Adherence of *Haemophilus somnus* to Tumor Necrosis Factor-α-stimulated Bovine Endothelial Cells in Culture

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ABSTRACT

Vascular thrombosis and tissue infarction is a principal lesion in *Haemophilus somnus* septicemia known also as thrombotic meningoencephalitis. This study was undertaken to examine whether tumor necrosis factor-α (TNF-α) can influence the adherence of *H. somnus* to cultured bovine aortic endothelial cells (BAEC).

Confluent BAEC were exposed to 0–100 nM of human recombinant TNF-α for 12-48 h. Suspensions of different strains of *H. somnus* (approximately 1.5–3 × 10^8) labelled with [methyl-^{3}H]-thymidine, were added to BAEC and incubated for 1.5 h. Initial studies with one pathogenic (P) strain and one non-pathogenic (NP) strain revealed that both strains adhered to normal endothelial cells but minimally to subendothelial matrix remaining after removal of BAEC. Adherence to BAEC was reduced by an excess of unlabelled *H. somnus* of the same strain. Adherence was enhanced for both strains by exposure of BAEC to TNF-α in a manner that increased with TNF-α concentration and with duration of exposure to TNF-α prior to addition of bacteria. A survey of adherence of six live P strains and six NP strains demonstrated considerable variation but no difference in adherence between P and NP strains to normal or to TNF-α-stimulated BAEC. However, TNF-α consistently increased adhesion of each strain to BAEC. Both P and NP strains caused more severe cytotoxic changes in TNF-α-treated BAEC. Tumor necrosis factor-α also increased adhesion of formalin-killed bacteria of P and NP strains. Killed bacteria did not cause cytotoxic changes in BAEC.

These studies demonstrate that various strains of *H.somnus* adhere to BAEC in culture and that adherence is enhanced by exposure of BAEC to TNF-α, but adherence does not correlate with pathogenicity. These studies suggest that intrinsic adhesive properties of *H. somnus* to BAEC do not explain clinical pathogenicity, but provide evidence that TNF-α might enhance bacterial adhesion to vascular endothelium if infection is established.

RÉSUMÉ

Des thromboses vasculaires et des infarcti tissulaires représentent les principales lésions retrouvées lors de septicémies à *Haemophilus somnus* aussi connues sous le nom de méningo-encéphalite thrombo-embolique. Le but de la présente étude était de déterminer si le facteur de nécrose tumorale α (TNF-α) peut influencer l'adhérence d'*H. somnus* à des cellules endothéliales aortiques d'origine bovine maintenue en culture.

Un tapis confluent de cellules endothéliales a été exposé à 0–100 nM de TNF-α recombinant humain pour une période de 12 à 48 h. Des suspensions de différentes souches d'*H. somnus* (contenant approximativement 1.5–3 × 10^8 bactéries) marquées à la thymidine tritiée ont été ajoutées aux cellules endothéliales et le tout incubé durant 1.5 h. Les travaux préliminaires ont montré qu'une souche pathogène et une souche non-pathogène ont adhéré aux cellules endothéliales normales mais très peu à la matrice subendothéliale. L'adhérence aux cellules endothéliales a été réduite par l'addition d'un excès de cellules d'*H. somnus* non-marquées de la même souche. L'adhérence des deux souches a été augmentée suite à une exposition des cellules endothéliales au TNF-α; cette augmentation était dépendante de la concentration de TNF-α et de la période entre l'exposition des cellules au TNF-α et l'addition des cellules bactériennes. Six souches pathogènes et six souches non-pathogènes ont montré un niveau d'adhérence variable, mais aucune différence n'a été notée entre l'adhérence des souches pathogènes et celle des non-pathogènes. Toutefois, le TNF-α a augmenté l'adhérence aux cellules endothéliales de toutes les souches testées. Toutes les souches ont également montré des effets cytotoxiques plus sévères chez les cellules endothéliales exposées au TNF-α. Le TNF-α a aussi augmenté l'adhérence des cellules bactériennes formolées. Les cellules bactériennes mortes n'ont pas causé d'effets cytotoxiques aux cellules endothéliales.

Ces résultats démontrent que différentes souches d'*H. somnus* adhèrent aux cellules endothéliales maintenues en culture et que cette adhérence est augmentée par une exposition des cellules endothéliales au TNF-α; aucune corrélation entre l'adhérence et la pathogénicité des souches n'a été observée. Les résul-

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tats suggèrent que les propriétés d'adhérence d'*H. somnus* n'expliquent pas la pathogénicité observée en clinique, mais démontrent que le TNF-α peut augmenter l'adhérence bactérienne à l'endothélium vasculaire une fois l'infection établie. (***Traduit par Dr Mario Jacques***)

INTRODUCTION

Haemophilus somnus, a gram-negative bacterial rod causes several disease syndromes in cattle including thrombotic meningoencephalitis (TME) (1–4) following bacteremia/septicemia, suppurative bronchopneumonia (3,5,6), suppurative myocarditis (7), arthritis (3,4), sporadic mastitis (8,9), and suppurative lesions in the reproductive tract of cows (10). The lesions caused by this organism in spontaneous (5) and experimentally induced (11,12) pneumonia, and the experimental meningitis induced after intracisternal (I/C) inoculation into the cisterna magna of calves (13), have been well characterized, but the primary pathogenic mechanisms are unclear. Pulmonary lesions found in calves 24 h after intrabronchial inoculation with a pneumonic strain of *H. somnus*, consisted of vasculitis, widespread serofibrinous alveolar effusion, and massive leukocytic infiltration with degenerate macrophages and neutrophils (12). Phagocytosis of *H. somnus* caused degeneration of monocytes but did not increase the release of superoxide anions in bovine macrophages (14). Opsonized *H. somnus* were readily ingested by bovine neutrophils but were not killed and the potency of the bactericidal respiratory burst was reduced (15,16). These influences of pathogenic strains of *H. somnus* on phagocyte function likely contribute to their successful establishment in the systemic circulation, but other virulence factors are probably also involved (10,17).

Various observations suggest a role for adherence of *H. somnus* to endothelial cells (EC) in the pathogenesis of thrombosis and vasculitis in TME. The predominant lesion in spontaneous (1) and experimental TME (18,19) is vasculitis and thrombosis. *Haemophilus somnus* adhered to endothelium of segments of bovine carotid artery, and caused EC to retract and expose subendothelial matrix in intercellular spaces (20). Similar effects occurred with several strains of live but not dead *H. somnus* in cultured bovine aortic endothelial cells (BAEC) (21). Although *H. somnus* does not contain a polysaccharide capsule or pili (22–24), it does adhere to bovine endothelium (20), nasal turbinate cells (24), embryos with an intact zona pellucida (25), and spermatozoa (26). These findings suggest intrinsic adherence mechanisms of *H. somnus* to EC and other cells but the factors governing these responses are unknown.

Stimulation of vascular endothelium by various proinflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) or by bacterial endotoxin which acts through IL-1 and TNF-α, can induce expression of various leukocyte adhesion receptors involved in adherence and transendothelial migration of leukocytes (27–30). Activated endothelium has increased procoagulant activity (27,31) and also serves as an adhesive surface for blood borne microorganisms; *Candida albicans* expresses a protein functionally analogous to CD11b/CD18 integrin, an adhesin for attachment of neutrophils to endothelial cells (32). Intercellular adhesion molecule-1 (ICAM-1), a receptor for the adherence of leukocytes (30), which is expressed on the surface of activated endothelium, is also a receptor for adhesion of *Plasmodium falciparum* (33), and human rhinovirus (34). Cytokine-mediated expression of endothelial surface molecules involved in adhesion of bacteria such as *H. somnus* has not been demonstrated.

The purpose of the present work was to compare adhesion of representative pathogenic and nonpathogenic isolates of *H. somnus* (13) to bovine endothelium. Isolates of *H. somnus* have been characterized as pathogenic (P) or nonpathogenic (NP), depending on the condition from which they were isolated and their ability to cause meningoencephalitis experimentally in calves (10). Several factors of virulence have been proposed (10,17) but their definitive role in the contribution to the distinction between pathogenic and nonpathogenic strains has not been characterized. We compared these P and NP strains with respect to their adherence to cultured bovine endothelial cells stimulated by human recombinant TNF-α (hrTNF-α), to determine if bacterial adherence might be inducible by this macrophage-derived cytokine which is implicated as a cause of endothelial activation in various endotoxemic diseases (27), and to determine if pathogenicity correlates with enhanced adhesiveness to BAEC.

MATERIALS AND METHODS

BACTERIAL SUSPENSION

Two genital strains of *H. somnus* were selected in the first stage of this work, a pathogenic (P) strain, 88159, which causes meningitis in calves in an intracisternal (I/C) assay, and a nonpathogenic (NP) strain, 88541, which does not (13). Subsequently, six additional P strains and six NP strains were used in assays of adherence to cultured BAEC. These strains were stored in aliquoted frozen egg yolk of five to seven day old embryonating chicks at −70°C. Portions of the egg yolk were plated on a solid medium with 7% bovine blood enriched with brain heart infusion yeast extract (BHI-agar) (13), cultured for 16–20 h, at 37°C and 5% CO_2 in a humid atmosphere. A number of colonies were scraped from each agar plate and suspended separately in plastic sterile tubes 17 × 100 mm (Fisher Scientific Ltd., Whitby, Ontario) containing 2 mL of a defined medium designed for growing *H. somnus* (35) supplemented with 40 μCi (148 MBq) of [methyl-3H]-thymidine with specific activity of 70–85 Ci/mM (ICN Biomedicals Inc., Irvine, California). The log phase of the bacterial growth occurred within the period of 4.5 h in culture. The bacterial suspension at 4.5 h was then centrifuged at 10,000 × *g* for 5 min, the supernatant removed and the pellet resuspended, and washed three times in warm HBSS, and adjusted to $A_{625\ nm} = 0.3$. This was equivalent to $6.1 \pm 2.2 \times 10^8$ CFU/mL for strain 88159 (P) and $3.6 \pm 0.7 \times 10^8$/mL for strain 88541 (NP) subsequently determined by plate counts. The rate of incorporation of [methyl-^{3}H]-thymidine (^{3}H-TdR) by bacteria was determined for each suspension sampled in

triplicate by liquid scintillation counting. The average specific activity of incorporated ^{3}H-TdR of bacterial suspensions was 4.24 (± 1.4) × 10^5 dpm/absorption unit, it varied between strains and between experiments for each of the assayed strains. Suspensions of each strain were added for endothelial adherence studies (see below).

TABLE I. Specific activity of strains of *H. somnus* labelled with ^{3}H-thymidine in standardized suspensions (A_{625} = 0.3) of 0.5 mL used for the endothelial adherence assay

Strain number	Counts per minute		Number experiments
	average	stand. dev.	
88159	162438	34634	31
88541	108279	25641	31
70986	145108	26484	3
88546	91533	26391	3
87121531	124037	8294	3
8711337	86730	20317	3
8712155	137182	18697	3
43826	187751	70342	3
88318	150961	53335	3
885104	92742	4858	3
26-16	99749	11463	3
88514	223054	35187	3
8851018	71398	17976	3
S-20	116683	8864	3

ENDOTHELIAL CULTURE

Endothelial cells were isolated from the aorta of an adult steer by collagenase digestion in a method described previously (36) and maintained in Medium 199 with Hanks' salts supplemented with aminoacids, vitamins, penicillin 100 IU/mL, streptomycin 100 mg/mL, 10% heat inactivated (56°C for 60 min) fetal bovine serum, 10% heat inactivated (56°C, 1 h) calf serum (Gibco BRL, Burlington, Ontario), and 20 mM N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid (HEPES, Gibco BRL) with pH 7.4 (37). Bovine aortic endothelial cells were identified by their cobblestone appearance in confluent culture, expression of von Willebrand factor in an indirect fluorescent antibody assay (38), and internalization of fluorescein-labelled acetylated low density lipoprotein (39). Endothelial cells passaged 5 to 15 times were harvested with trypsin-EDTA and seeded into 24-well tissue culture plates (Corning, New York), 4 × 10^5 cells per well and grown to confluency within two days. The medium was then removed and monolayers washed three times with warm Hanks' balanced salt solution (HBSS, Gibco BRL) with 20 mM HEPES. Cells were cultured in a medium (0.5 mL) as above but devoid of antibiotics and calf serum. Human recombinant TNF-α (a gift of Genentech Inc., San Francisco, California), was added (0–100 nM) to confluent monolayers and incubated for 12, 24 and 48 h. Each concentration of TNF-α and medium with no TNF-α were added to wells in quadruplicate for each of two bacterial strains examined.

ASSAY OF BACTERIAL ADHERENCE

At the end of the various periods of incubation, the medium was removed from monolayers and 0.5 mL of a suspension of ^{3}H-labelled *H. somnus*, adjusted to $A_{625\ nm}$ = 0.3 was added and incubated for 1.5 h at 3°C, 5% CO_2 and 85% relative humidity. Then, the supernatant was removed and the monolayers were washed three times with 0.5 mL of warm HBSS, to remove unattached bacteria. The plates were then agitated with 1 mL of 1N NaOH on a rotating shaker for approximately 15 h. The solubilized content of the wells was diluted with 1 mL of distilled water and then 0.5 mL sampled for scintillation counting. The samples of the bacterial suspensions and of the solubilized monolayers were added to EcoLite(+) scintillation cocktail (ICN Biomedicals Inc.) and the amount of radioactivity determined in a Beckman® LS-3133 scintillation counter (Beckman Instruments Inc., Irvine, California). The amount of radioactivity in the endothelial solubilizate in each of four wells was determined and expressed as a percentage of total radioactivity added in the initial bacterial suspension to calculate percentage of attachment according to the following formula: % adherence = (cpm well solubilizate × 4^a × 0.75^b/cpm added × 10^c) × 100; where ^{a} indicates 0.5 mL sampled from 2 mL of the solubilizate, ^{b} is a coefficient indicating quenching of radioactivity counted in the sample of 0.5 mL as compared with the sample of 50 μL, and ^{c} indicates volume of 50 μL sampled from the volume of 0.5 mL of bacterial suspension added to each well. The experiments for each of four concentrations of TNF-α and each of three incubation times were repeated twice. The mean percent attachment values from each of three experiments were then expressed as the mean with standard deviation (SD) in the figures presented.

MORPHOLOGICAL EVALUATION

Endothelial monolayers with or without preexposure to TNF-α, were cultured with strains 88541 and 88159 for 1.5 h, washed with HBSS, fixed with 1 mL of 10% buffered formaldehyde and photographed using a Nikon Diaphot inverted microscope with FE2 Nikon camera (Nikon Kogaku K.K., Tokyo, Japan).

ATTACHMENT OF *H. SOMNUS* TO SUBENDOTHELAL MATRIX

Confluent EC after two days of culture in the absence of TNF-α were nonenzymatically removed from the substrate with Dulbecco's PBS free of Ca^{2+} and Mg^{2+} (Gibco BRL) containing 2 mM EDTA. Adhesion of both isolates of *H. somnus* to residual subendothelial matrix was then assayed, as above, to estimate the requirement for intact endothelial cells for attachment.

COMPETITION FOR ADHERENCE BY UNLABELLED *H. SOMNUS*

Adherence of *H. somnus* strains to BAEC untreated or treated with 10 nM TNF-α for 48 h was evaluated in the presence or absence of 20 × 10^8 CFU of unlabelled *H. somnus* in 0.5 mL of HBSS combined with 0.5 mL of the standard inoculum of radiolabelled bacteria to determine if adherence of radiolabelled organisms was saturable.

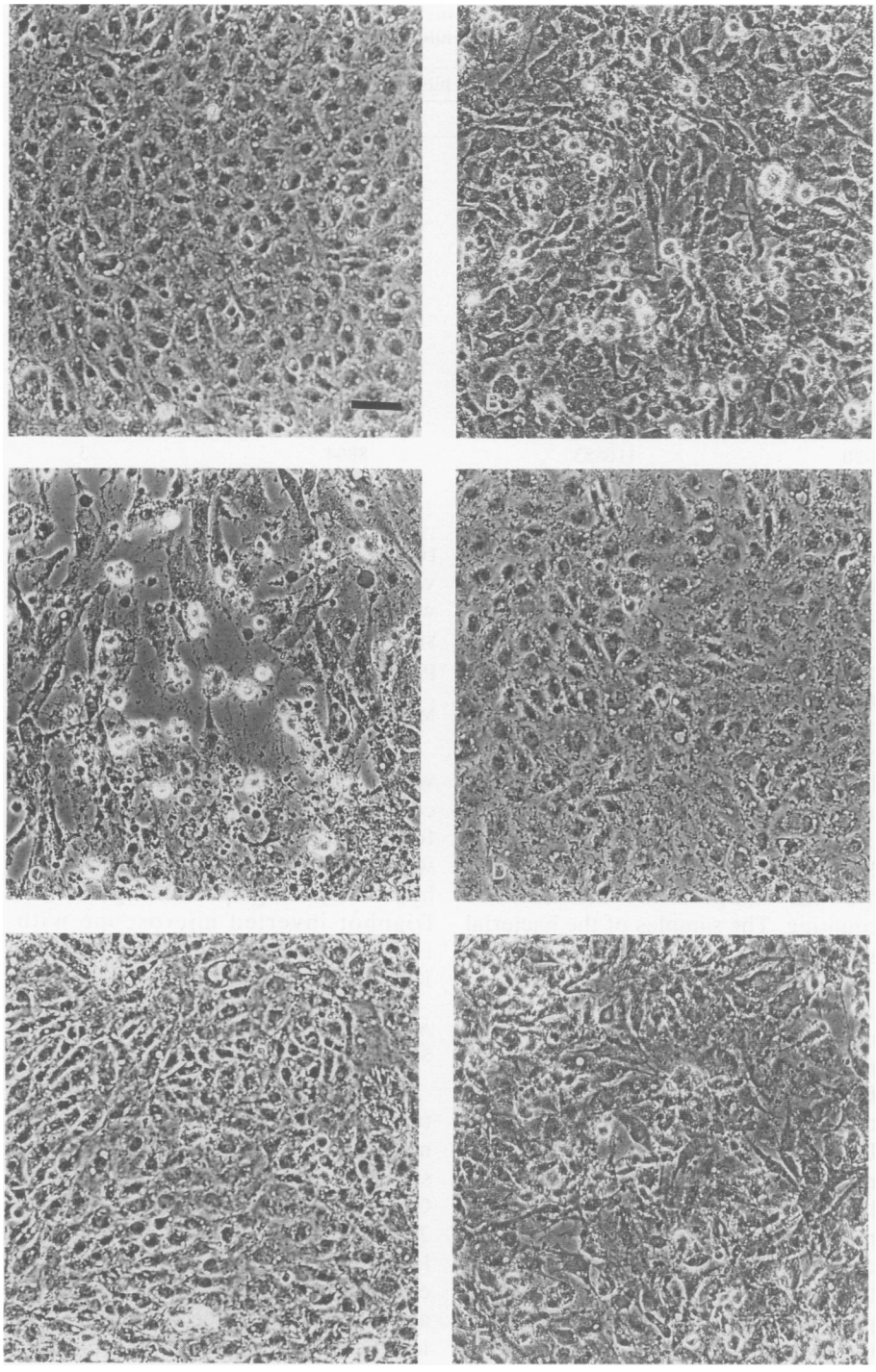

Fig. 1. Bovine aortic endothelial cells inoculated with an adjusted suspension, $A_{625\ nm}$ = 0.3, of live (A, B, C) and killed with formalin (D, E, F) pathogenic strain 88159 of *H. somnus* for 1.5 h. BAEC exposed to 0 (A, D), 10 (B, E), and 100 nM (C, F) of human recombinant tumor necrosis factor-α for 48 h. Bar 142 mm.

ATTACHMENT OF FORMALINIZED *H. SOMNUS* TO BAEC

Haemophilus somnus bacterial suspensions of strains 88541 and 88159 were killed with buffered formaldehyde (0.5%, pH 7.4) by incubation on the rotating shaker (180 rpm for 1 h). The suspensions were then centrifuged, washed, optical density adjusted as described before, and promptly used in the adherence assay. Bacterial killing was confirmed by absence of colony growth after plating 50 μL of the adjusted suspension on the BHI-agar for two days. The sampling for the scintillation counting and calculation of the proportion of adherence were performed on triplicate experiments as described above. Representative assayed monolayers were fixed in 10% formaldehyde and photographed.

ADHERENCE OF VARIOUS STRAINS OF *H. SOMNUS* TO BAEC

Twelve additional strains of *H. somnus* including six pathogenic and six nonpathogenic isolates, determined previously by I/C inoculation in calves (13,21,40), were cultured, radiolabelled, adjusted to A_{625} = 0.3 in suspension, and assayed for attachment to BAEC treated with 0 or 10 nM TNF-α for 48 h as described above. These strains included: 43826 (P) isolated from the brain of a case of TME (19), 70986 (P) isolated from *H. somnus* pneumonia (41), 26–16 (NP) isolated from a normal prepuce (42), S-20 (NP) isolated from semen (42), and eight strains isolated from the reproductive tract of cows; namely 8711337 (P), 8712155 (P), 87121531 (NP), 88318 (P), 88546 (NP), 885514 (NP), 885104 (P), and 8851018 (NP) (13). The experiments for each strain were performed in triplicate. Measurements and calculations of the radioactivity added and recovered, and percentage of adherence were performed as described above (Table I).

STATISTICAL ANALYSIS

Mean percent of bacterial adherence values were compared by analysis of variance using SuperAnova® software (Abacus Concepts, Inc., Berkeley, California).

RESULTS

ADHERENCE OF TWO LIVE STRAINS OF *H. SOMNUS* TO BAEC

Treatment of BAEC with 1 nM concentration of TNF-α or higher for 24 h or 48 h caused the cells to acquire a fusiform appearance and long, slender, frequently overlapping processes (43), which was more prominent at 48 h of incubation. When incubated with 100 nM TNF-α for 48 h, the cells had small blebs and

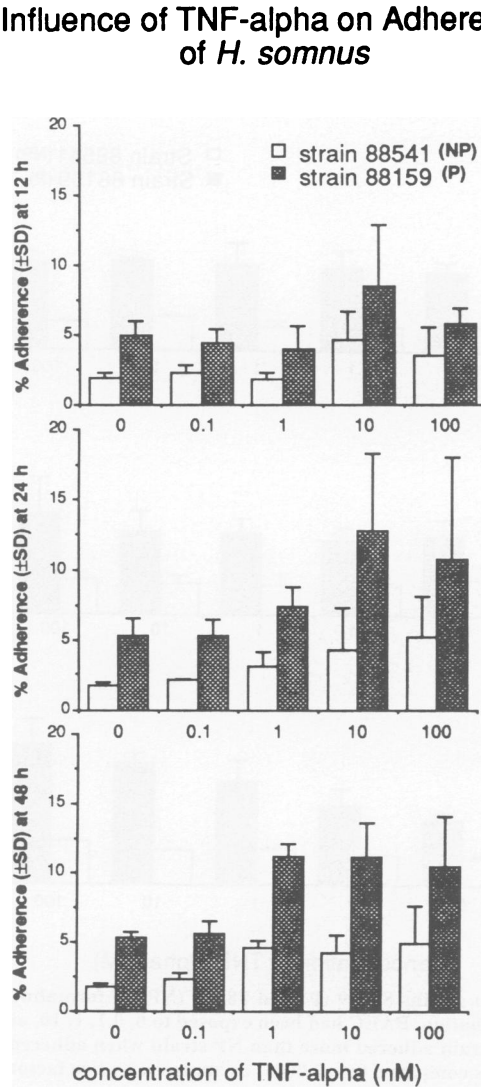

Fig. 2. Adherence of two live strains of *H. somnus* 88159 (P) and 88541 (NP) to BAEC after 1.5 h of incubation. BAEC had been exposed to 0, 0.1, 1, 10, and 100 nM of hrTNF-α for 12 (A), 24 (B), and 48 h (C). P strain adhered more than NP strain when the adherence in all tested concentrations of TNF was compared ($p < 0.0001$) as analyzed by two factor ANOVA.

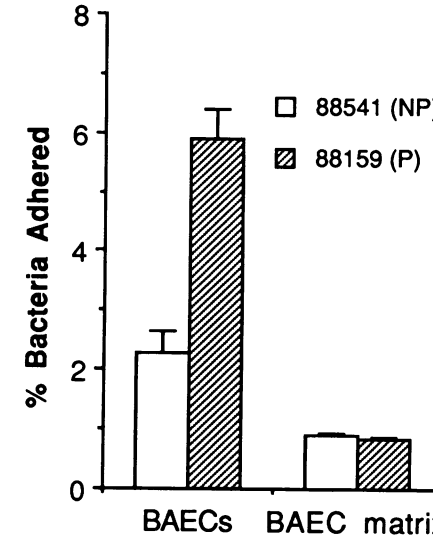

Fig. 3. Adherence, after 1.5 h of incubation of two strains, 88159 (P) and 88541 (NP) of *H. somnus* to BAEC (TNF untreated) and subendothelial matrix prepared by removing untreated confluent BAEC with PBS without Ca^{2+} and Mg^{2+} supplemented with 2 mM EDTA. Adherence of P and NP strains to BAEC was higher than to BAEC matrix ($p < 0.0001$) as analyzed by two factor ANOVA.

were moderately retracted revealing intercellular spaces. After the incubation with either strain of *H. somnus*, 88159 (P) or 88541 (NP), for 1.5 h the endothelial monolayers, in the absence of TNF-α, had vacuolated cytoplasm and occasional, scattered blebs. By comparison, the monolayers pretreated with TNF-α had more pronounced degrees of cellular blebbing and retraction of the cell borders with formation of intercellular spaces, and scattered karyorrhexic cells after incubation with live *H. somnus* for 1.5 h. These changes increased in severity with the concentration of TNF-α (Fig. 1 A, B, C) and the duration of exposure to TNF-a before the addition of bacteria.

Viewed under the light microscope, single or chains of bacterial rods were observed attached randomly to the surface of endothelial cells and some bacteria were present in the intercellular spaces (Fig. 1C, 1F). Bacterial organisms of strain 88159 (P) were more numerous than of strain 88541 (NP).

The quantitative results of adherence of live ^{3}H-labelled *H. somnus* of both strains to BAEC are presented in Fig. 2. Adherence to control monolayers (no TNF-α) by strain 88159 (P) was greater than for strain 88541 (NP) (Fig. 2). These values are expressed as a percentage of organisms inoculated and are corrected for the numbers of CFU added to the cultures. This difference in rates of adhesion to control monolayers was evident at all durations of culture of BAEC with TNF-α (12–48 h) (Fig. 2). Concentrations of TNF-α above 1 nM increased adhesion of both strains, the percentages of which were greater after 24 or 48 h of exposure of BAEC to TNF-α (Fig. 2). Adherence to monolayers treated with 100 nM TNF-α likely has been reduced (Fig. 2) due to retraction of EC and a concommitant reduction in available endothelial surface (Fig. 1C, 1F).

Confluent BAEC, not treated with TNF-α, were nonenzymatically removed from the substrate to evaluate adhesion of *H. somnus* to subendothelial matrix. In these experiments, rates of adhesion were much lower and similar for both strains (Fig. 3), suggesting that the higher rates of adhesion in the presence of BAEC is due to adhesion to BAEC rather than to exposed subendothelial matrix. Adherence rates to subendothelial matrix were 0.85 ± 0.02% for 88159 (P) and 0.91 ± 0.03% for

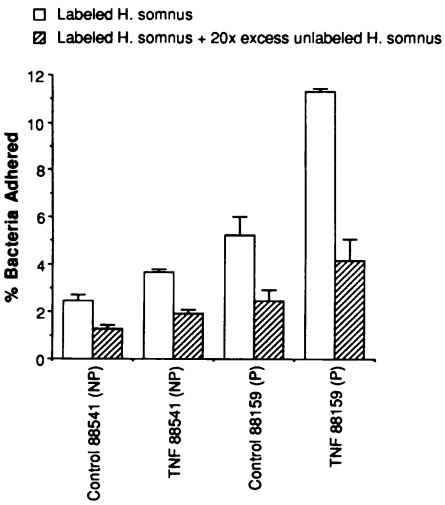

Fig. 4. Adherence of two strains, 88159 (P) and 88541 (NP) of *H. somnus* after 1.5 h incubation to BAEC which had been exposed to 0 and 10 nM hrTNF-α for 48 h. Adherence of ^{3}H-TdR-labelled standard bacterial inoculum, $A_{625\ nm}$ = 0.3 (10^8 CFU) is compared with the same inoculum supplemented with 20 × 10^8 CFU of unlabelled bacteria. Addition of excess unlabelled *H. somnus* decreased the adherence of labelled bacteria, and treatment with TNF-α increased bacterial adherence ($p < 0.0001$) as analyzed by two factor ANOVA.

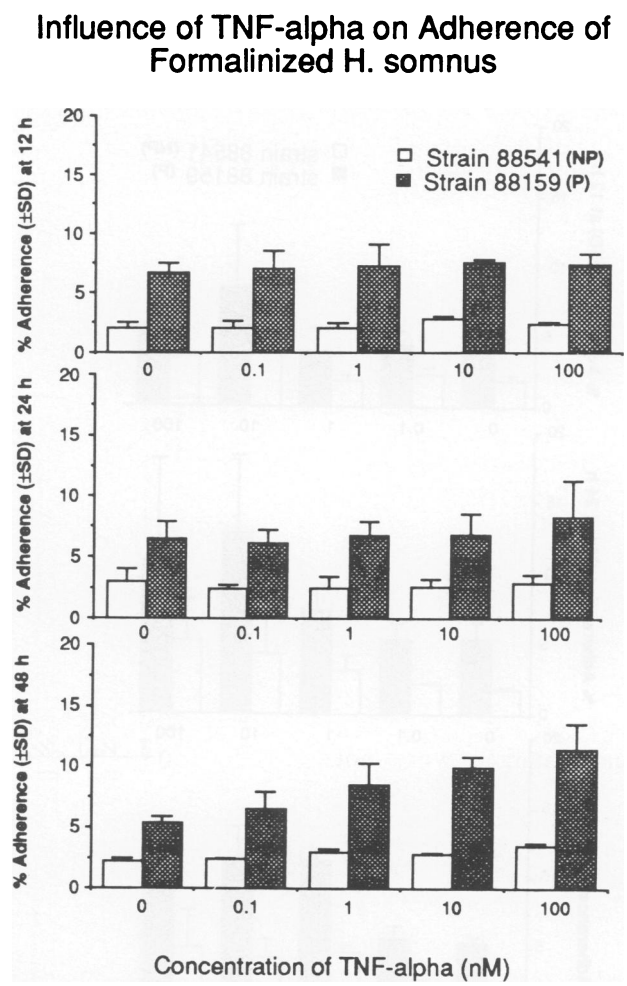

Fig. 5. Adherence of two strains 88159 (P) and 88541 (NP) of formalin-killed *H. somnus* to BAEC after 1.5 h of incubation. BAEC had been exposed to 0, 0.1, 1, 10, and 100 nM hrTNF-α for 12, 24, and 48 h. P strain adhered more than NP strain when adherence in all tested concentrations of TNF-α was compared ($p < 0.0001$) as analyzed by two factor ANOVA.

88541 (NP) (n = 2) (Fig. 3). Adherence of both strains to BAEC appeared to be saturable. Addition of 10^9 CFU of unlabelled organisms to control monolayers (no TNF-α), resulted in lower percentages of adhesion of radiolabelled *H. somnus*, namely 2.3 ± 0.4% for 88159 (P) and 0.8 ± 0.2% for 88541 (NP) (n = 2). Adherence of radiolabelled *H. somnus* to BAEC cultured with TNF-α (10 nM, 48 h) was also reduced by addition of 20 × 10^8 CFU of unlabelled *H. somnus* for each strain (Fig. 4).

ADHERENCE OF FORMALINIZED *H. SOMNUS* TO BAEC

Formalin-killed organisms of the strain 88159 (P) adhered in higher numbers than of the strain 88541 (NP). Adherence of killed organisms (88159) (P) to BAEC treated with TNF-α for 48 h increased in a concentration dependent manner (Fig. 5), which was essentially similar to adherence of live organisms (see Fig. 2). A similar but less pronounced increase in adherence of the formalin-killed strain 88541 (NP) was observed in monolayers treated with up to 100 nM TNF-α for 48 h (Fig. 5).

ADHERENCE OF VARIOUS STRAINS OF *H. SOMNUS* TO BAEC

All strains adhered more to endothelial monolayers treated with 10 nM TNF-α for 48 h than to BAEC not exposed to TNF-α (Fig. 6). All strains caused marked cytotoxic morphological changes in BAEC exposed to TNF-α and negligible or mild changes in TNF-α-untreated BAEC similar to already described changes caused by strains 88541 (NP) and 88159 (P). However, there was no indication that the pathogenic strains were more adherent to control or TNF-α-exposed BAEC than were the nonpathogenic strains.

DISCUSSION

We have demonstrated in this work that *H. somnus*, a common bovine pathogen, adheres to BAEC in vitro and this adherence is enhanced by pretreatment of endothelium with hrTNF-α. These studies do not explain the molecular basis for this adherence, nor do they indicate that the intrinsic adherence properties of *H. somnus* organisms demonstrated in this model is a basis for virulence of the pathogenic isolates studied. However, the results are consistent with the hypothesis that TNF-α mediates increased adherence of all strains of *H. somnus* to BAEC. The adherence of *H. somnus* to BAEC increased as the concentration and time of exposure of these cells to TNF-α increased. Adherence of *H. somnus* to dishes with exposed subendothelial matrix prepared by nonenzymatic removal of BAEC was substantially lower than observed in the presence of BAEC, suggesting that the adhesion observed is to EC rather than to intercellular spaces. When a 20×

excess of unlabelled organisms were added with the standard inoculum of radiolabelled bacteria, adherence of the radiolabelled *H. somnus* decreased, consistent with competition by unlabelled organisms for saturable binding sites of *H. somnus* on BAEC. Two strains killed with formalin adhered to BAEC in a fashion essentially similar to that observed in adherence of live strains. The adhesins involved in attachment to cultured BAEC in these experiments are not known. The results suggest however, that the adhesive properties are present in formalin-killed *H. somnus*, because TNF-α-dependent and time-dependent adhesion of killed bacteria resembled that observed for live organisms.

In BAEC, live strains of *H. somnus* caused toxic morphological changes, the severity of which increased with increased concentration and duration of TNF-α exposure (Fig. 1). Such morphological changes were not induced by eight P and eight NP isolates in the absence of TNF-α, nor in TNF-α-exposed BAEC incubated with formalin killed *H. somnus*. Mechanisms by which TNF augments cellular toxicity of live *H. somnus* are unknown.

Since TNF-α enhanced the adherence of *H. somnus* to BAEC, it is possible that monocyte-derived TNF-α might be involved in procoagulant activation of endothelium and microvascular injury during *H. somnus* bacteremia. However, this interpretation is subject to some possible limitations in the model used. In our studies, hrTNF-α was used rather than bovine TNF-α. Definitive evidence for a role of leukocyte-derived TNF-α in the pathogenesis of microvascular injury in *H. somnus* septicemia will require demonstration that pathogenic strains induce bovine monocytes to generate larger and cytotoxic concentrations of TNF-α. Other endothelium activating cytokines (27–29,31) might also play an important role in the process of *H. somnus* cytotoxicity in vivo. Also, the present observations relate to bacterial adherence to endothelium of large vessels, however, other microvascular endothelial cells need to be evaluated to more clearly define the role of endothelial adherence of *H. somnus* in TME in vivo.

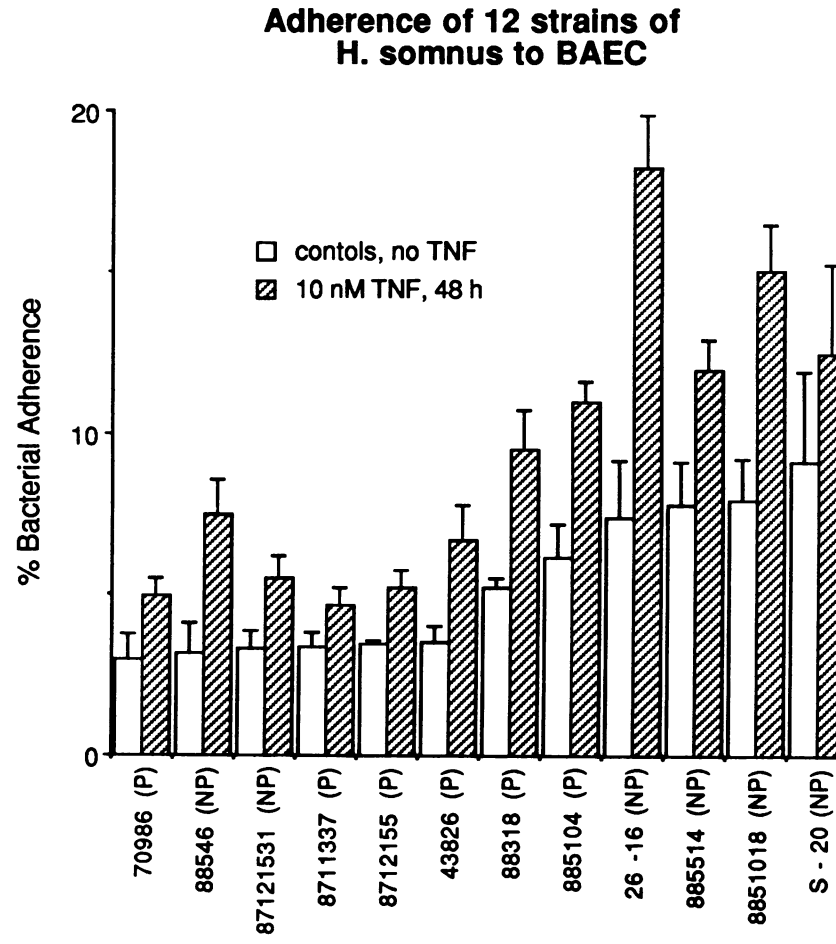

Fig. 6. Adherence of 12 various isolates of *H. somnus* after 1.5 h of incubation to BAEC treated with 0 and 10 nM hrTNF-α for 48 h.

There is some evidence that *H. somnus* does adhere to other cells. This organism is not encapsulated and does not have pili or fimbriae (22–24), but it can adhere to zona pellucida-intact bovine embryos (25), bovine spermatozoae (26), and the endothelium of bovine aortic explants (20). Surface receptors for the Fc fragment of immunoglobulins (Ig) have been suggested to be important in the interaction of *H. somnus* with bovine cells (17,44,45). *Haemophilus somnus* in immune complexes involving specific anti-*H. somnus* IgG and fragment C1 of complement are implicated in adhesion to bovine spermatozoa (46). In our model, adhesion of *H. somnus* was measured in the presence of 10% fetal bovine serum which might contain Ig or other proteins involved in *H. somnus* adhesion.

A capsule-deficient isolate of *Haemophilus influenzae* type b adhered more to human umbilical vein endothelial cells (HUVEC) than a capsulated isolate in one study (47). The adherence of *Staphylococcus aureus*, to cultured human (48), and bovine (49) endothelium, was found to be facilitated by multiple bacterial proteins (50) and a component specific for *S. aureus* binding to HUVEC has been recently identified (51). The attachment of *S. aureus* to HUVEC was enhanced in the presence of fibrinogen (52) and to BAEC in the presence of fibronectin (53). Although the adherence of *S. aureus* did not increase after stimulation of HUVEC by lipopolysaccharide (LPS) or interleukin-1 (IL-1) in one study (54), both these mediators of inflammation increased this adherence in another, differently designed study (55). Recombinant human tumor necrosis factor-α increased adherence of *S. aureus* to HUVEC but the presence of plasma was a prerequisite for this increase to occur (56). Among

gram-negative bacterial pathogens capable of causing septicemia, a strain of *Salmonella minnesota* has been found to adhere to bovine pulmonary endothelial cells (BPEC) and C1q component of complement increased this adherence (57).

The evidence of adherence of *H. somnus* to BAEC and enhancement of this adherence by TNF-α may suggest the direction of future research on pathogenicity of this organism towards investigation of specific interactions of *H. somnus* with vascular endothelium involving bacterial and cellular receptors. The role of cytokines in this putative interaction should also be addressed.

REFERENCES

1. **HUMPHREY JD, STEPHENS LR.** "*Haemophilus somnus*": A review. Vet Bull 1983; 53: 987–1004.
2. **KENNEDY PC, BIBERSTEIN EL, HOWARTH JA, FRAZIER LM, DUNGWORTH DL.** Infectious meningoencephalitis in cattle caused by a *Haemophilus*-like organism. Am J Vet Res 1960; 21: 403–409.
3. **PANCIERA RJ, DAHLGREN RR, RINKER HB.** Observations on septicemia of cattle caused by a *Haemophilus*-like organism. Pathol Vet 1968; 5: 212–226.
4. **STEPHENS LR, LITTLE PB, WILKIE BN, BARNUM DA.** Infectious thromboembolic meningoencephalitis in cattle: A review. J Am Vet Med Assoc 1981; 178: 378–384.
5. **ANDREWS JJ, ANDERSON TD, SLIFE LN, STEVENSON GW.** Microscopic lesions associated with isolation of *Haemophilus somnus* from pneumonic bovine lungs. Vet Pathol 1985; 22: 131–136.
6. **CORBEIL LB, WIDDERS PR, GOGOLEWSKI R, ARTHUR J, INZANA TJ, WARD ACS.** *Haemophilus somnus*: Bovine reproductive and respiratory disease. Can Vet J 1986; 27: 90–93.
7. **GUICHON PT, PRITCHARD J, JIM GK.** *Haemophilus somnus* myocarditis in a feedlot steer. Can Vet J 1988; 29: 1012–1013.
8. **ARMSTRONG KR, OSBORNE AD, JANZEN ED.** *Haemophilus somnus* mastitis in a dairy cow. Can Vet J 1986; 27: 211–212.
9. **HIGGINS R, MARTIN JR, LAROUCHE Y, GOYETTE G.** Mastitis caused by *Haemophilus somnus* in a dairy cow. Can Vet J 1987; 28: 117–119.
10. **KWIECIEN JM, LITTLE PB.** *Haemophilus somnus* and reproductive disease in the cow: A review. Can Vet J 1991; 32: 595–601.
11. **GOGOLEWSKI RP, LEATHERS CW, LIGGITT HD, CORBEIL LB.** Experimental *Haemophilus somnus* pneumonia and immunoperoxidase localization of bacteria. Vet Pathol 1987; 24: 250–256.
12. **GOGOLEWSKI RP, SCHAEFER DC, WASSON SK, CORBEIL RR, CORBEIL LB.** Pulmonary persistence of *Haemophilus somnus* in the presence of specific antibody. J Clin Microbiol 1989; 27: 1767–1774.
13. **KWIECIEN JM, LITTLE PB.** Isolation of pathogenic strains of *Haemophilus somnus* from the female bovine reproductive tract. Can J Vet Res 1992; 56: 127–134.
14. **LEDERER JA, BROWN JF, CZUPRYNSKI CJ.** "*Haemophilus somnus*", a facultative intracellular pathogen of bovine mononuclear phagocytes. Infect Immun 1987; 55: 381–387.
15. **CZUPRYNSKI CJ, HAMILTON HL.** Bovine neutrophils ingest but do not kill *Haemophilus somnus* in vitro. Infect Immun 1985; 50: 431–436.
16. **HUBBARD RD, KAEBERLE ML, ROTH JA, CHIANG YW.** *Haemophilus somnus*-induced interference with bovine neutrophil functions. Vet Microbiol 1986; 12: 77–85.
17. **CORBEIL LB.** Molecular aspects of some virulent factors of *Haemophilus somnus*. Can J Vet Res 1990; 54: S57–S62.
18. **STEPHENS LR, LITTLE PB, HUMPHREY JD, WILKIE BN, BARNUM DA.** Vaccination of cattle against experimentally induced thromboembolic meningoencephalitis with a *Haemophilus somnus* bacterin. Am J Vet Res 1982; 43: 1339–1342.
19. **STEPHENS LR, LITTLE PB, WILKIE BN, BARNUM DA.** Humoral immunity in experimental thromboembolic meningoencephalitis in cattle caused by *Haemophilus somnus*. Am J Vet Res 1981; 42: 468–473.
20. **THOMPSON KG, LITTLE PB.** Effect of *Haemophilus somnus* on bovine endothelial cells in organ culture. Am J Vet Res 1981; 42: 748–754.
21. **HUMPHREY JD.** PhD thesis. University of Guelph, Guelph, Ontario, 1982.
22. **SILVA P, T MOK, LITTLE PB.** The development of protein A-gold electron microscopy for immunological studies of *Haemophilus somnus*. Vet Microbiol 1991; 27: 25–37.
23. **STEPHENS LR, LITTLE PB.** Ultrastructure of *Haemophilus somnus*, causative agent of bovine infectious thromboembolic meningoencephalitis. Am J Vet Res 1981; 42: 1638–1640.
24. **WARD GE, NIVARD JR, MAHESWARAN SK.** Morphologic features, structure, and adherence to bovine turbinate cells of three *Haemophilus somnus* variants. Am J Vet Res 1984; 45: 336–338.
25. **THOMSON MS, STRINGFELLOW DA, LAUERMAN LH.** Adherence of *Haemophilus somnus* to bovine embryos after in vitro exposure. Am J Vet Res 1988; 49: 63–66.
26. **CHELMONSKA A.** The influence of *Haemophilus somnus* on bull sperms examined in vitro. Pol Arch Weter 1990; 30: 141–154.
27. **CYBULSKY MI, CHAN MKW, MOVAT HZ.** Acute inflammation and microthrombosis induced by endotoxin, interleukin-1, and tumor necrosis factor and their implication in gram-negative infection. Lab Invest 1988; 58: 365–378.
28. **MANTOVANI A, BUSSOLINO F, DEJANA E.** Cytokine regulation of endothelial function. FASEB J 1992; 6: 2591–2599.
29. **MANTOVANI A, DEJANA E.** Cytokines as communication signals between leukocytes and endothelial cells. Immunol Today 1989; 10: 370–375.
30. **OSBORN L.** Leukocyte adhesion to endothelium in inflammation. Cell 1990; 62: 3–6.
31. **GIMBRONE MA, BEVILACQUA MP.** Vascular endothelium, functional modulation at the blood interface. In: Simionescu N, Simionescu M, eds. Endothelial Cell Biology in Health and Disease. New York: Plenum Press, 1988: 255–273.
32. **GUSTAFSON KS, VERCELLOTTI GM, BENDEL CM, HOSTETTER MK.** Molecular mimicry in *Candida albicans*. J Clin Invest 1991; 87: 1896–1902.
33. **BERENDT AR, SIMMONS DL, TANSEY J, NEWBOLD CI, MARSH K.** Intercellular adhesion molecule-1 is an endothelial adhesion receptor for *Plasmodium falciparum*. Nature 1989; 341: 57–59.
34. **GREVE JM,DAVIS G, MEYER AM, FORTE CP, YOST SC, MARLOR CW, KAMARCK ME, McCLELLAND A.** The major human rhinovirus receptor is ICAM-1. Cell 1989; 56: 839–847.
35. **INZANA TJ, CORBEIL LB.** Development of a defined medium for *Haemophilus somnus* isolated from cattle. Am J Vet Res 1987; 48: 366–369.
36. **MACARAK EJ, HOWARD BV, KEFALIDES NA.** Properties of calf endothelial cells in culture. Lab Invest 1977; 36: 62–67.
37. **VALDIVIESO-GARCIA A, ROSENDAL S.** Endothelial cell cytotoxicity assay: A model for pathogenicity studies for *Mycoplasma mycoides* subspecies *mycoides*. Int J Microbiol (Suppl.) 1990; 20: 406–415.
38. **RYAN US.** Immunofluorescence and immunocytochemistry of endothelial surface antigens. J Tissue Cult Methods 1986; 10: 27–29.
39. **VOYTA JC, VIA DP, BUTTERFIELD CE, ZETTER BR.** Identification and isolation of endothelial cells based on their increased uptake of acetylated-low density lipoprotein. J Cell Biol 1984; 99: 2034–2040.
40. **GROOM SC.** PhD thesis. University of Guelph, Guelph, Ontario, 1990.
41. **GROOM SC, LITTLE PB, ROSENDAL S.** Virulence differences among three strains of *Haemophilus somnus* following intratracheal inoculation of calves. Can J Vet Res 1988; 52: 349–354.
42. **HUMPHREY JD, LITTLE PB, BARNUM DA, DOIG PA, STEPHENS LR, THORSEN J.** Occurrence of *Haemophilus somnus* in semen and in the prepuce of bulls and steers. Can J Comp Med 1982; 46: 215–217.
43. **STOLPEN AH, GUINAN EC, FIERS W, POBER JS.** Recombinant tumor necrosis factor and immune interferon act singly and in combination to reorganize human vascular endothelial cell monolayers. Am J Pathol 1986; 123: 16–24.

44. **WIDDERS PR, DORRANCE LA, YARNALL M, CORBEIL LB.** Immunoglobulin-binding activity among pathogenic and carrier isolates of *Haemophilus somnus*. Infect Immun 1989; 57: 639–642.
45. **YARNALL M, GOGOLEWSKI RP, CORBEIL LB.** Characterization of two *Haemophilus somnus* Fc receptors. J Gen Microbiol 1988; 134: 1993–1999.
46. **CHELMONSKA A.** PhD thesis. University of Agriculture, Wroclaw, Poland, 1991.
47. **VIRJI M, KAYTHY H, FERGUSON DJP, ALEXANDRESCU C, MOXON ER.** Interactions of *Haemophilus influenzae* with cultured human endothelial cells. Microb Pathog 1991; 10: 231–245.
48. **OGAWA SK, YURBERG ER, HATCHER VB, LEVITT MA, LOWY FD.** Bacterial adherence to human endothelial cells in vitro. Infect Immun 1985; 50: 218–224.
49. **HAMILL RJ, VANN JM, PROCTOR RA.** Phagocytosis of *Staphylococcus aureus* by cultured bovine aortic endothelial cells: Model for postadherence events in endovascular infections. Infect Immun 1986; 54: 833–836.
50. **TOMPKINS DC, BLACKWELL LJ, HATCHER VB, ELLIOTT DA, O'HAGAN-SOTSKY C, LOWY FD.** *Staphylococcus aureus* proteins that bind to human endothelial cells. Infect Immun 1992; 60: 965–969.
51. **TOMPKINS DC, HATCHER VB, PATEL D, ORR GA, HIGGINS LL, LOWY FD.** A human endothelial cell membrane protein that binds *Staphylococcus aureus* in vitro. J Clin Invest 1990; 85: 1248–1254.
52. **CHEUNG AL, KRISHNAN M, JAFFE EA, FISCHETTI VA.** Fibrinogen acts as a bridging molecule of *Staphylococcus aureus* to cultured human endothelial cells. J Clin Invest 1991; 87; 2236–2245.
53. **VANN JM, HAMILL RJ, ALBRECHT RM, MOSHER DF, PROCTOR RA.** Immunoelectronmicroscopic localization of fibronectin in adherence of *Staphylococcus aureus* to cultured bovine endothelial cells. J Infect Dis 1989; 160: 538–542.
54. **BLUMBERG EA, HATCHER VB, LOWY FD.** Acidic fibroblast growth factor modulates Staphylococcus aureus adherence to human endothelial cells. Infect Immun 1988; 56: 1470–1474.
55. **THOMAS PD, HAMPSON FW, HUNNINGHAKE GW.** Bacterial adherence to human endothelial cells. J Appl Physiol 1988; 65: 1372–1376.
56. **CHEUNG AL, KOOMEY JM, LEE S, JAFFE EA, FISCHETTI VA.** Recombinant human tumor necrosis factor alpha promotes adherence of *Staphylococcus aureus* to cultured human endothelial cells. Infect Immun 1991; 59: 3827–3831.
57. **RYAN US, SHULTZ DR, GOODWIN JD, VANN JM, SELVARAJ MP, HART MA.** Role of C1q in phagocytosis of Salmonella minnesota by pulmonary endothelial cells. Infect Immun 1989; 57: 1356–1362.