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Influence of Blood Proteins in the *in vitro* Adhesion of *Staphylococcus epidermidis* to Teflon, Polycarbonate, Polyethylene and Bovine Pericardium

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The influence of human plasma proteins (fibrinogen, albumin and fibronectin) on the adherence of *Staphylococcus epidermis* to teflon, polyethylene, polycarbonate and bovine pericardium was studied in an *in vitro* quantitative assay by scintillation counting. Bacterial adhesion was generally reduced by the presence of protein during the adherence assay except in the case of bovine pericardium, in which adherence remained almost unaffected. The effect of these plasma proteins on bacterial surface properties resulted in strong increases of surface charge as measured by ion-exchange chromatography and with no effect on hydrophobicity, estimated as contact angles. Adherence was not found to be correlated with these two properties, suggesting that bacteria-surface interactions must not be simplified to the influence of interfacial forces.

Key words: Adhesion, *Staphylococcus epidermidis*, Biomaterials.

The most serious complications associated with the increasingly frequent implantation of foreign devices into the human body are thrombus formation and infections, mostly due to *Staphylococcus epidermidis* (7). Microbial adhesion to artificial surfaces is considered an initial event in the pathogenesis of such infections (2). Many studies report bacterial adherence to solid surfaces in salt solutions (6, 11, 15), however macromolecules present in the liquid medium affect the adhesion process through their association with the bacterial cell surface, the solid surface or both (22). Most of the temporal and permanent prosthetic devices are in contact with blood or other body fluids, and in these

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circumstances, biomaterial surfaces become rapidly coated with proteins (5). These protein layers may mediate surface interactions with formed elements of blood, tissue cells and microorganisms. On the other hand, specific or non-specific binding of macromolecules by bacterial cells can alter their ability to adhere (21). In principle, any plasma protein might influence the adherent interaction, but some of them seem to be better candidates. Albumin is the most abundant protein in plasma and was generally reported to have an inhibitory effect in cell or bacterial adherence to synthetic materials (10). This protein is adsorbed on biomaterials (27) and certain bacteria (14) surfaces. The possible use of albumin-coated materials to avoid their thrombogenic and adhesive properties is currently being investigated (12). Fibrinogen is a fundamental glycoprotein in the coagulation cascade, an immediate blood reaction to the presence of foreign materials. It is quickly adsorbed on variety of biomaterials (23), and can be specifically bound by different bacterial species (13, 14). Fibronectin is found in plasma and other body fluids, and it has binding sites for macromolecules, cells and bacteria (13, 14). Its capacity to bind to platelets and fibrin (25) contributes to the thrombogenicity of the surfaces (9). It also adsorbs on artificial surface (2), thereby surely influencing adherent interactions.

Our purpose was to investigate the influence of albumin, fibrinogen and fibronectin in the adherence of *S. epidermidis* to teflon, polyethylene, policarbonate and bovine pericardium, studying as well, the alteration produced by these proteins in bacterial surface properties.

Materials and Methods

Bacteria. — Seven bacterial strains isolated in the Hospital General de Galicia (Santiago de Compostela, Spain) from hemocultures and identified as *Staphylococcus epidermidis* using the Api Staph Gallery (API Systems, S. A., Montalieu Vercieu, France) were used in this study. Strains stored at −20 °C in skim milk were grown on Brain Heart Agar plates and maintained at 4 °C for one month.

Biomaterials. — Teflon 140-N type A (copolymer of tetrafluoroethylene and hexafluoropropylene), polyethylene (Hostalen) and polycarbonate (Pokalon) films were gifts from Du Pont de Nemours International, S. A. (Geneva, Switzerland), Fa. Kalle (Wiesbaden, FRG) and Lonza-Werke (D-7858, Weil am Rheim, FRG), respectively. Bovine pericardium in the form of pericardial closure patch, was generously donated by Shiley Incorporated (Irvine, CA, USA). Circular samples (6 mm diameter) were cut from synthetic polymer fils and 5 × 5 mm squares were cut from the pericardial patch. All pieces were impaled on pins to avoid their flotation during the adhesion experiments. Synthetic biomaterials were washed with 1 % (w/v) sodium lauryl-sulphate for 10 min, deionizated water, ethanol and finally deionizated water again, in agitation. Bovine pericardium was extensively rinsed with deionizated water.

Plasma proteins. — Albumin and fibrinogen were purchased from Sigma and freshly dissolved for each experiment in saline phosphate buffer (PBS, 0.33 M NaCl, 3 mM KCl, 8.4 mM Na_2HPO_4, 1.6 mM KH_2PO_4, pH 7.2) to concentrations similar to those in blood, 40 mg/ml and 4 mg/ml respectively. Fibronectin was purified from human plasma by gelatin-sepharose affinity chromatography following the method described by MIEKKA *et al.* (16). Purified fibronectin was freshly dissolved in PBS to 40 μg/ml before the experiment. It has been proved that there is a range of protein concentrations (inferior to those those used here within which the

protein does not influence the number of adhered bacteria (10, 26).

Adherence assay. — Bacterial adhesion to biomaterials was assayed by scintillation counting as previously described (6). Briefly, bacteria grown for 18 h at 37 °C in Mueller Hinton Broth containing 1µl/ml of D-[^{14}C(U)]-Glucose (0.1 mCi/ml, 3.7 mCi/mmol) (Du Pont) were centrifuged and washed twice (10.000 × g, 4 °C, 10 min) in PBS and suspended in the same buffer. Bacterial clusters were dispersed by aspirating and expelling the suspensions twice through a 25 gauge steel needle attached to a syringe. Optical density of suspensions was adjusted at 540 nm to 5 × 10^8 colony forming units/ml (CFU/ml). Bacterial suspensions (250 µl) were then incubated with the pieces of biomaterials for 1 h at 37 °C with 90 rpm constant shaking. Subsequently, biomaterial pieces were washed once with 5 mil of PBS and prepared for scintillation counting. Each experiment was carried out in PBS (control) and in every protein solution in PBS. At least triplicates were done. The number of bacteria attached per mm^2 of biomaterial was calculated as:

Bacteria/mm^2 =

$$(EX\text{-}CI) \times 1.25 \times 10^8/CT \times S$$

where EX = cpm associated to the material piece and the pin; CI = cpm associated to the pin; CT = cpm associated to 250 µl of bacterial suspension containing 5 × 10^8 CFU/ml; and S = effective surface of the material piece.

Bacterial surface charge. — Negative surface charge was determined by batch ion-exchange chromatography using Dowex 1 × 8 resin (100-200 mesh, chloride form, Sigma) as previously described (6). After growth and processing for the determination of adherence, bacteria were suspended is PBS to a density of 7.5 × 10^8 CFU/ml. Aliquots of bacterial suspensions were incubated in PBS (controls) and in protein solutions in PBS for 1 h at 37 °C with 90 rpm constant shaking. They were then, centrifuged and washed twice in PBS (10,000 × g, 4 °C, 10 min) and 1 ml amounts were mixed with 0.5 ml aliquots of resin (1 g/ml in PBS). After 10 min at room temperature with constant mild shaking, 1 ml amounts of PBS were added and the resin was allowed to settle for 5 min. Subsequently, 0.5 ml amounts of supernatants were prepared for liquid scintillation counting. Negative surface charge values were calculated as:

Charge (%) =

$$100 - [(EX \times 2.5 \times 100)/CT]$$

being EX = cpm in experiments, and CT = cpm in 0.5 ml of bacterial suspensions containing 7.5 × 10^8 CFU/ml.

Bacterial surface hydrophobicity. — Surface hydrophobicity was estimated by calculation of contact angles following the sessile drop method as described (6). Five µl distilled water drops deposited on layers of bacteria were photographed within 5 s. Developed negatives were projected on a screen, heights (H) of the drops and widths (W) of the dropbacterial layer interfaces were measured and contact angles calculated from:

$$\text{Angle } (°) = 2 \times \text{arctg } 2H/W$$

Bacterial layers were obtained from bacteria grown in Mueller Hinton Broth for 18 h at 37 °C and processed as follows: They were centrifuged and washed twice in PBS (10,000 × g, 4 °C, 10 min) and the optical density at 540 nm was adjusted to 8 × 10^8 CFU/ml. Aliquots of these suspensions were incubated in PBS (controls) or in protein solution for 1 h at 37 °C. Afterwards, they were centrifuged and washed twice in PBS and suspended in the same buffer. Then, bacterial suspensions were collected on 0.45 µm pore diameter filters to a density of 10^8 cells/mm^2. Filters with bacteria were maintained for 30 min in Petri dishes on the surface of a layer of 1 % (w/v) agar in water containing 10 % (v/v) glycerol (3).

Statistics. — Statistical analysis of the data were performed by means of the SPSS/PC + statistical package (SPSS Inc. & Microsoft Corp.). Comparisons between groups were done by application of the Mann-Whitney U test. Correlations were tested by calculation of the Tau-B Kendall's coefficient (19). Signification limit was set to 0.05.

Results

The adherence of the *S. epidermidis* strains to the different biomaterials both in the absence and presence of human serum proteins is graphically shown in fig. 1. The effects of proteins on adherence were always evaluated by comparison with controls parallelly processed. The extent of the variations induced by the presence of proteins during the adherence experiments, in the cases in which they are statistically significant, are shown in table I. In most cases, the effect produced is a decrease in the adherence values. Treatment of the bacteria with proteins always induced an increase in the surface charge whereas hydrophobicity was weakly affected and only in few cases (table I).
An absence of correlation between hydrophobicity, charge and adherence to the

Table I. *Variations induced by proteins in the surface hydrophobicity, surface negative charge and adherence to biomaterials of* Staphylococcus epidermidis *strains.*
Data (means from three experiments) are expressed as percentages of variation with respect to controls. Standard errors of the means were always less than 5 % of the mean.

			Adherence to				
Protein	Strain	HY[a]	CH	TF	PE	BP	PC
Albumin	S6	33	∞[c]	−68	−70	NS	NS
	S8	NS	326	NS	−89	NS	−85
	S20	−46	93	−75	−69	NS	NS
	S31	−35	327	−99	−65	NS	NS
	S39	22	169	−99	NS	NS	−95
	S42	NS	168	−99	−65	103	NS
	S45	NS	229	−100	−93	NS	−93
Fibrinogen	S6	NS[b]	4689	−91	−92	−71	−52
	S8	NS	834	−88	−69	139	−77
	S20	NS	252	−90	−93	NS	−94
	S31	−36	643	−95	NS	NS	−90
	S39	NS	310	−78	−99	NS	−99
	S42	NS	1518	−97	−95	−22	−93
	S45	−41	322	NS	−87	−42	NS
Fibronectin	S6	NS	316	−100	−97	NS	−90
	S8	NS	111	NS	−61	NS	−65
	S20	NS	160	NS	NS	NS	NS
	S31	NS	149	NS	−63	NS	−63
	S39	NS	169	−65	−67	NS	−71
	S42	NS	797	NS	NS	NS	NS
	S45	NS	185	NS	−80	NS	−68

[a] HY, hydrophobicity; CH, surface negative charge; TF, teflon; PE, polyethylene; BP, bovine pericardium; PC, polycarbonate.
[b] NS, variation not statistically significant at p = 0.05.
[c] Infinity (control charge was null).

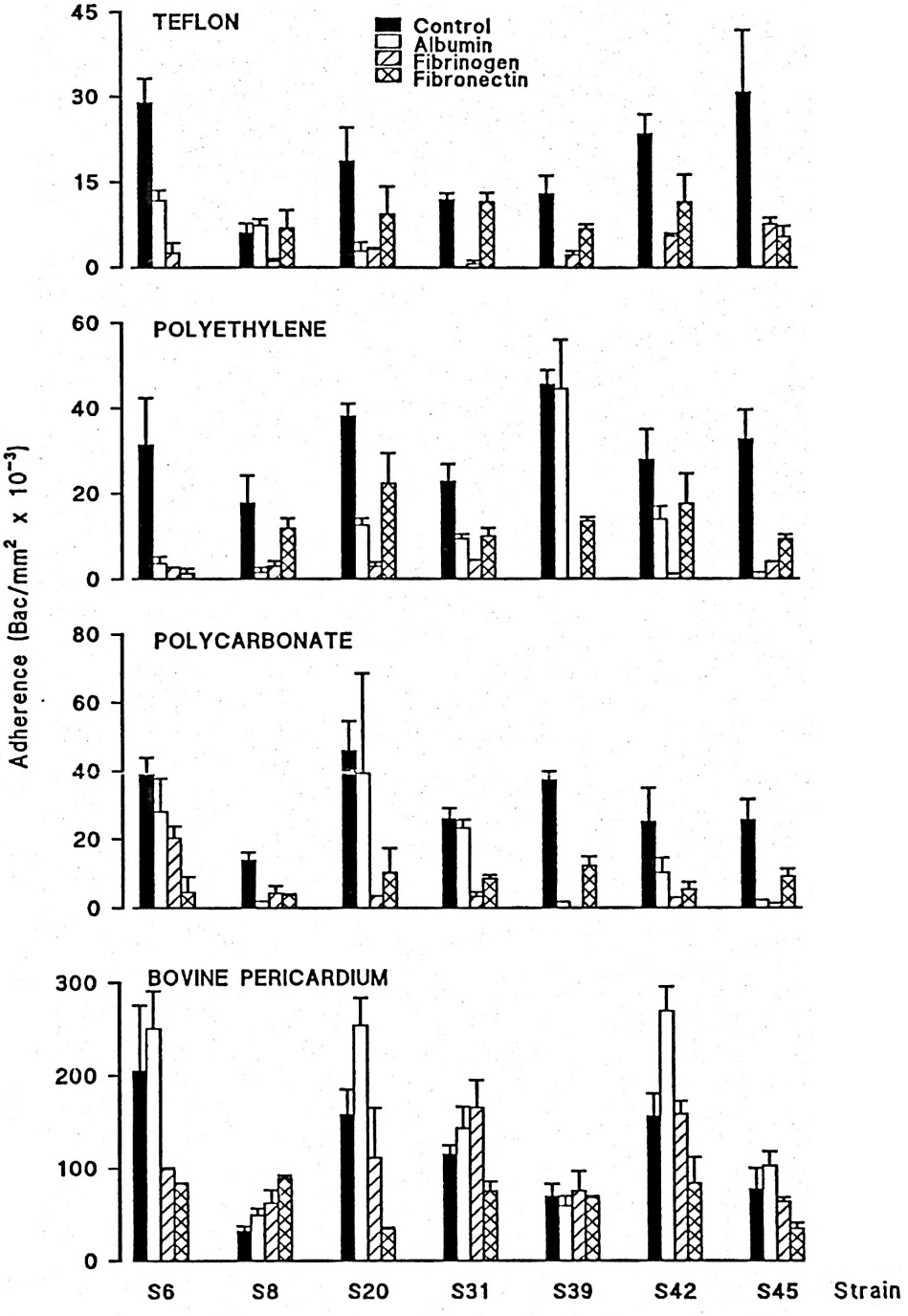

Fig. 1. *Adherence of* Staphylococcus epidermidis *strains to teflon, polyethylene, polycarbonate and bovine pericardium in the absence (control) and presence of albumin, fibrinogen and fibronectin.* Bars indicate mean ± standard error of the number of adhering bacteria per mm^2.

four biomaterials was found both in the presence or absence of proteins, except for charge and adherence to polyethylene in the presence of fibronectin (Kendall's Tau B = 0.62, p = 0.021) and hydrophobicity and adherence to polycarbonate in the presence of fibronectin (Kendall's Tau B = −0.52, p = 0.03).

Discussion

Results of this study demonstrate that albumin, fibrinogen and fibronectin affected staphylococcal adherence to Teflon, polyethylene, polycarbonate and bovine pericardium when they were present in the medium during the adherence assay. The influences of these proteins resulted in strong inhibition of adherence, except in the case of bovine pericardium, in which fibronectin had no effect, albumin increased the number of adhered bacteria of one strain and fibrinogen decreased attachment values of three strains and increased the attachment value of one. These different effects of proteins in the adherence of bacteria to synthetic materials and bovine pericardium may be due to the fact that the latter is a natural tissue, and its interaction with bacteria, therefore, ought likely to be examined from a very different point of view, as pericardium is a denatured collagen matrix with a very irregular surface made of fibrillar material which can offer a higher surface area than the equivalent size of plastic biomaterials. The factors affecting protein-tissue interaction may be also different in nature or degree from those controlling protein adsorption on synthetic materials. GOËAU-BRISSONIÈRE *et al.* (8) studied *in vivo* adherence of *Staphylococcus aureus* to expanded polytetrafluoroethylene and Dracon velour grafts and to bovine heterografts. They exposed these grafts to circulating blood in a dog model for two hours or two months and then inoculated bacteria. The proteins adsorbed on bovine heterografts did not have much influence in the adherence since the number of bacteria bound were similar after the two different periods of implantation. This is consistent with our finding that adherence of bacteria to bovine pericardium was not affected by the presence of proteins in most cases. The generally recognised fact of serum albumin inhibition of adherence (1, 10, 21, 26) which is explained by the alteration of bacterial and material surface properties has been confirmed.

Several authors (10, 18, 26) have recently reported enhanced staphylococcal adhesion to *in vivo* or *in vitro* fibrinogen-coated surfaces. They postulated that staphylococci specifically bind fibrinogen, its presence in the surface being a substrate for bacterial adhesion, which is not in accordance with our results. Since our experiments were carried out by incubating bacteria, protein and solid surfaces for one hour, thus allowing fibrinogen to adsorb in the surface and bacteria to bind fibrinogen, an increase in adherence could be expected. Our results can be explained from the fact that staphylococci bind fibrinogen from the medium, the fibrinogen binding molecules being, therefore, occupied to adhere to the fibrinogencoated surface. The configurations of the protein adsorbed on the surface and free in the medium might also be different and bacteria might blind preferably to one of them. The data of KUUSELA *et al.* (13) suggest that fibronectin-fibronectin interactions may occur and lead to the adherence of fibronectin-coated bacteria to fibronectin-coated surfaces. Fibronectin binds *S. epidermidis* strains (10, 24) and adsorbs on variety of biomaterials (2), so fibronectin-fibronectin interactions and increased adherence could be expected in our experimental conditions. The lack of such results may be due to the presence of the remaining fibronectin in the adherence medium which impede the interactions between molecules of fibronectin bound to the bacteria and to the surfaces.

Physicochemical bacterial surface properties like surface charge and hydrophobicity are known to contribute to bacterial adherence (6, 11, 15). Very few studies on the influence of adsorbed proteins in the surface characteristics of bacteria have been published, while there is an extensive literature concerning the alteration of material surface properties by the adsorption of proteins (21, 27). Bacteria was treated with serum albumin, fibrinogen and fibronectin and the change in bacterial surface charge and hydrophobicity produced by protein bound to the bacterial surface was investigated. The three proteins tested caused a strong increase in surface charge, as might have been expected, since all of them are acidic proteins. Fibronectin had no effect in bacterial contact angles while albumin and fibrinogen had a heterogeneous influence depending on the bacterial strain. These results are consistent with those of MIÖRNER *et al.* (17), who detected changes in bacterial partition in polymer two phase systems after protein binding. Variations in contact angles after bacterial treatment with human serum albumin and IgG were also described (1).

When a synthetic material contacts with blood, plasma proteins are adsorbed onto its surface, fibrinogen being the first one deposited. The protein adsorption is followed by coagulation and platelets adhesion, leading to thrombus formation in which fibronectin and leucocytes are also present (4, 25). Albumin-coated surfaces have been reported to be less prone to the adhesion of platelets and fibrinogen-coated surfaces to intensify platelet adhesion (4, 12). This finding is being used to improve biocompatibility of vascular grafts, since albumin-coated Dracon grafts are less thrombogenic and less adherent. It can be inferred (10, 26) that albumin-coated materials are also less susceptible to bacterial adherence. When bacteria approach the surface, they can encounter a clean surface, a protein layer deposited on it, and fibrin clots or thrombi on the surface. It is not yet known if bacteria are able to attach to the surface in all of these circumstances.

Our results suggest that bacteria-surface interactions must not be simplified by correlating adherence to specific bacteria-protein or surface-protein interactions. Many dissolved proteins, cells and other components of the body fluids could be of great importance in bacterial adherence to implanted surfaces. More *in vitro* and *in vivo* studies are needed to dilucidate the role and interrelations of all these factors.

Resumen

Se estudia la influencia de proteínas de plasma humano (fibrinógeno, albúmina y fibronectina) en la adherencia de *Staphylococcus epidermidis* a teflon, polietileno, policarbonato y pericardio bovino por contaje de centelleo líquido. La presencia de proteínas durante el ensayo de adherencia reduce la adherencia bacteriana, a excepción del caso de adherencia a pericardio bovino que no se ve afectada. Los efectos de estas proteínas del plasma en las propiedades de superficie de las bacterias se traducen en fuertes incrementos de la carga de superficie medida por cromatografía de intercambio iónico, mientras que la hidrofobicidad, estimada por medida de ángulos de contacto, no se ve afectada. Se encontró que la adherencia no se correlaciona con estas dos propiedades, lo que sugiere que las interacciones bacteria-superficie no deben simplificarse atribuyéndolas solamente a las fuerzas interfaciales.

Palabras clave: Adherencia, *Staphylococcus epidermidis*, Biomateriales.

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