is unknown.

Some of the problems to establish immunological differences are inherent to the antibodies and others to the antigens. With regard to the nature of the anticapsular antibodies used in different studies, Loeffler and Norcross (1987) observed that 75 per cent of the DCM strains isolated from mastitis milk change to CCM after the addition of anticapsular type A sera obtained against the E Smith-diffuse strain after growth in m110. It might be possible that these sera contain not only antibodies against capsules but also antibodies against slime. Furthermore, Caputy and Costerton (1984) considered that many studies (of electron microscopy and immunological) on identification of capsules and slime may have been misinterpreted, since slime inducing media were used to grow bacteria in both cases. However, the possibility that antigens similar to those of E strains (criteria Y) are also present in other strains with DCM isolated from mastitis milk should not be discarded. In fact, immunological cross-reactivity between the slime of K93 strain and the capsule of the Smith diffuse strain has been shown (Karakawa and Kane, 1972).

The results of Sutra et al. (1990a; 1990b) suggest that the better method for exopolysaccharide detection is the direct serotyping of the capsular antigen. With their technique and using monoclonal antibodies against types 5 and 8, the authors did not find any antigenic differences between bacteria growing in m110 agar, Columbia agar, skim-milk agar or BHI agar. However, when washing bacteria after growth in an exopolysaccharide inducing medium (m110), capsular polysaccharide was detected in the washing supernatant of 83 per cent of type 8 strains, but in only 28 per cent of type 5 strains. There was therefore a removal of exopolysaccharide from the bacterial surface during washing but it was imcomplete, since upon autoclaving of washed bacteria (sediment) a substantial amount of exopolysaccharide was released (Sutra et al., 1990b). The antigens of supernatant and those released by the autoclaved bacteria were of the same type. These results suggest that the stable exopolysaccharide layer (remaining on bacteria after washing) may be the capsule or pseudocapsule and that the labile layer (in the washing supernatants) may be the slime.

9. PRODUCTION OF SLIME BY COAGULASE NEGATIVE STAPHYLOCOCCI

Coagulase negative staphylococci have acquired great importance in human clinical medicine because of their capacity to adhere to different

materials used in surgery (Quie and Belani, 1987). This capacity has also been shown in some *S. aureus* strains (Mayberry-Carson et al., 1986; Barth et al., 1989; Vaudaux et al., 1989). Several studies suggest that slime production facilitates this binding (Christensen et al., 1985) and protects bacteria from phagocytosis and from antimicrobial agents, including antibiotics (Quie and Belani, 1987). The slime production for coagulase negative staphylococci can be estimated either by the procedure developed by Christensen et al. (1985), in which the capacity of bacteria to form biofilms on the walls of a tube is estimated, or by the colonial morphology in Congo red agar (Freeman et al., 1989). Similarly, morfology on mucoid maintenance agar can be applied to determine the mucoid variants *Pseudomona aeruginosa* (Terry et al., 1991).

10. CONSEQUENCES OF CAPSULATION ON INFECTION

10.a. Resistance of S. aureus to phagocytosis

In ruminat mastitis, phagocytosis mediated by polymorphonuclear neutrophils (PMN) is likely the most important defence mechanism of the mammary gland. These PMN have opsonin receptors, specifically for the C3b complement component and for the Fc portion of IgG2 but not for other immunoglobulin isotypes (Craven and Williams, 1985).

Different workers have shown that the bacterial cell wall peptidoglycan exposed on bacterial surfaces activates the complement alternative pathway (Peterson et al., 1978a; Verbrugh et al., 1980; Table 1). On the other hand, the majority of mammalian sera contain antibodies directed against the *S. aureus* cell wall components (Bell et al., 1987; Wergeland et al., 1989), resulting in the activating of the complement classical pathway. These antibodies are commonly directed against peptidoglican. This high frequency is expected, considering the immunological cross-reactivity between different Gram-positive bacterial peptidoglicans.

The fact that in E strains the bacterial cell wall remains "hidden" by the capsule, does not limit the binding of opsonins (immunoglobulins and complement) to this wall (Peterson et al., 1987b; Wilkinson et al., 1979b; King and Wilkinson, 1981; Fig. 1), but it may result in the inhibition of the interaction between the bound opsonins and the corresponding receptors located on phagocytes (Wilkinson et al., 1979a).

Some slime-associated effects could be similar to those attributed to true capsules, since like capsules, abundant slime may mask the cell wall antigens (Caputy and Costerton, 1982; Johne et al., 1989) Furthermore, immunologically, slime antigens have a low immunopotency when compared to those of the cell wall (Caputy and Costerton, 1982), resulting in a poor opsonization and hindrance for phagocytosis (Table 1, Fig. 1).

The mechanism of resistance to phagocytosis has been shown with strains (Smith-diffuse and M) showing large capsules (criteria Y) but it may not neccesarily be applicable to strains with microcapsules, as shown by Albus and co-workers (1991). Lee and co-workers (1987b), showed that only strains provided with large capsules need specific antibodies against these capsules for correct opsonization, whereas strains with microcapsules are efficiently phagocytized simply in the presence of complement (likely involved via the alternative pathway). It has also been suggested that in the strains with microcapsules, antibodies directed against the bacterial cell wall may also play a role in opsonization (Karakawa and Young., 1979). Furthermore, the latter strains are also opsonized with antibodies obtained against strains with large capsules (Lee et al., 1987b).

Keeping in mind that an extensive capsular exopolysaccharide layer does not allow the activation of complement alternative pathway, the presence of specific antibodies against capsular antigens becomes necessary to activate the classical pathway, according to Verbrugh et al. (1982) and Lee et al. (1987a; 1987b). These authors suggest that complement may be crucial to eliminate "in vivo" the E strains of *S. aureus*. It would seem reasonable to pay attention to complement levels in mammary secretions. These levels are high in colostrum and at the end of lactation (between 1 and 5 per cent of the serum concentration). In mastitis, milk complement levels are also high (3 to 12 per cent of the serum concentration; Craven and Williams, 1985).

However, it has been shown that even though E strains require high normal bovine serum and whey concentrations for "in vitro" opsonization, these concentrations are still lower than those corrresponding to normal physiological conditions (Peterson et al., 1978b; Anderson and Williams, 1985). Furthermore, many serum components pass to milk as soon as mastitis-associated inflammation begins, thus increasing the opsonizing capacity of milk. Anderson and Williams (1985) observed that diluted (50 per cent v/v) delipidized milk efficiently opsonized E strains in the majority of animals (57 out of 62). These authors concluded that there is little need to supply additional opsonins to milk by immunization against capsular antigens. However, Verbrugh and co-workers (1982) observed that the encapsulated Smith-diffuse strain can be opsonized by human normal serum, in contrast to the M strain, which is more heavily encapsulated. These observations are in agreement with those of Lee et al. (1987b).

Virtually all sera from healthy human donors possess immunoglobulins against capsular exopolysaccharides (Wilkinson 1983). Albus et al. (1988) found high antibody titers against microcapsular antigens (types 5 and 8, criteria K), either in healthy individuals or in cystic fibrosis patients. In cattle, milk antibodies against *S. aureus* exopolysaccharides are frequently found (Norcross and Opdebeeck, 1983; Watson and Colditz, 1983; Anderson and Williams, 1985; Opdebeeck and Norcross, 1985b; Loeffler et al., 1989; Watson, 1989b). These data suggest that in humans, and likely in cattle, prolonged exposures to capsular antigens are taking place.

Given that the composition of *S. aureus* capsular polysaccharides is very similar to that of other Gram-positive and negative bacteria, it might be expected that these capsular antigens provide a natural cross-reactive immunity (Arbeit et al., 1984).

Altogether, these findings help understanding the fact that upon bacterial inoculations in mice *S. aureus* E strains are found more virulent than NE strains (Yokomizo et al., 1977). This is obviously attributable to the higher resistance of E strains to phagocytosis (Peterson et al., 1978b; Wilkinson et al., 1979a; Verbrugh et al., 1982; Karakawa et al., 1988). Similarly, strains showing DCM after growth in whey also show a higher resistance to phagocytosis in comparison with these same strains after growth in regular media (which do not induce DCM; Jonsson et al., 1989). Mamo and co-workers (1991b) have reported that whey-grown *S. aureus* strains were phagocytosed to a lower extent and bound less complement-factor C3 than their TSB-grown counterparts. Moreover, in this study 5 out of 6 *S. aureus* strains grown in milk whey were significantly more resistant to in vivo clearance from the peritoneal cavity of mice than their homologous strains grown in TSB. Yoshida and Ekstedt (1968) found that *S. aureus* is more virulent for mice after growth in m110 rather than growth in normal laboratory media. This greater virulence was also attributed to a greater resistance to phagocytosis due to the slime production. Similarly, Brock and his group (1973), carrying out intradermal inoculation studies in cattle, also observed a greater virulence when bacteria were previously grown in m110 or in milk.

10.b. Resistance to intracellular killing by PMN

Buggy and co-workers (1984) suggested that the continuous *S. aureus* peritoneal infections that appear in patients subjected to peritoneal dialysis, could be due to the survival of bacteria within the PMN. Williams and his group (1984) also working with *S. aureus* strains, observed that bovine PMN kill 90% of the phagocytized bacteria after 2 hours of incubation. Adam et al., (1971), found a greater resistance to intracellular killing when the strains were grown "in vivo". It has been suggested that staphylococci surviving within the cell are responsible for the chronic inflammatory reactions (Anderson and Williams, 1985). Some authors, however, propose that the presence of a capsule does not seem to increment the intracellular survival, once the bacteria have been phagocytized (Anderson and Williams, 1985).

10.c. "In vivo" antigenic types

For Mackie and co-workers (1979) the physiological consequence of exopolysaccharide production is that bacteria present a greater resistance to phagocytosis and, a greater capacity to adhere to cell surfaces, properties which may help to bacteria survive (Table 1). Thus, when the selection pressures favoring these two properties disappear under certain (regular) growth conditions, the presence of exopolysaccharides is not advantageous and this extracellular layer disappears. Hence, growth in regular culture media (BHI, THB, etc) implies that bacteria have different virulence and immunologic properties and are potentially more vulnerable to the immune system attack, when compared to those freshly obtained after "in vivo" growth (Brown and Williams, 1985; Lorian, 1989). Thus, some "in vivo" produced antigens may not be detectable after "in vitro" growth in regular media (Watson, 1989b). Some of these antigens may however be shared by strains grown in nutrient broth supplemented with milk whey and, altough these antigenic structures do not constitute a true capsule, they confer the bacteria some resistance to PMN mediated phagocytosis, according to findings in sheep (Watson, 1982; 1989b).

In any case, it is easily understood why in general live attenuated vaccines are more efficient than inactivated vaccines, even in cases where the bacteria used have been grown in media which resemble some of the "in vivo" conditions (milk whey; Watson et al., 1978; Watson, 1982; 1989b).

Besides the antigenic differences between the strains grown "in vivo" and "in vitro", there may be quantitative differences (Power et al., 1990).

These may also influence the strains virulence. Thus, the capsular size and the amount of mucus vary among strains and they may likely be related to virulence and/or invasiveness (Wiley, 1968; Yoshida and Ekstedt, 1968; Chomarat et al., 1989; Yoshida, 1987).

10.d. Resistance to agglutination

Kapral (1966) observed that after intraperitoneal inoculation in mice, NE *S. aureus* strains rapidly agglutinate (due to fibrinogen, according to the author) and later, the resulting aggregates are surrounded by PMN. These bacteria cannot freely multiply, nor liberate appreciable amounts of toxins to the medium and therefore the inoculated mice survive. In E strains this agglutination does not take place, bacteria resist phagocytosis and, after the liberation of toxins, the infected mouse dies (Table 1).

Baselga and Amorena (1990a) showed that during bacterial growth, aggregation of bacteria can be induced when supplementing media with ovine or bovine whey. In cattle, Gudding and co-workers (1984), two hours after inoculation of *S. aureus* into the mammary gland, observed aggregates of neutrophils and bacteria which could easily be eliminated during milking. These aggregates, however, were not observed 18 hours after inoculation. It is possible that bacteria become adapted to the "in vivo" conditions producing appreciable amounts of exopolysaccharide, impeding with it the agglutination or the easy elimination during milking, and thus favouring the bacterial survival within the mammary gland.

The strong migratory response of the PMN towards the bovine mammary gland during infection suggests the participation of chemotactic mediators (Craven and Williams, 1985). This chemotactic attraction becomes inefficient in the luminal surfaces of the epithelium. Once PMN enter the gland, they are incapable to produce an active locomotion and depend on random collisions to contact bacteria. For this reason, high PMN concentrations are necessary to produce an efficient defence (Craven and Williams, 1985). Obviously, the existence of small bacterial aggregates or the absence of agglutinability may difficult phagocytosis since they difficult the contact PMN-bacteria (White et al., 1980). Other authors (Lam, 1985) speculate that the presence of large aggregates difficults their elimination by phagocytosis due to the large aggregate volume. Both mechanisms (formation of large and small aggregates), are not necessarily mutually exclusive, they can act simultaneously.

10.e. Microcolony formation

The observation of bacterial populations in infectious processes shows that the majority of bacteria form microcolonies adherent to tissues. Thus, after the initial adhesion of free bacteria to tissues, bacterial cells grow within an exopolysaccharide matrix, in microcolonies giving rise to an irreversible binding (Mayberry-Carson et al., 1986, Costerton et al., 1987). This mechanism could be present in the "in vivo" infection. Thus, Chan and collaborators (1982) suggest for *E. coli* an "in vivo" mechanism of adherence to the intestinal epithelium, whereby adhesins (fimbriae) are responsible for the initial adhesion and "capsular" exopolysaccharides are responsible for the formation of microcolonies in which bacteria multiply. Compatible with this hypothesis are, on one hand, the findings of Ryden et al. (1987; 1989), who found a specific bone sialoprotein, to which *S. aureus* strains isolated from osteomyelitis patients bind selectively and, on the other hand, the fact that *S. aureus* slime is involved in "in vivo" bacterial adherence to cartilage and bone and in microcolony formation (Mayberry-Carson et al., 1984; Speers and Nade, 1985; Power et al., 1990).

In *S. aureus*, the initial binding is favoured by the presence of plasma and basal membrane proteins (Vaudaux et al., 1989). Concerning mastitis, *S. aureus* could adhere to mammary gland ducts and alveoli via basal membrane proteins (Mamo et al., 1988) or epithelial cells (Amorena et al., 1990), to form microcolonies later on. Two proteins derived from the cell wall of *S. aureus*, were able to bind to bovine lactiferus sinus and human buccal epithelial cells. One of them was identified as fibronectin-binding protein (Lindahl et al., 1990). Gudding and co-workers (1984) observed that "in vivo" *S. aureus* was bind to mammary gland epithelial cells but this binding is not mediated by fimbriae or similar structures. The authors suggest that exopolysaccharide molecules could be involved in this binding.

It is not yet clear whether extracellular slime functions as a "glue" in the initial adhesion of bacteria, or whether it is produced only after the bacteria have adhered and have been subjected to metabolic stress (Karakawa and Kane, 1972; Terry et al., 1991). In fact, antibody obtained against slime inhibits the "in vitro" and "in vivo" adherence of slime producer coagulase negative bacteria to silicon elastomer catheters (Kojima y cols., 1990). Our results (Iturralde et al., 1991) suggest the possibility that "slime" may act as an adhesin to epithelial cells.

Many chronic infections imply a bacterial growth in the form of adherent colonies within a large exopolysaccharide matrix (Brown et al., 1988). Thus, the resulting microcolonies are not susceptible to phagocytosis by macrophages and PMN because of their size (Lam et al., 1980). These colonies form foci of humoral and cellular inflammatory reactions which are usually sufficent to eliminate individual bacterial cells that are shed off the surface. However, the defence mechanisms may sometimes fail, specially in the stressed or weak animals, where these liberated cells can disseminate and exert a pathogenic role (Costerton et al., 1983). These phenomena could explain some cases of *S. aureus* chronic mastitis.

The growth within microcolonies may also explain the great resistance of chronic mastitis animals to antibiotic treatment, given the difficulty of antibiotics to reach inhibitory concentrations within the colony (Marrie et al., 1982; Brown et al., 1988). In fact, there is a correlation between the "in vitro" exopolysaccharide production of *Streptococcus*, from heart vegetations in rabbits with experimental aortic-valve endocarditis, and the failure to erradicate the infection with antibiotics (Dall and Herndon, 1989). Davenport et al. (1986) also found that only 32% of infections caused by slime producer coagulase-negative staphylococci, in patients with a prosthetic device, in contrast to 100% of infections caused by slime-non producer organisms were improved by treatment with antibiotics.

The formation of an exopolysaccharide matrix "in vivo" may have important metabolic implications, favoring the sequestration of bacterial exoenzymes in these colonies nearby the bacteria which produce them (Caputy and Costerton, 1982). These enzymes may thus facilitate the access of degradation products of different milk components to bacteria. In this line of work, Mattila et al. (1988) found that strains with DCM grew more rapidly than those with CCM in bovine milk whey. The opposite situation was found when growth was taking place in milk whey of mastitis affected animals. According to these authors, it is possible that in these diseased animals there is an abundance of extra-nutrients existing in milk whey (degradation products of caseins, hemo-products, etc), meaning that these nutrients are not any more limiting factors for bacterial growth and thus, in these cases the elaboration of exopolysaccharides would be a load instead of an advantage for bacteria. How this suggestion fits the hypothesis proposed by other authors on "in vivo" associated slime production (Caputy and Costerton, 1982) in mastitis affected animals is unknown. Both hypotheses are compatible if it is assumed that bacterial cells may change the surface properties throughout the different stages of the disease, according to the microenvironments created within the mammary gland during these stages.

11. CONCLUSIONS

The conceptual differences between the terms capsule, microcapsule, pseudocapsule and slime have not always been clear in the literature: they have indistinctly been used on many occasions without immunologic and/or biochemical characterizations that endorse these differences. In any case, the implications of these layers on the enhacement of mastitis virulence are evident.

Further studies on identification and immunopotentiation of *S. aureus* extra-cellular layers and on the way to monitor the exopolysaccharide-dependent virulence mechanisms involved in mastitis, may help to clarify the possible molecular events underlying this disease (or other *S. aureus* invasions) and design new approaches for prophylaxis and treatment of the disease.

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Tabla 1. Major physiologycal implications associated with an extensive exopolysaccharide matrix in *S. aureus**

1. Hindered exposure of cell wall and coagulase molecules
2. Impaired aggregate formation mediated by adhesins (immunoglobulins, fibrinogen, fibronectin, etc.)
3. Reduced interaction of bacteria-bound opsonins (immunoglobulins, complement,etc.) with the corresponding phagocyte receptors
4. Lack of activation of the complement alternative pathway by cell wall peptidoglycan: Decreased bacterial opsonization
5. Greater success in bacterial multiplication within microcolonies
6. Greater resistance to phagocytosis (consequence of points 1 to 4)
7. Decreased access of antibiotics to bacteria
8. Higher virulence (consequence of points 1 to 7)

* The mentioned properties correspond to cases in which the exopolysaccharide matrix (capsule, microcapsule, pseudocapsule, slime, etc.,) is abundant

Table 2. *S. aureus* envelopes according to the strain types and culture media used: denominations applied by different authors

Growth medium	Type of envelope	
	Truly encapsulated strains (E)	Non-truly encapsulated strains
In vivo	*Capsule and slime* (Caputy and Costerton, 1982; 1984)	*Capsule or Microcapsule*:- Karakawa's criteria (Albus et al.,1988; Arbeit and Dunn, 1987a; Boutonnier et al., 1989) Yoshida and Minegishi,1976) *Pseudocapsule* (Watson, 1989a) *Slime* (Johne et al., 1989)
Special media (m110, Columbia, Bovine whey)	*Capsule and slime* (Caputy and Costerton, 1982; 1984;Yoshida, 1971; Yoshida and Ekstedt, 1968)	*Capsule or Microcapsule*: -Karakawa's criteria (Hockeppel et al 1987; Karakawa and Vann, 1982; Karakawa et al., 1985) -Yoshida's criteria (Chomarat et al. 1989;Norcross and Opdebeeck, 1983; Yoshida et al., 1979) *Pseudocapsule* (Watson, 1982; 1989a;) *Slime* (Caputy and Costerton, 1982;1984; Johne et al., 1989, Watson, 1989a)
Regular media (BHI, THB, etc.)	*Capsule* (Caputy and Costerton, 1982; 1984; Yoshida and Ekstedt, 1968)	Strain types: -*With capsular antigens* (Chomarat et al., 1989; Yoshida and Minegishi, 1976) -*Without capsular antigens* (Yoshida and Minegishi, 1976)

Table 3. Chemical composition of capsule and slime layers from some *S. aureus* strains

Strain	Composition
Capsule	
M (Murthy et al, 1983) (Type 1, criteria K)	2-acetamido-2-deoxy-D-galacturonic acid, 2-acetamido-2-deoxy-D-fucose, taurine
SA1 mucoid (Lee et al., 1987)	2-acetamido-2-deoxy-a-galacturonic acid, 2-acetamido-2-deoxy-a-fucose, taurine
T (Williams et al., 1984)	2-acetamido-2-deoxy-D-mannuronic acid, 2-acetamido-2-deoxy-D-fucose
Smith-diffuse (Hannessian et al., 1964; Type A, criteria Y)	2-acetamido-2-deoxy-D-glucuronic acid, 2-acetamido-2-deoxy-L-alanyl glucuronic acid
Becker (Fournier et al., 1984; Type 8, criteria K)	O-acetyl, N-acetylfucosamine groups and aminuronic acids similar to N-acetylgalactosaminuronic acid
Reynolds, 840843 and 850052 (Fournier et al., 1987; Type 5, criteria K)	N-acetylfucosamine, N-acetylhexosaminuronic acid
Slime	
K93 (Karakawa et al., 1972)	Galacturonic acid, galactose, mannose, phosphorus
Several strains (Rozgonyi et al., 1985)	D-galacturonic acid, D-glucuronic acid, D-galactosamine, D-galactose, D-mannose, D-xylose

Fig. 1. Barrier effects created in strains with an extensive exopolysaccharide matrix (option A) in relation to strains with a low or null exopolysaccharide content (option B). Two consecutive phases may be considered. In phase I, different compounds (immunoglobulins, fibrinogen, fibronectin and complement components) interact with the bacterial cell wall in both options, but thereafter they remain hidden when the mentioned matrix is present (Option A). As a consequence, if these (option A) bacteria are grown in serum soft agar, aggregates (CCM) can not be formed and a diffuse colony morphology (DCM) is observed. On the other hand, if these bacteria encounter mammalian phagocytes (phase II) with C3 or Fc immunoglobulin receptors, an inhibition of the normal interaction between bacteria and phagocytes is observed, favouring virulence.