INFECTION AND IMMUNITY, Aug. 1996, p. 3016–3022 Vol. 64, No. 8
0019-9567/96/$04.00+0

Cytokine Production by Human Epithelial and Endothelial Cells following Exposure to Oral Viridans Streptococci Involves Lectin Interactions between Bacteria and Cell Surface Receptors

ALINE VERNIER,[1] MOUNA DIAB,[1] MARTINE SOELL,[1] GISÈLE HAAN-ARCHIPOFF,[2] ALAIN BERETZ,[2] DOMINIQUE WACHSMANN,[1] AND JEAN-PAUL KLEIN[1]*

Faculté de Pharmacie, Institut National de la Santé et de la Recherche Médicale Unité 392,[1] and Unité de Recherche Associée 600, Centre National de la Recherche Scientifique,[2] F-67401 Illkirch Cedex, France

Received 13 December 1995/Returned for modification 20 February 1996/Accepted 20 May 1996

In order to examine the possible implication of human epithelial and endothelial cells in the pathogenesis of various diseases associated with oral viridans streptococci, we tested the immunomodulatory effects of 11 representative strains of oral viridans streptococci on human epithelial KB cells and endothelial cells. We then examined the possible role of two major adhesins from oral viridans streptococci, protein I/II and rhamnose-glucose polymers (RGPs), in this process. In this study we demonstrate that oral viridans streptococci are potent stimulators of interleukin-8 (IL-8) production from KB cells and of IL-6 and IL-8 production from endothelial cells. The ability of protein I/II and RGPs to contribute to these effects was then examined. Using biotinylated protein I/IIf and RGPs from *Streptococcus mutans* OMZ 175, we showed that these adhesins bind to KB and endothelial cells through specific interactions and that the binding of these molecules initiates the release of IL-8 from KB cells and of IL-6 and IL-8 from endothelial cells. These results suggest that protein I/IIf and RGPs play an important role in the interactions between bacteria and KB and endothelial cells in that similar cytokine profiles are obtained when cells are stimulated with bacteria or surface components. We also provide evidence that protein I/IIf binds to and stimulates KB and endothelial cells through lectin interactions and that *N*-acetyl neuraminic acid (NANA) and fucose present on cell surface glycoproteins may form the recognition site since binding and cytokine release can be inhibited by dispase and periodate treatment of cells and by NANA and fucose. These results demonstrate that oral viridans streptococci, probably by engaging two cell surface adhesins, exert immunomodulatory effects on human KB and endothelial cells.

Oral viridans streptococci, which comprise several species, are major commensal bacteria of the human oral and pharyngeal cavity (8). Although infection by oral viridans streptococci is a model of mucosal bacterial pathogenesis, these microorganisms have been associated with invasive diseases such as sepsis and toxic shock. Bochud et al. (6) have recently shown that in immunocompromised patients, oral viridans streptococci have progressively become one of the most frequently isolated blood-borne pathogens, particularly in patients with chemotherapy- or radiotherapy-induced neutropenia. In addition, they can induce chronic inflammatory diseases such as subacute endocarditis, subacute glomerulonephritis, and rheumatic fever (33). Despite the fact that the mechanisms of pathogenesis remain undefined, several reports have suggested that the production of excess proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), IL-6, and IL-8, may be involved in the development of these diseases (5, 30).

The adherence of oral viridans streptococci to tissues, which is an initial and essential step in pathogenesis, is mediated by several bacterial adhesins that bind either to salivary receptors adsorbed onto buccal surfaces (10, 11) or to epithelial cell surface receptors. Two kinds of adhesins have been described: cell-wall polysaccharides (34) and a family of immunologically and structurally related cell surface proteins, termed antigens I/II (21, 27), with molecular masses of approximately 180 to 200 kDa. The wide distribution of both adhesins among the different oral species suggests that they serve an important function for the bacteria and may be important virulence factors in bacterial pathogenicity. In fact, previous studies in our laboratory have demonstrated that protein I/IIf as well as a polysaccharide formed by a poly-L-rhamnose backbone with D-glucose side chains (RGPs) (20, 26) from *Streptococcus mutans* OMZ 175, a member of the oral viridans streptococci, exerts in vitro immunomodulatory effects on human monocytes, such as the induction of TNF-α, IL-1β, and IL-6 (5, 30), and can therefore be implicated in the initiation and development of immunopathological reactions associated with oral viridans streptococci. However, mononuclear phagocytes are probably not the primary target cells for oral viridans streptococci, and a large body of evidence (13, 32) has recently suggested that the epithelium, which constitutes the first site of colonization, also has the potential to synthesize a variety of cytokines, e.g., TNF-α, IL-1β, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor, and transforming growth factor β, which may contribute to immunopathological disorders if they are secreted in excess, in response to microbial challenge. Moreover, previous studies on the pathogenesis of oral viridans streptococcal diseases seem to imply that oral viridans streptococci are likely to become hematogeneously

* Corresponding author. Faculté de Pharmacie, INSERM Unité 392, 74 route du Rhin, F-67401 Illkirch Cedex, France. Phone: (33) 88 67 68 28. Fax: (33) 88 67 92 42. Electronic mail address: jpklein@pharma.u-strasbg.fr.

TABLE 1. IL-6 and IL-8 production by KB and endothelial cells after stimulation with oral viridans streptococcal strains[a]

Streptococcal strain	Amt (ng/ml) of cytokine in:			
	KB cells		Endothelial cells	
	IL-6	IL-8	IL-6	IL-8
Control	0.23 ± 0.02	0.27 ± 0.03	0.45 ± 0.05	0.55 ± 0.07
S. mutans OMZ 175	0.27 ± 0.03	1.04 ± 0.08	3.20 ± 0.40	1.49 ± 0.20
S. milleri A 1784	0.31 ± 0.03	0.62 ± 0.03	3.93 ± 0.35	2.00 ± 0.22
S. salivarius ATCC 9759	0.29 ± 0.02	1.00 ± 0.09	3.60 ± 0.28	1.87 ± 0.16
S. gordonii P4A7	0.30 ± 0.03	0.76 ± 0.04	3.62 ± 0.32	1.80 ± 0.11
S. mitis ATCC 903	0.32 ± 0.04	1.12 ± 0.09	3.57 ± 0.33	1.72 ± 0.20
S. downei MFe 28	0.26 ± 0.04	0.52 ± 0.03	3.49 ± 0.36	1.70 ± 0.18
S. sobrinus 6715	0.22 ± 0.02	0.76 ± 0.04	3.45 ± 0.31	1.71 ± 0.21
S. constellatus NCTC 10714	0.24 ± 0.02	0.86 ± 0.04	3.50 ± 0.32	1.66 ± 0.18
S. gordonii challis	0.25 ± 0.03	1.40 ± 0.10	3.19 ± 0.30	1.45 ± 0.12
S. anginosus NCTC 10713	0.25 ± 0.04	1.35 ± 0.09	3.07 ± 0.33	1.30 ± 0.15
S. intermedius 415-87	0.28 ± 0.04	0.76 ± 0.04	3.00 ± 0.25	1.25 ± 0.09

[a] Supernatants were tested at 20 h of culture, and IL-6 and IL-8 levels were determined by a heterologous two-site sandwich ELISA. The means ± standard deviations for three determinations carried out in triplicate are shown.

disseminated. Thus, endothelial cells may be among the first host cells to interact with these organisms as they invade, and it is highly probable that endothelial cells may contribute to the pathology, particularly in toxic shock (4, 24).

In order to provide further evidence for these suggestions, we have investigated the ability of 11 strains of oral viridans streptococci, all of which reacted with anti-protein I/IIf serum (21) to exert immunomodulatory effects on cells of a human epithelioid carcinoma cell line (KB cells) and on human saphenous vein endothelial cells. This investigation was followed by examination of the possible contributions of two major adhesins of *S. mutans* OMZ 175, protein I/IIf and RGPs, to this process.

MATERIALS AND METHODS

Reagents. Cell culture media (M 199 and RPMI 1640), L-glutamine, penicillin, streptomycin, gentamicin, amphotericin B, trypsin-EDTA solutions (0.5 g of trypsin per liter, 0.2 g of EDTA per liter, 0.85 g of NaCl per liter), and fetal calf serum were obtained from Life Technologies (Cergy-Pontoise, France). Cell-culture media had an endotoxin content which never exceeded 0.04 ng/ml as tested by the *Limulus* chromogenic assay. Polymyxin B, biotinamidocaproyl hydrazide, *N*-acetyl neuraminic acid (NANA), and L-fucose were obtained from Sigma Chemical Co. (Saint-Quentin-Fallavier, France). Dispase was obtained from Boehringer (Mannheim, Germany), and NHS-LC biotin was purchased from Pierce (Oud-Beijerland, the Netherlands). Alkaline phosphatase-streptavidin was obtained from Dako (Trappes, France). Recombinant human TNF-α was kindly given by Knoll (BASF, Ludwigshafen, Germany), and rabbit polyclonal anti-recombinant human TNF-α and the corresponding biotinylated immunoglobulin G were prepared as previously described (5). The enzyme immunoassay kits for IL-1β and IL-6 were from Immunotech (Marseille, France), and those for IL-8 were from Euromedex (Schiltigheim, France). Purified factor VIII and human normal serum (HNS) prepared from a pool of serum specimens from 13 to 15 healthy blood donors negative for hepatitis B virus and immunodeficiency virus were obtained from the Centre Regional de Transfusion Sanguine, Strasbourg, France. The serum was inactivated at 56°C for 30 min (heat-inactivated HNS [HI-HNS]).

Bacterial strains and preparation of protein I/IIf, and RGPs. *S. mutans* OMZ 175 (serotype f), *Streptococcus sobrinus* 6715 (serotype g), and *Streptococcus downei* MFe28 (serotype h) have been previously described (3, 21). The following viridans *Streptococcus* strains were also examined: *S. mitis* ATCC 903 and *S. salivarius* ATCC 9759, *S. milleri* A 1784 (from K. L. Ruoff, Harvard Medical School, Boston, Mass.), *S. anginosus* NCTC 10713, *S. constellatus* NCTC 10714, *S. intermedius* 415-87 (from R. A. Whiley, London Hospital Medical College, United Kingdom), *S. gordonii* (formerly *S. sanguis*) P4A7 (from J. S. Van Der Hoeven, University of Nijmegen, Nijmegen, The Netherlands), and *S. gordonii* Challis (from our laboratory collection). The strains were grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.). Recombinant protein I/IIf of *S. mutans* OMZ 175 (23) was purified from pHB*sr*-1-transformed *Escherichia coli* cell extract by gel filtration and immunoaffinity chromatography as described by Ackermans et al. (1). The polysaccharide antigen of *S. mutans* OMZ 175, formed of RGPs has been described previously and was prepared according to the method described by Benabdelmoumene et al. (5). Purified protein I/IIf and RGPs were labelled with NHS-LC biotin and biotinamidocaproyl hydrazide, respectively, according to the manufacturer's procedure (Pierce, Oud-Beijerland, the Netherlands).

Preparation of cells and solubilization of membrane proteins. Cells of the human epithelioid carcinoma cell line KB (ECACC 86103004) were cultured as previously described (29). Briefly KB cells were routinely grown in RPMI 1640 medium containing 2 mM L-glutamine, gentamicin (50 μg/ml), and 10% heat-inactivated fetal calf serum at 37°C in a humidified atmosphere of 5% CO_2 in air, until they reached confluence. Cells monolayers were then treated with trypsin-EDTA (5 min, 25°C) and centrifuged at 500 × *g* for 10 min, and the cell pellet was suspended in fresh medium. The cell suspension (200 μl) was added to each well of 96-well microtiter plates (Nunc, Roskilde, Denmark) (10^5 cells per well), and the cells were allowed to grow until confluence (48 h), to a final density of $2.2 \times 10^5 \pm 0.2 \times 10^5$ cells per well.

Human saphenous vein endothelial cells were collected as previously described by Klein-Soyer et al. (19) and grown in M 199-RPMI 1640 (1:1) containing 2 mM L-glutamine, penicillin (100 IU/ml), streptomycin (100 μg/ml), amphotericin B (0.25 μg/ml), and 20% HI-HNS. Between passages 3 and 10, the cells were grown to confluence in factor VIII-coated 96-well plates (8 days), to a final concentration of $1.5 \times 10^4 \pm 0.2 \times 10^4$ cells per well.

The number of cells and cell viability were examined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] test as described elsewhere (22). Prior to binding or activation experiments, cells were intensively washed with serum-free RPMI 1640, before the addition of the appropriate stimuli diluted in serum-free RPMI 1640.

KB and endothelial cells membrane proteins were solubilized by incubating 3×10^7 cells with 600 μl of phosphate-buffered saline containing 1% Nonidet P-40 and antiproteases for 5 min on ice as previously described (14). After centrifugation (30,000 × *g*, 15 min), the protein concentrations in the supernatant were determined by using a dye reagent (Bio-Rad, Ivry sur Seine, France) according to the manufacturer's instructions. Cell extracts were stored until use at −80°C.

Binding assays. Protein I/IIf and RGP binding activities in human KB and endothelial cells were analyzed at 4°C in serum-free RPMI 1640. Microtiter plates coated with KB cells (2.2×10^5 cells per well) or endothelial cells (1.5×10^4 cells per well) were first incubated with 250 μl of RPMI 1640 containing 0.5% gelatin for 30 min. After being washed, the cells were incubated for 20 h at 4°C with 50 ml of various concentrations of biotinylated protein I/IIf or biotinylated RGPs in RPMI 1640. After washing and fixation of the cells with methanol, bound ligands were detected by sequential incubations with alkaline phosphatase-streptavidin (1 h, 37°C) followed by a 1-h incubation with 50 μl of *p*-nitrophenylphosphate in 10% diethanolamine buffer (pH 9.8; (1 mg/ml). The plates were read at A_{405} with an Anthos Labtec (Salzburg, Austria) spectrophotometer. Nonspecific binding was determined in the same way, in the presence of a 20-fold excess of unlabelled protein I/IIf or RGPs. The level of specific binding was obtained by subtracting nonspecific binding from total binding. In parallel, binding was tested in the presence of various concentrations of HI-HNS. In competitive assays, minimal saturating amounts of biotinylated protein I/IIf in RPMI 1640 were mixed with NANA or fucose (50 mM), incubated 30 min at 37°C, and then added to the cells monolayers. The results were expressed as the percent inhibition of labelled protein I/IIf binding without competitor: % inhibition = 100 − [(binding activity with competitor/binding activity without competitor) × 100].

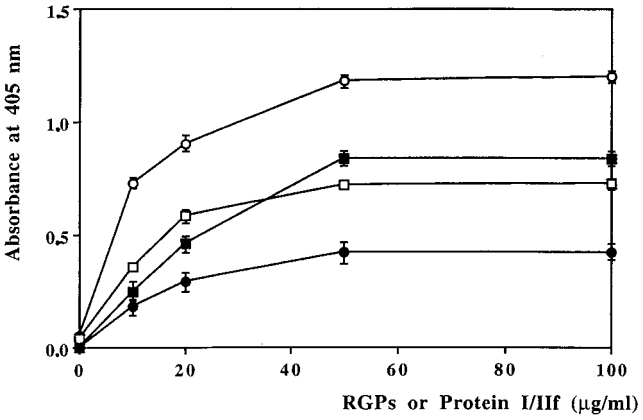

FIG. 1. Dose-response analysis of the specific binding of protein I/IIf and RGPs to human KB and endothelial cells. Plates coated with KB (squares) and endothelial (circles) cells were incubated for 20 h at 4°C with increasing amounts of biotinylated protein I/IIf (open symbols) or biotinylated RGPs (closed symbols). Each point represents the mean for three determinations carried out in triplicate; error bars indicate standard deviations.

Activation of cells. KB cells (2.2×10^5 cells per well) or endothelial cells (1.5×10^4 cells per well) were incubated at 37°C with 200 μl of RPMI 1640 containing 2×10^7 bacteria per well (28) or various amounts of protein I/IIf or RGPs in the presence or absence of HI-HNS. After a 20-h incubation period, which was found to be optimal for cytokine secretion, culture supernatants were harvested, centrifuged (3,000 × *g*, 10 min), and used to estimate TNF-α, IL-1β, IL-6, and IL-8 release by a heterologous two-site sandwich enzyme-linked immunosorbent assay (ELISA) as previously described (5, 29). The readings were related to standard curves, and the level of sensitivity was as high as 100 pg. In some experiments, polymyxin B (2 μg/ml) was added to confirm that the observed effects were not due to possible lipopolysaccharide contamination. In competition experiments, protein I/IIf was first preincubated for 30 min at 37°C in the presence of NANA or fucose (50 mM).

Enzymatic and periodate treatment of cells. KB and endothelial cells were incubated with various amounts of either dispase (0 to 200 μg/ml) or $NaIO_4$ (0 to 20 mM) for 1 h at 37°C. The cells were then washed, and binding and activity were assayed with protein I/IIf as previously described.

RESULTS

Cytokine release by human KB and endothelial cells after stimulation with oral viridans streptococci. In order to investigate the active role of KB and endothelial cells in response to bacterial stimuli, with regard to release of proinflammatory cytokines TNF-α, IL-1β, IL-6, and IL-8, cells were incubated at time zero in RPMI 1640 alone or with bacteria at a concentration of 2×10^7 per well. The stimulus was maintained for 20 h. Cytokine activities in the supernatants of cells exposed to bacteria were determined by a two-site sandwich ELISA and were subsequently compared with the constitutive cytokine levels from the same experiment. In the absence of bacteria, KB and endothelial cells release small amounts of TNF-α, IL-1β, IL-6, and IL-8, with the four cytokines making up between 0.20 and 0.55 ng/ml. TNF-α, IL-1β, and IL-6 were not generated above constitutive levels from bacteria stimulated KB cells, whereas secretion of IL-8 was markedly increased (Table 1). As shown in Table 1 upregulation of IL-8 release was variable among the oral viridans streptococcal strains used in this study. Bacterial activation experiments were also performed with endothelial cells. It was also found that incubation of endothelial cells with bacteria did not enhance the secretion of IL-1β and TNF-α above constitutive levels. In contrast, bacteria induced an increase of both IL-6 and IL-8 release. The results for IL-6 and IL-8 release by endothelial cells (Table 1) indicate that upregulation occurred at similar orders of magnitude among the strains tested. Taken together, these results provide evidence that oral viridans streptococci may exert immunomodulatory effects, such as the induction of IL-6 and IL-8 release, on human KB and endothelial cells.

Specific binding of protein I/IIf and RGPs to human cells. As recent studies suggested that protein I/IIf and RGPs, two major adhesins from oral viridans streptococci, were powerful inducers of inflammatory cytokines from stimulated monocytes, the present study was undertaken to examine the possible contribution of protein I/IIf and RGPs to the immunomodulatory effects of oral viridans streptococci on human KB and endothelial cells. We first studied the binding of protein I/IIf and RGPs from *S. mutans* OMZ 175 serotype f to human KB and endothelial cells. The binding of either biotinylated protein I/IIf or RGPs was tested over the concentration range of 0 to 100 μg/ml, in serum-free conditions and in the absence or presence of a 20-fold excess of unlabelled ligand to establish the specificity of binding. As shown in Fig. 1, KB and endothelial cells exhibited specific and saturable binding of both adhesins and saturation was achieved at 50 μg of ligands per ml. Nonspecific binding never exceeded 20%. These results were confirmed by competition studies which showed that binding of protein I/IIf and RGPs to KB or endothelial cells is inhibited by the corresponding Nonidet P-40 cell extracts (data not shown). As previous observations had shown that serum components could interfere with the binding of RGPs to human monocytes (31), it was of interest to test the effect of HI-HNS on the binding of protein I/IIf or RGPs to the cells. Cells monolayers were incubated with 50 μg of either protein I/IIf or RGPs per ml in medium containing increasing amounts of HI-HNS (2 to 10%). The results shown in Fig. 2 indicate that HI-HNS inhibited the binding of protein I/IIf and RGPs to both kind of cells, and maximal binding-inhibiting activity between 70 and 80% was obtained with 10% HI-HNS.

Cytokine release by human KB cells and endothelial cells after stimulation with protein I/IIf and RGPs. To determine if the same array of cytokines that are released after stimulation with whole bacteria can be produced by KB and endothelial cells stimulated with protein I/IIf and RGPs, cells were incubated in vitro for 20 h in presence of various concentrations of protein I/IIf and RGPs. The levels of TNF-α, IL-1β, IL-6, and IL-8 in the culture supernatants of stimulated human KB and endothelial cells were determined by a two-site sandwich ELISA. As described above, TNF-α and IL-1β were never

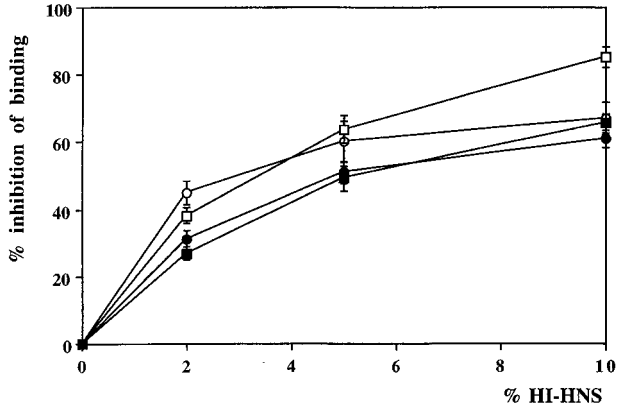

FIG. 2. Inhibition of binding of biotinylated protein I/IIf (open symbols) or RGPs (closed symbols) to KB (squares) and endothelial (circles) cells by increasing amounts of human serum (2 to 10%). The results are expressed as the percent inhibition of protein I/IIf or RGPs binding without serum and are the means for triplicate determinations from three different experiments; error bars indicate standard deviations.

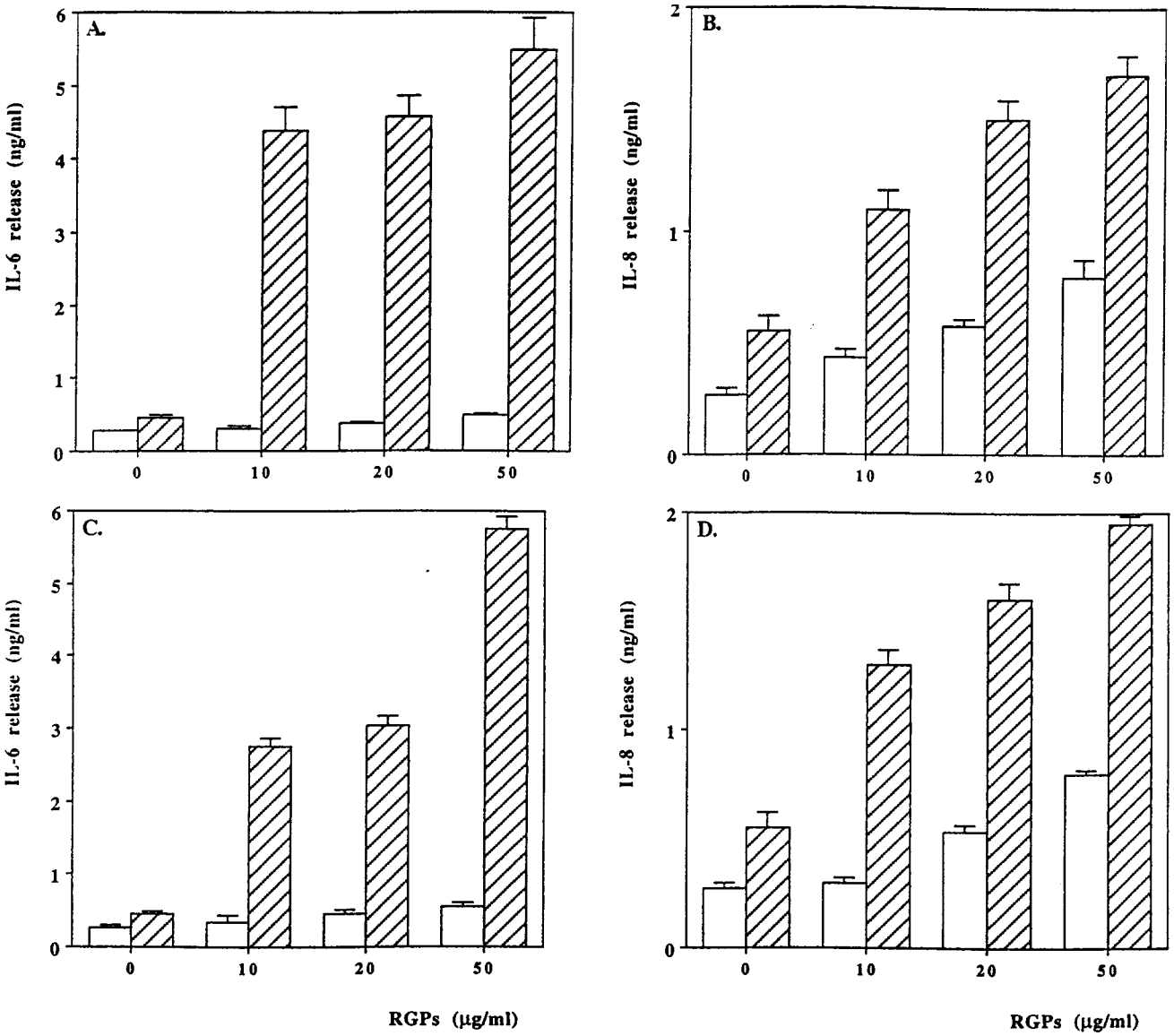

FIG. 3. Dose-dependent production of IL-6 (A and C) and IL-8 (B and D) by endothelial (▨) and KB (□) cells stimulated for 20 h with increasing amounts of protein I/IIf or RGPs (10, 20, and 50 μg/ml). Supernatants were tested at 20 h of culture, and IL-6 and IL-8 levels were determined by a heterologous two-site sandwich ELISA. Data are expressed as means for triplicate cultures and the results are representative of three different experiments; error bars indicate standard deviations.

upregulated in response to protein I/IIf or RGPs. In contrast, the addition of increasing amounts of protein I/IIf, ranging from 0 to 50 μg/ml, enhanced in a dose-dependent fashion the secretion of IL-6 and IL-8 by endothelial cells, with maximal values for IL-6 and IL-8 of 5.8 and 1.7 ng/ml, respectively (Fig. 3A and B). This IL-8 secretion profile was also obtained with KB cells (Fig. 3B). In contrast, stimulation of KB cells with protein I/IIf did not modify the secretion of IL-6 above the constitutive levels (Fig. 3A). We next determined if RGPs was able to stimulate IL-6 and IL-8 secretion by KB and endothelial cells. As shown in Fig. 3C and D, RGPs are potent stimuli for the secretion of IL-6 and IL-8 by endothelial cells, with maximal values of 6 and 2 ng/ml, respectively. Furthermore, RGPs also induced the production of IL-8 by KB cells (Fig. 3D) but did not influence IL-6 release by these cells (Fig. 3C). In addition, to determine if serum components triggered KB and endothelial cells IL-6 and IL-8 release in response to protein I/IIf or RGPs, monolayers of cells were incubated with one concentration of protein I/IIf or RGPs and various concentrations of HI-HNS ranging from 2 to 10%. As previously noted for binding experiments, HI-HNS reduced in a dose-dependent fashion the release of IL-6 and IL-8 in response to 50 μg of protein I/IIf or RGPs per ml. Maximal inhibitions of IL-8 release of 60 and 90% were obtained when KB and endothelial cells were stimulated with RGPs and protein I/IIf, respectively, in the presence of 10% of HI-HNS (Fig. 4A). Furthermore, the results shown in Fig. 4B indicate that HI-HNS also inhibited the RGP- and protein I/IIf-induced IL-6 production by endothelial cells. A maximal inhibition of 60% was obtained with 10% HI-HNS.

Protein I/IIf binds and activates KB and endothelial cells through lectin interactions. Recent studies in our laboratory have demonstrated that activation of monocytes resulted from specific binding of protein I/IIf to cell surface glycoproteins

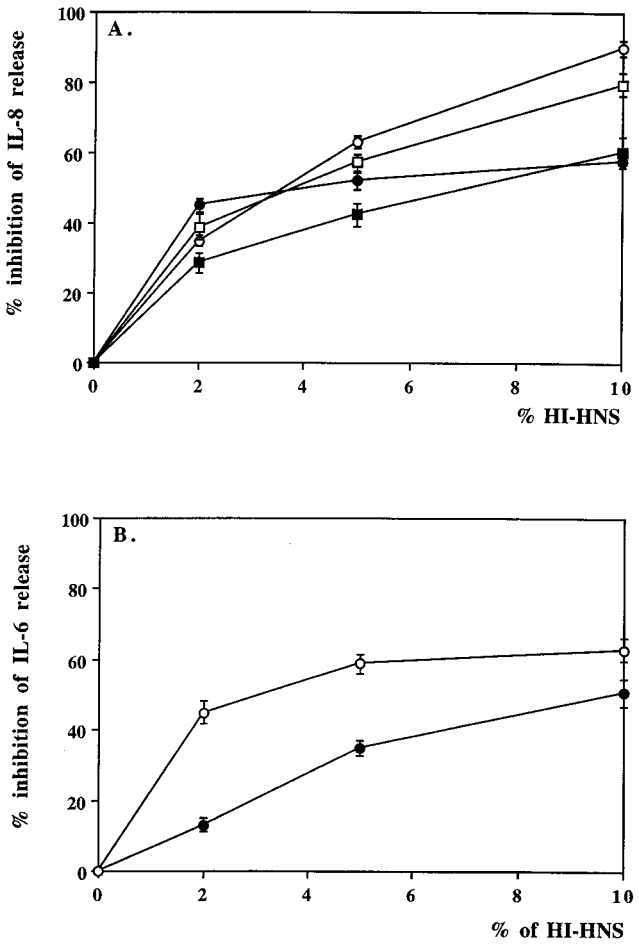

FIG. 4. Serum-mediated inhibition of IL-8 (A) and IL-6 (B) release by KB (squares) or endothelial cells (circles) each stimulated with 50 μg of protein I/IIf (open symbols) or RGPs (closed symbols) per ml. The results are expressed as the percent inhibition of cytokine release and are the means for triplicate determinations from three different experiments; error bars indicate standard deviations.

(30). To further determine if identical mechanisms were implicated in protein I/IIf stimulation of KB and endothelial cells, we first pretreated cells with various concentrations of dispase prior to binding or stimulation experiments. As shown in Table 2, increasing concentrations of dispase from 50 to 200 μg/ml caused a significant reduction of protein I/IIf binding to KB and endothelial cells, and inhibition of binding activities of 51 and 64% were obtained with KB and endothelial cells, respectively, on digestion with 200 μg of dispase per ml. The digestion of cells with dispase markedly inhibited secretion of IL-8 by both kind of cells (Table 2).

Consistent with previous results which suggested that protein I/IIf interacts with monocytes through lectin interactions via NANA and fucose residues (30), we investigated whether $NaIO_4$ treatment or the addition of fucose or NANA to the medium could influence (i) the binding of protein I/IIf to KB and endothelial cells and (ii) the release of IL-8 by the stimulated cells. The results depicted in Fig. 5 show that pretreatment of endothelial cells with 20 mM of $NaIO_4$ induced a 75% inhibition of binding (Fig. 5A) and a 60% inhibition of IL-8 secretion (Fig. 5B). Similar effects of $NaIO_4$ treatment on binding and cytokine release were also observed for KB cells. Moreover, the addition of 50 mM of NANA or fucose to the medium was also able to inhibit the binding and the secretion of IL-8 by KB and endothelial cells incubated with 50 μg of protein I/IIf per ml. However, as shown in Table 3, NANA-mediated inhibition of protein I/IIf binding and IL-8 production was more effective for endothelial cells than for KB cells. Conversely, fucose seems to be more important for binding and activation of KB cells. Similar results were obtained for IL-6 with endothelial cells (data not shown). Taken together, these results indicate that NANA and fucose present on cell surface glycoproteins are needed for the binding of protein I/IIf to KB and endothelial cells and for the release of IL-8 and IL-6.

DISCUSSION

It is now generally accepted that during the course bacterial infection, cells that line mucosal surfaces or endothelium, aside of their ability to form a mechanical barrier, can provide signals that are essential for the initiation and amplification of inflammatory responses. Among these signals, cytokine expression and release are considered early events that participate to the cascade of events leading to inflammation. As part of this study, we have analyzed the human KB epithelial and endothelial cell cytokine response elicited by oral viridans streptococci. In this study, we demonstrated that 11 representative strains of oral viridans streptococci, all of which reacted with anti-protein I/IIf serum, were potent stimulators of IL-8 production from KB cells and that this ability was variable among the bacterial strains considered. This variability could be due to the degree of exposure of components on the bacterial surface that are able to stimulate cytokine release from KB cells. In contrast, the secretion of other proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, after stimulation of human monocytes with the same strains (unpublished data) was not upregulated. These results are in agreement with previous observations by Agace et al. (2), who showed that epithelial cells have a more selective cytokine response profile than do circulating monocytes, with this restricted profile probably serving to limit the consequences of microbial exposure at the mucosal surface. However, these and other investigators (15, 16) demonstrated that epithelial cells generally were also able to produce IL-6 in response to bacterial stimuli. One explanation could be that they used epithelial cell lines originating from the urinary tract exposed to gram-negative bacteria. At the present time, there is no conclusive evidence that IL-8 is the only cytokine whose level is upregulated after stimulation of buccal epithelial cells with oral viridans streptococci; however, it should be noted that KB cells are transformed cells and in this regard may differ from normal buccal epithelial cells. Indeed, Jung et al. (18) have shown that IL-6 is produced by freshly

TABLE 2. Dose-dependent dispase-mediated inhibition of binding of biotinylated protein I/IIf to KB and endothelial cells and of IL-8 production by human KB and endothelial cells

Dispase (μg/ml)	% Inhibition of[a]:			
	Binding		IL-8 release	
	KB cells	Endothelial cells	KB cells	Endothelial cells
50	13.84 ± 1.12	46.44 ± 3.12	37.72 ± 3.65	61.50 ± 4.89
100	25.39 ± 2.63	60.22 ± 3.04	60.03 ± 4.37	77.50 ± 3.72
200	51.30 ± 2.54	64.72 ± 4.42	76.89 ± 4.08	83.38 ± 4.13

[a] Each value is the mean for triplicate determinations ± standard deviation and is representative of three different experiments.

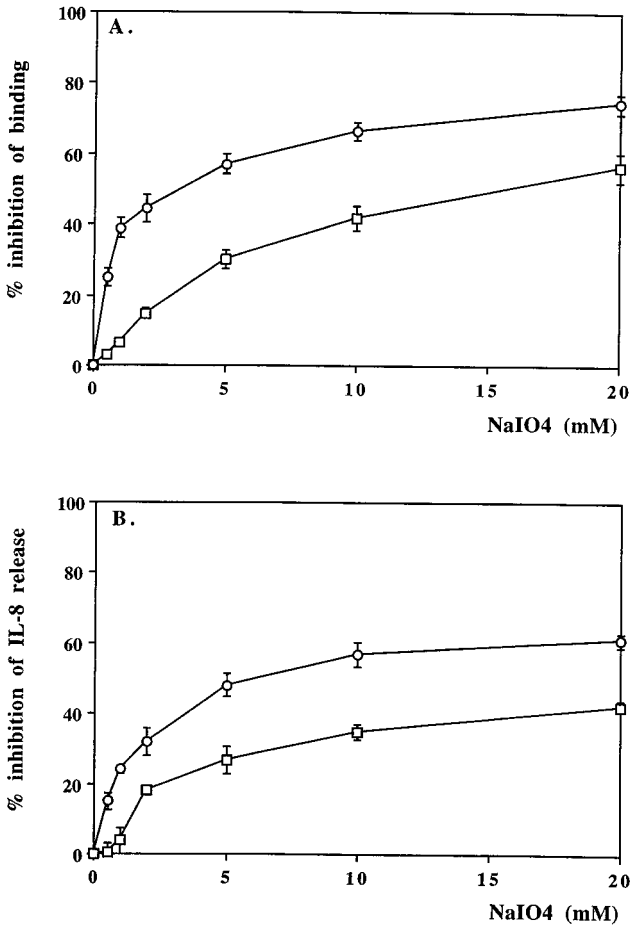

FIG. 5. Dose-dependent sodium periodate-mediated inhibition of binding of biotinylated protein I/IIf (50 μg/ml) to KB (□) and endothelial cells (○) (A) and of IL-8 production by KB (□) and endothelial cells (○), stimulated with 50 μg of protein I/IIf per ml. Each value is the mean for triplicate determinations and is representative of three different experiments; error bars indicate standard deviations.

isolated healthy colon epithelial cells but not by colonic epithelial cell lines in response to invasive gram-negative bacteria.

In studies parallel to those with KB cells, we found that following stimulation with oral viridans streptococci, endothelial cells produced IL-6 and IL-8. As for KB cells, oral viridans streptococcal stimulation of endothelial cells did not elicit a general proinflammatory cytokine response since TNF-α and IL-1β secretion, which is often enhanced principally by gram-negative bacteria, is not affected. Our findings, consistent with those of prior studies by Yao et al. (35), failed to detect TNF-α release by endothelial cells following infection with *Staphylococcus aureus*; however, secretion of IL-1β was upregulated. These results suggest that the characteristic array of proinflammatory cytokines produced by endothelial cells exhibits some important differences according to the bacterial stimuli. However, the fact that endothelial cells in response to oral viridans streptococci were able to release IL-8 and IL-6, which have been shown to be important mediators in sepsis caused by infections with gram-negative bacteria (7, 25), supports the concept that if oral viridans streptococci enter the bloodstream they may contribute to the pathogenesis of oral viridans streptococcal shock syndrome through cytokine release.

There is now evidence that interactions between bacteria and many types of eukaryotic cells are under the control of their surface-bound adhesins (17). In the present work, we demonstrate that protein I/IIf and RGPs, two major adhesins from *S. mutans* OMZ 175, specifically bind to KB and endothelial cells in a dose-dependent, saturable fashion in the absence of serum. Furthermore, the binding of these molecules initiates the cellular response which results in the release of IL-8 from KB cells and of IL-8 and IL-6 from endothelial cells. These data suggest that protein I/IIf and RGPs play a very important role in the interactions between bacteria and KB or endothelial cells insofar as similar cytokine profiles were obtained when cells were stimulated with whole bacteria or surface components. Moreover, aside of the pivotal role these adhesins play as cell surface components, they can be released from the cell surface and act per se at a distance with the potential capacity of accelerating the inflammatory process by stimulating monocytes and epithelial and endothelial cells. However, at present time there is no conclusive evidence that protein I/IIf or RGPs are translocated through the mucosal barrier, apart from the fact that Challacombe et al. (9) showed that protein I/IIf seemed to enter the mucosal barrier since it could induce the production of natural antibodies. Our results also confirm the fact that human serum is not necessary to the binding of both adhesins to KB and endothelial cells. Moreover, we found that human serum was able to inhibit the cellular response to protein I/IIf and RGPs. These results are in agreement with those of Soell et al. (29), who showed that human serum inhibits the binding and stimulating activities of two capsular polysaccharides, types 5 and 8, of *Staphylococcus aureus* on KB and endothelial cells. An explanation could be that human serum contains components which neutralize the biological activities of these two streptococcal adhesins by forming inactive complexes. This possibility is consistent with the results of recent studies by Dentener et al. (12), who showed that the bactericidal permeability-increasing protein binds to lipopolysaccharide and prevents cytokine synthesis by stimulated monocytes. Accordingly, Soell et al. (31) reported that the mannan-binding protein interacts with several polysaccharides and directs them towards the C1q receptor without

TABLE 3. Sugar-mediated inhibition of binding of biotinylated protein I/IIf to KB and endothelial cells and IL-8 production by KB and endothelial cells stimulated for 20 h with 50 μg of protein I/IIf per ml

Monosaccharide (50 mM)	% Inhibition of[a]:			
	Binding		IL-8 release	
	KB cells	Endothelial cells	KB cells	Endothelial cells
NANA	59.71 ± 2.21	64.66 ± 5.03	45.25 ± 5.23	84.48 ± 4.36
Fucose	68.38 ± 1.02	22.83 ± 8.89	64.42 ± 5.86	19.33 ± 4.72

[a] Each value is the mean of triplicate determinations ± standard deviation and is representative of three different experiments.

cytokine synthesis. However, at the present time, the serum components that inhibit the binding and activation of KB and endothelial cells remain unidentified.

In the present work we also provide evidence that protein I/IIf binds to and stimulates KB and endothelial cells through lectin interactions and that NANA and fucose may form the recognition sites that mediate protein I/IIf binding which allows these cells to secrete inflammatory cytokines. These data confirm previous experiments showing that NANA and fucose present on glycoproteins are needed for the immunomodulation of human monocytes by protein I/IIf and other bacteria (30).

In conclusion, the results of the present study demonstrate that oral streptococci, probably by engaging two cell surface adhesins, protein I/IIf and RGPs, exert immunomodulatory effects on KB and endothelial cells that allow them to secrete IL-6 and IL-8. Therefore, they may be putative components in the pathogenesis of the various disorders associated with oral viridans streptococci. However, the mechanisms as well as the exact nature of the cell receptors which are implicated are as yet unknown, and further experiments will provide important insights into the mechanisms of disease by these bacteria.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the CAMPLP.
Aline Vernier and Mouna Diab are equivalent authors.

REFERENCES

1. **Ackermans, F., J. P. Klein, J. A. Ogier, H. Bazin, F. Cormont, and R. M. Frank.** 1985. Purification and characterization of a saliva interacting cell surface protein from *Streptococcus mutans* serogroup f using monoclonal antibody immunoaffinity chromatography. Biochem. J. **228:**211–217.
2. **Agace, W., S. Hedges, U. Andersson, J. Andersson, M. Ceska, and C. Svanborg.** 1993. Selective cytokine production by epithelial cells following exposure to *Escherichia coli*. Infect. Immun. **61:**602–609.
3. **Beighton, D., R. R. B. Russell, and H. Hayday.** 1981. The isolation and characterization of *Streptococcus mutans* serotype h from dental plaque of monkeys (*Macaca fascicularis*). J. Gen. Microbiol. **124:**271–278.
4. **Beilke, M. A.** 1989. Vascular endothelium in immunology and infectious disease. Rev. Infect. Dis. **11:**273–283.
5. **Benabdelmoumene, S., S. Dumont, C. Petit, P. Poindron, D. Wachsmann, and J. P. Klein.** 1991. Activation of human monocytes by *Streptococcus mutans* serotype f polysaccharide: immunoglobulin G Fc receptor expression, tumor necrosis factor, and interleukin-1 production. Infect. Immun. **59:**3261–3266.
6. **Bochud, P. Y., P. Eggiman, T. Calandra, G. Van Melle, L. Saghafi, and P. Francioli.** 1994. Bacteremia due to viridans streptococci in neutropenic patients with cancer. Clin. Infect. Dis. **18:**25–31.
7. **Bone, R. C.** 1991. The pathogenesis of sepsis. Ann. Intern. Med. **115:**457–469.
8. **Bouvet, A.** 1994. Taxonomie des streptocoques et des enterocoques. Bull. Soc. Fr. Microbiol. **9:**273–277.
9. **Challacombe, S. J., L. A. Bergmeier, C. Czerkinsky, and A. S. Rees.** 1984. Natural antibodies in man to *Streptococcus mutans*: specificity and quantification. Immunology **52:**143–147.
10. **Demuth, D. R., E. E. Golub, and D. Malamud.** 1990. Streptococcal-host interactions: structural and functional analysis of a *Streptococcus sanguis* receptor for a human salivary glycoprotein. J. Biol. Chem. **265:**7120–7126.
11. **Demuth, D. R., M. S. Lammey, M. Huck, E. T. Lally, and D. Malamud.** 1990. Comparison of *Streptococcus mutans* and *Streptococcus sanguis* receptors for human salivary agglutinin. Microb. Pathog. **9:**199–211.
12. **Dentener, M. A., E. J. U. von Asmuth, G. J. M. Francot, M. N. Marra, and W. A. Buurman.** 1993. Antagonist effects of lipopolysaccharide binding protein and bactericidal/permeability-increasing protein on lipopolysaccharide-induced cytokine release by mononuclear phagocytes. J. Immunol. **151:**4258–4265.
13. **Eckmann, L., M. F. Kagnoff, and J. Fierer.** 1993. Epithelial cells secrete the chemokine interleukin-8 in response to bacterial entry. Infect. Immun. **61:**4569–4574.
14. **Fargeas, C. A., M. Scholler, A. Pini, D. Wachsmann, P. Poindron, and J. P. Klein.** 1990. Purification and partial characterization of rat macrophage Fc receptor and binding factor for IgA. Biochim. Biophys. Acta **1037:**344–350.
15. **Hedges, S., P. de Man, H. Linder, C. van Kooten, and C. Svanborg-Eden.** 1990. Interleukin-6 is secreted by epithelial cells in response to Gram negative bacterial challenge, p. 144–148. *In* T. MacDonald (ed.), Advances in mucosal immunology. Kluwer, London.
16. **Hedges, S., M. Svensson, and C. V. Svanborg.** 1992. Interleukin-6 response of epithelial cell lines to bacterial stimulation in vitro. Infect. Immun. **60:**1295–1301.
17. **Hoepelman, I. M., and E. I. Tuomanen.** 1992. Consequences of microbial attachment: directing host cell functions with adhesins. Infect. Immun. **60:**1729–1733.
18. **Jung, H. C., L. Eckmann, S. K. Yang, A. Panja, J. Fierer, E. Morzycka-Wroblewska, and M. F. Kagnoff.** 1995. A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. J. Clin. Invest. **95:**55–65.
19. **Klein-Soyer, C., A. Beretz, R. Millon-Collard, J. Abecassis, and J. P. Cazenave.** 1986. A simple *in vitro* model of mechanical injury of confluent culture endothelial cells to study quantitatively the repair process. Thromb. Haemostasis **56:**232–235.
20. **Linzer, R., M. S. Reddy, and M. J. Levine.** 1987. Structural studies of the serotype-polysaccharide antigen from *Streptococcus mutans* OMZ 175. Infect. Immun. **55:**3006–3010.
21. **Moisset, A., N. Schatz, Y. Lepoivre, S. Amadio, D. Wachsmann, M. Schöller, and J.-P. Klein.** 1994. Conservation of salivary interacting and human immunoglobulin G cross-reactive domains of the I/II antigen in oral streptococci. Infect. Immun. **62:**184–193.
22. **Mosmann, T.** 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods **65:**55–63.
23. **Ogier, J. A., A. Pini, P. Sommer, and J. P. Klein.** 1989. Purification and characterization of the expression product of the sr gene of *Streptococcus mutans* OMZ 175. Microb. Pathog. **6:**175–182.
24. **Parillo, J. E.** 1993. Pathogenetic mechanisms of septic shock. N. Engl. J. Med. **328:**1471–1477.
25. **Pober, J. S., and R. S. Cotran.** 1990. The role of endothelial cells in inflammation. Transplantation **50:**537–544.
26. **Pritchard, D. G., S. M. Michalek, J. R. McGhee, and R. L. Furner.** 1987. Structure of the serotype f polysaccharide antigen of *Streptococcus mutans*. Carbohydr. Res. **166:**123–131.
27. **Russell, M. W., and T. Lehner.** 1978. Characterization of antigens extracted from cells and culture fluids of *Streptococcus mutans* serotype c. Arch. Oral Biol. **23:**7–15.
28. **Sharma, S. A., M. K. R. Tummuru, G. C. Miller, and M. J. Blaser.** 1995. Interleukin-8 response of gastric epithelial cell lines to *Helicobacter pilori* stimulation in vitro. Infect. Immun. **63:**1681–1687.
29. **Soell, M., M. Diab, G. Haan-Archipoff, A. Beretz, C. Herbelin, B. Poutrel, and J. P. Klein.** 1995. Capsular polysaccharide types 5 and 8 of *Staphylococcus aureus* bind specifically to human epithelial (KB) cells, endothelial cells, and monocytes and induce release of cytokines. Infect. Immun. **63:**1380–1386.
30. **Soell, M., F. Holveck, M. Schöller, D. Wachsmann, and J. P. Klein.** 1994. Binding of *Streptococcus mutans* SR protein to human monocytes: production of tumor necrosis factor, interleukin 1, and interleukin 6. Infect. Immun. **62:**1805–1812.
31. **Soell, M., E. Lett, F. Holveck, M. Schöller, D. Wachsmann, and J.-P. Klein.** 1995. Activation of human monocytes by streptococcal rhamnose glucose polymers is mediated by CD14 antigen, and mannan binding protein inhibits TNF-α release. J. Immunol. **154:**851–860.
32. **Stadnyk, A. W.** 1994. Cytokine production by epithelial cells. FASEB J. **8:**1041–1047.
33. **Stinson, M. W., B. Albini, and R. J. Nisengard.** 1986. Adverse effects of *Streptococcus mutans* antigens on host tissues, p. 307–318. *In* S. Hamada, S. M. Michalek, H. Kiyono, L. Menaker, and J. R. McGhee (ed.), Molecular microbiology and immunobiology of *Streptococcus mutans*. Elsevier Science Publishing, Inc., Amsterdam.
34. **Van Houte, J.** 1980. Bacterial adhesion in the mouth, p. 69–100. *In* S. A. Leach (ed.), Dental plaque and surface interactions in the oral cavity. Information Retrieval, Ltd., London.
35. **Yao, L., V. Bengualid, F. D. Lowy, J. L. Gibbons, V. B. Hatcher, and J. W. Berman.** 1995. Internalization of *Staphylococcus aureus* by endothelial cells induces cytokine gene expression. Infect. Immun. **63:**1835–1839.

Editor: J. R. McGhee