Glucosyltransferases of Viridans Streptococci Are Modulins of Interleukin-6 Induction in Infective Endocarditis

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The glucosyltransferases (GTFs) of viridans streptococci, common pathogens of infective endocarditis, are extracellular proteins that convert sucrose into exopolysaccharides and glucans. GTFs B, C, and D of *Streptococcus mutans* are modulins that induce, in vitro and in vivo, the production of cytokines, in particular interleukin-6 (IL-6), from monocytes. The roles of *S. mutans* GTFs in infectivity and inflammation in situ were tested in a rat experimental model of endocarditis. No significant differences in infectivity, in terms of 95% infective dose and densities of bacteria inside vegetations, were observed between laboratory strain GS-5 and two clinical isolates or isogenic mutant NHS1DD, defective in the expression of GTFs. In aortic valves and surrounding tissues, IL-6 was detected by Western blots and immunostaining 24 h after GS-5 infection, was maintained over 72 h, and was followed by production of tumor necrosis factor alpha but not IL-1 β . Animals infected with NHS1DD showed markedly lower levels of IL-6 (less than 5% of that of parental GS-5-infected rats), while tumor necrosis factor alpha was unaffected. In contrast, animals infected with NHR1DD, another isogenic mutant expressing only GtfB, showed a much smaller reduction (down to 56%). These results suggest that GTFs are specific modulins that act during acute inflammation, inducing IL-6 from endothelial cells surrounding the infected valves without affecting bacterial colonization in vegetations, and that IL-6 might persist in chronic inflammation in endocarditis.

The pathogenesis of infective endocarditis is characterized by the formation of endocardial vegetations resulting from an inflammatory process. These vegetations consist of a clot of fibrin, containing platelets and inflammatory cells, in which the causative microorganisms are embedded and multiply (4). During infection, bacteria in vegetations may grow to reach densities of 10^9 to 10^{10} cells per gram, and the organisms may become metabolically dormant, causing resistance to the bactericidal activity of β -lactam and glycopeptide antibiotics (25). Accompanied by vegetation formation, the pathogenesis of infective endocarditis is characterized histopathologically by an inflammatory reaction, greatest at the site of attachment or base of the vegetation. The intensity of inflammation and tissue destruction may vary, depending on the bacterial species and clinical onset, classified as acute or subacute, as well as the relative numbers and distribution of inflammatory infiltrate (5, 7). Nonetheless, the cellular or bacterial components involved in endocardial inflammation and tissue damage remain unclear.

Members of the viridans streptococci are common causes of infective endocarditis in humans (13, 35). *Streptococcus mutans*, an oral pathogen of human dental caries (27), accounts for 1.7 to 14% of cases of streptococcal endocarditis (42). *S. mutans* induces endocarditis primarily in elderly patients with underlying heart diseases, such as idiopathic hypertrophic sub-

aortic stenosis (42), and can cause quadric valvular endocarditis (6). *S. mutans* also induces endocarditis in animals with experimentally induced heart valve damage and its infectivity may be enhanced by existing exopolysaccharides, glucan, and fructan (31).

Glucosyltransferases (GTFs) are enzymes responsible for the synthesis of glucans, and isozymes also have been identified in other viridans streptococci, such as *Streptococcus sanguis*, *Streptococcus gordonii*, and *Streptococcus salivarius* (30). These GTFs, around 150 kDa, share conserved structural and functional domains near both the N and C termini of the proteins, although each exhibits distinct enzymatic activities. Genetic (3, 18, 19) and genomic (1) analysis confirmed that *S. mutans* expresses three GTFs, B, C, and D, with an amino acid sequence identity of over 50%. GtfG, a GTF of *S. gordonii*, can act as an adhesin and interact directly with endothelial cells in vitro (43). GTFs are highly immunogenic, and naturally occurring anti-GTF-specific immunoglobulin G (IgG) in serum or IgA in saliva may be detected readily in children and young adults (11). In addition, GTFs can directly stimulate T-cell proliferation and modulate cytokine production from monocytes (12). IL-6, in particular, along with other cytokines, was induced readily when GTFs were administered in vitro in cell culture or challenged in vivo (10).

Induction of proinflammatory cytokines, such as IL-6, IL-1, and tumor necrosis factor alpha (TNF- α), through pathogen-associated molecular patterns or modulins, is a characteristic innate immune response to eliminate and/or constrain microorganisms at the site of entrance (20). IL-6, as both a pro- and anti-inflammatory cytokine (16), plays important roles in the

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cardiac innate immune response and also in the development of cardiac injury due to infection or ischemia (34, 40). Plasma concentrations of IL-6 but not TNF- α or IL-1 also were found to be associated with inflammation in infective endocarditis (2, 33). Although TNF- α has been demonstrated in human endocarditis specimens (15), the induction of IL-6 surrounding vegetations in situ has not been studied, and the modulins responsible for induction of cytokines from viridans streptococci still have not been identified unequivocally. The wide distribution and conserved features of GTFs, as well as preferential IL-6 stimulation, suggest a common role as modulins in the inflammatory reaction to systemic infection by viridans streptococci.

In this study, we sought to delineate the role of *S. mutans* GTFs in experimental endocarditis in rats. We tested the hypothesis that GTFs are specific modulins that induce IL-6 preferentially at the infected valves and surrounding tissue. We found that IL-6 was induced earlier than TNF- α during acute inflammation and persisted to the chronic stage of infection in experimental endocarditis. During the acute stage of inflammation, GTFs are specific and major modulins responsible for the induction of IL-6 and are released primarily from the endothelial lining of the valvular regions.

MATERIALS AND METHODS

Bacteria. One laboratory strain of *S. mutans*, GS-5 (26), and two clinical isolates, NTU-5526 and NTU-4312, were grown and maintained in brain-heart infusion broth (BHI, Difco Laboratories Inc., Detroit, MI). Strain NTU-5526 was isolated at the Department of Infectious Diseases, National Taiwan University Hospital, from the peripheral blood of a patient suffering from infective endocarditis. Strain NTU-4312 is an oral isolate from a patient with rampant caries from the Department of Dentistry, National Taiwan University Hospital. Two isogenic mutants derived from GS-5, NHR1DD ($\Delta gtfC$, inactivated *gtfD*) and NHS1DD ($\Delta gtfB/C$, inactivated *gtfD*), which differ from the wild type in the expression of two or three *gtf* genes (41), were grown in BHI supplemented with erythromycin (10 μ g/ml) and tetracycline (25 μ g/ml). Before inoculation in vivo, strains were subcultured in TTY medium (9) free of sucrose, to reach their final density of around 0.9 at an optical density of 600 nm.

Phenotypic and genetic characterization. A series of biochemical tests was performed to confirm the identity of the parental and mutant phenotypes used in this study (44). Briefly, bacteria were tested for hydrolysis of esculin, the production of acid from inulin, sucrose, lactose, mannitol, sorbitol, meliobiose, raffinose, trehalose, the production of α -D-galactosidase, β -D-glucosidase, acetoin, and growth tolerance of bacitracin. The colonial morphology and hemolytic characteristics were determined after overnight growth on mitis-salivarius agar (Difco) plates. Growth characteristics were performed by inoculating each organism into BHI broth at 10^8 CFU/ml. Plate counts were made to determine the number of viable bacteria per milliliter in each dilution. The optical density values of a 1:10 dilution of each organism were taken at 530 nm (spectrophotometer; Pharmacia) over a period of several hours until the stationary phase was reached. Growth curves were plotted according to absorbance or viable bacteria versus time.

To confirm the genetic stability of NHS1DD and NHR1DD after a single in vivo passage from vegetation in the absence of the selected antibiotics, chromosomal DNA from the parent and mutants was purified and digested with EcoRI, PstI, or SphI. Southern blot analysis was carried out by hybridization with α - ^{32} P-labeled 1.6-kb HindIII-digested fragment specific to *gtfB/C* or 1.3-kb BamHI-digested fragment specific to *gtfD*. Hybridization and preparation of isotope labeled probes were carried out as described previously (8).

Protein and enzymatic analysis. Extraction and preparation of cell wall-associated or extra-cellular proteins from *S. mutans* were described previously (9). Cell wall-associated protein antigens were prepared with 8 M urea extraction fluid for 1 h at 25°C. The cell wall extracts or culture supernatants were subsequently concentrated by 60% (saturation) ammonium sulfate precipitation. The proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) followed by silver staining or transferred electrophoretically to a Hybond-P super membrane (Amersham). For Western blot analysis, the blotted membrane was exposed to rabbit polyclonal antibody PJS-2,

which recognized three GTFs of *S. mutans* as described previously (9). Protein concentrations were determined using bicinchoninic acid as the colorimetric detection reagent (Pierce) and enzymatic activities of GTFs from the wild-type or isogenic mutants were determined using [^{14} C]sucrose as described previously (9).

Rat model of endocarditis. A modified rat model of endocarditis described by Santoro and Levinson (36) and Heraief et al. (21) was used. Approval for animal use was obtained from the National Taiwan University Institutional Animal Care and Use Committee prior to initiation of experiments. Male or female Wistar rats, weighing 450 to 500 g, were anaesthetized with ketamine (100 μ g per gram body weight) after premedication with penicillin and spectinomycin (50 μ g per gram body weight). The left carotid artery was exposed through an anterior incision of the skin above the clavicles and was sutured closed at the cephalic end. To create injury at aortic valves, a polyethylene tube (0.4 mm in internal diameter and 0.8 mm in external diameter; Natsume-Seisakusyo, Tokyo, Japan), with a stainless steel wire embedded inside, was inserted through a small incision in the carotid and advanced toward the chest until resistance was met and characteristic pulsation of the catheter was observed. The purpose of the stainless wire was to facilitate the insertion of the tubing and to act as an indicator for the X radiograph.

The carotid artery with the catheter in place was tied with a suture at the base to secure catheter placement inside the left ventricle. Following approximation of the neck tissues, closure of the neck incision, and before recovery from anesthesia, an X radiograph was taken so that the proper placement of the catheter could be confirmed. The catheter remained in place throughout the course of the experiment by fixation with sutures around the skin. Twenty-four hours following catheter placement, rats were assigned randomly to test groups receiving an inoculation of selected bacterial strains in 0.5 ml phosphate-buffered saline (PBS, pH 7.4), via the tail vein, or control groups (PBS injection only). Animals were sacrificed by CO₂ inhalation at various time intervals (1 to 3 days) and the heart was removed and opened.

Proper catheter placement and the presence or absence of vegetations on the aortic valve were scored by examination under stereoscope at a magnification of 10x. The aortic valves and associated vegetations were removed, and their weights were determined. After removing the vegetation, the surrounding tissues at the base of the vegetation were carefully dissected under the stereoscope and were frozen immediately in liquid nitrogen. To estimate the number of colonized bacteria, removed vegetations were homogenized in a sterile disposable tissue grinder, serially diluted in PBS, and plated on BHI to determine CFU per gram of vegetation.

Assessment of virulence in vivo. The virulence of *S. mutans*-induced systemic infection in vivo was assessed by determining the ability of the organism to colonize and proliferate within vegetation formed on the damaged aortic valves and to disseminate to the spleen. The ID₉₅, the number of bacteria necessary to infect 95% of the exposed animals, recently used as a virulence parameter of endocarditis in animal models, was adopted (46). To define the ID₉₅ inoculum of the different parental and GTF mutant strains in the endocarditis model, we performed preliminary studies in our laboratory in which each strain individually was injected intravenously into animals with indwelling transvalvular catheters. For these studies, an inoculum range of 10^5 to 10^9 CFU per catheterized rat was utilized for the parental and mutant strains, the relative capacities of these strains to induce experimental endocarditis and cause metastatic abscesses in spleen were evaluated and compared 24 h after catheterization. Preliminary results indicated this inoculum range encompassed the ID₉₅ for *S. mutans* in our experimental rat model of endocarditis. The ID₉₅ defined for the parental and GTF mutant strains were then utilized for the detection of inflammatory responses in subsequent studies.

Statistical evaluation. Differences between the proportions of animals infected by different challenge inocula were analyzed by the χ^2 test. Differences in mean vegetation weights and mean vegetation bacterial concentrations were evaluated by use of the *t* test for unpaired data.

Histologic examination and detection of cytokines. Histologic examinations were carried out using O.C.T. (optimal compound temperature)-embedded frozen sections of hearts and spleens from the animals and were examined with hematoxylin-eosin and Gram stain.

For detection of specific cytokine production in the rat specimens (frozen tissue), a series of polyclonal antibodies were purchased and used following the manufacturer's instructions (Santa Cruz Biotechnology, Inc.). IL-1 β , IL-6, and TNF- α in rat specimens were detected with sc-1251, sc-1265, and sc-1350, respectively. Immunohistochemical studies of the above-mentioned cytokines were performed using avidin-biotin-complex methods in rat tissues with commercial ABC kits (DAKO) and colorization with the chromogen diaminobenzidine. For detecting cytokines on Western blots, dissected tissues were homogenized immediately after freezing and treated with DNase and RNase in buffers containing

cocktails of proteinase inhibitors (Sigma). Western blots were probed as described previously using the polyclonal antibodies listed above (11). A control antigen, β -actin, was detected with polyclonal antibody sc-1616 (Santa Cruz Biotechnology). Relative amounts of the proteins were quantified by densitometric analysis using the Electrophoresis Documentation and Analysis System 120 (Scientific Imaging Systems, Eastman Kodak Co.).

RESULTS

Genetic and phenotypic characteristics of wild-type rats and isogenic mutants. We have demonstrated previously that GTFs are potent modulators, inducing IL-6 production from monocytes when added in vitro or challenged in vivo (10). To test the modulatory effect of GTFs presented in situ through a natural route, the infectivity of and the inflammatory response elicited by a laboratory strain, GS-5, and isogenic mutants defective in the expression of GTFs were compared in a rodent model of endocarditis. Isogenic mutants derived from *S. mutants* GS-5, NHR1DD, and NHS1DD, which differ from the wild type in two or three *gtf* genes, were constructed in another laboratory (18, 19, 41).

Genetically, the *gtfB* and *gtfC* genes are arranged tandemly, whereas *gtfD* is located distally. NHS1DD, carrying deletions in both *gtfB* and *gtfC* and also a tetracycline resistance cassette integrated in the middle of *gtfD*, expresses no detectable GTFs or related enzymatic activities. NHR1DD has an intact *gtfB* but an internal deletion and integration in *gtfC* and *gtfD*, respectively. Both isogenic mutant strains are resistant to erythromycin and tetracycline, but NHR1DD expresses biologically active GtfB molecules. Primer extension and promoter reporter fusion analysis indicated that each of the *gtf* genes is transcribed independently from its own promoter (37). Therefore, the allelic replacement found in *gtfB/C* or *gtfC* and integration mutation in the *gtfD* locus would not cause polar effects on the downstream genes in either isogenic mutant.

To exclude possible adverse effects attributable to the selective antibiotics, erythromycin and tetracycline, which might affect the expression of outer surface components and infectivity in vivo, the genetic stability of the parental GS-5 and NHS1DD and NHR1DD mutants was tested initially in a rat model of endocarditis without administration of antibiotics and compared to the parental strain (see below). Each strain was capable of inducing the formation of vegetations on the injured aortic valves. The bacteria colonizing the vegetations were isolated, and the various isolates were subcultured in vitro for subsequent analysis. Southern blot analysis confirmed that the genetic organization of *gtfB/C/D* in each isolate was identical before and after passage in vivo (representative results are shown in Fig. 1, panels A and B). In addition, Western blot analysis revealed the expected expression profiles of GTFs, corresponding to the genetic background for each isogenic mutant (Fig. 1D).

The recovered wild type or isogenic mutants exhibited similar grow rate, colonial morphology, hemolytic characteristics, and identical capacity for carbohydrate utilization. Enzymatic activity for glucan synthesis by incorporation of ^{14}C -labeled glucose confirmed that crude extracts (12) from wild-type GS-5 synthesized both soluble and insoluble glucans, extracts from NHR1DD synthesized exclusively insoluble glucan, whereas no glucan synthesis could be detected with the extracts from NHS1DD. No significant difference, other than glucan synthe-

FIG. 1. Southern and Western blot analysis of the parental GS-5 and isogenic mutants NHR1DD and NHS1DD. (A) Hybridization patterns of chromosomal DNA digested with EcoRI (lane 1) or PstI (lane 2) with [α - ^{32}P]dCTP (Amersham Pharmacia)-labeled DNA probes recognizing both *gtfB* and *gtfC*. The deletion of *gtfB/C* resulted in the absence of two EcoRI-digested bands (7.3 and 4.6 kb) and two PstI-digested bands (7.3 and 6.4) in NHS1DD. The deletion of *gtfC* resulted in the absence of a 7.3-kb EcoRI fragment and a 7.3 PstI fragment in NHR1DD. (B) Hybridization patterns of chromosomal DNA digested with HindIII (lane 1) or HpaI (lane 2) with [α - ^{32}P]dCTP (Amersham Pharmacia)-labeled DNA probes specifically recognizing *gtfD*. Insertional mutation in the *gtfD* with a tetracycline resistance cassette resulted in an increase (2.0 kb) in size of HindIII- or HpaI-digested fragments in both NHS1DD and NHR1DD. (C) Protein profiles of from GS-5 (lane 1), NHR1DD (lane 2), or NHS1DD (lane 3). Total proteins were resolved on a 7.5% polyacrylamide gel stained with silver. (D) Western blot analysis of GTFs. Total proteins from GS-5 (lanes 1 and 2), NHR1DD (lane 3), or NHS1DD (lane 4) were probed with purified anti-GtfB/C/D rabbit IgG (PJS-2). No GTFs were found in NHS1DD, and only GtfB was expressed on NHR1DD. Molecular size in kb or kDa is depicted in the left border of panels A, B, and C.

sis, was found between GS-5 and NHR1DD or NHS1DD in terms of biochemical and growth characteristics, after recovery from passage in vivo. These results confirmed that the genetic and phenotypic characteristics of both the parental and mutant strains were stable after passage in vivo in the absence of the selective pressure of an antibiotic.

Virulence assessment in rat endocarditis. To ensure the correct placement of the inserted catheter passing through the aortic valves without perforation of the endocardium, a modified method was developed in this study, using a stainless steel wire embedded inside the plastic catheter. During the surgical procedures, the position of the inserted catheter could be mon-

FIG. 2. Vegetation formation in rat experimental endocarditis. The correct placement of the inserted transvalvular catheter through aortic valves was confirmed by X radiograph (upper panel). A typical vegetation, mostly small with warty nodules, could be visualized 24 h after intravenous challenge with *S. mutans* GS-5 at an inoculum higher than 10^7 CFU (lower panel). Normal aortic valves are shown in the middle panel.

itored and adjusted simply by checking X radiographs to ensure the correct placement of the catheter (Fig. 2). Such a simple procedure could increase not only the survival but also the success rate of catheter insertion.

In these studies, animals with aortic catheters were chal-

lenged individually with the parental GS-5 or NHS1DD or NHR1DD mutant strain over a range of 10^5 to 10^9 CFU per rat. The results for GS-5 and NHS1DD from individual animals are depicted in Fig. 3 and summarized in Table 1. For the GS-5 parental strain, the ID_{95} for inducing infective endocarditis and concomitant disseminated infection in the spleen was $\approx 10^7$ and $\approx 10^6$ CFU, respectively. Bacteria could be recovered readily from the vegetations at 10^7 CFU inoculum challenges and from the spleens at 10^6 CFU inoculum challenges (Table 1). For the NHS1DD mutant strain, the ID_{95} for inducing infective endocarditis or disseminated infection in the spleen was similar to those found in the parental GS-5 (Table 1). Therefore, the virulence of the parental GS-5 and mutant strain NHS1DD in terms of inducing endocarditis and causing disseminated infection in the spleen did not differ statistically.

In addition, the densities of the NHS1DD mutant in the target tissues and the weights of vegetations were not significantly different from those of the parental strain (Table 1; $P > 0.05$ and > 0.05 , for vegetation and spleen, respectively) at inocula greater than 10^7 CFU. The only significant difference was the slightly higher densities of GS-5 over NHS1DD found in the spleen at 10^6 CFU inoculum (Table 1; $P < 0.01$). Therefore, no significant difference was found in the relative virulence of the GS-5 parental versus the NHS1DD mutant strain in experimental endocarditis. Two clinical isolates, NTU4312 (oral isolate) and NTU5526 (blood isolate), were tested subsequently in the rat models. For both strains, the virulence, in terms of ID_{95} , vegetation size, and densities of colonized bacteria, were similar to that of GS-5. Therefore, the GS-5 strain was selected for subsequent analysis of the inflammatory response and compared to the isogenic mutant strains NHS1DD and NHR1DD.

Histology and detection of proinflammatory cytokines in situ. On the basis of the above data and to ensure higher rates of endocarditis and disseminated infection of target tissue, we used a 10^9 CFU inoculum of each strain for subsequent challenge experiments in catheterized rats. The primary objective of this study was to test the hypothesis that GTFs might act as modulins in situ and induce IL-6 production after colonization in the vegetation or in the disseminated target organ. Frozen sections of aortic valves and spleens were prepared from catheterized rats, 24 or 72 h after bacterial challenge or mock challenge. Hematoxylin and eosin staining of the sections from uninfected control animals revealed a band of thrombus formation overlying the surface of traumatized valvular base regions. Mild inflammation accompanied by infiltration of neutrophils was detected at these base regions 24 or 72 h later. In animals which developed endocarditis following inoculation with 10^9 CFU of GS-5 or NHS1DD strains, typical vegetations attached to the damaged aortic valves were formed with deposits of bacteria surrounded by a vascular network of fibrin-platelets, visible after Gram staining. Severe inflammation with an intensity of neutrophil infiltration much greater than that of the uninfected controls was detected at the bases of the vegetations.

Serial sections of vegetations taken from different animals infected with either GS-5 or NHS1DD revealed indistinguishable histological pictures in terms of the severity of inflammation and distribution of infiltration of neutrophils. These histological findings confirmed our earlier gross observation that

FIG. 3. Infectivity and virulence of GS-5 and NHS1DD in rat experimental endocarditis. (A and B) Weight of vegetation and infectivity titration. Each point represents one rat and a dash represents the mean weight of vegetation formed. (C and D) Bacterial densities in vegetations. Each point represents one rat and a dash represents the mean bacterial density. The ID₀₅ for GS-5 and NHS1DD was similar, 10⁷ CFU. (E and F) Bacterial densities in the spleens. Each point represents one rat, and the bars represent the mean bacterial densities. No statistically significant difference was found in vegetation weights or bacterial densities recovered from either the vegetations or spleens between GS-5- and NHS1DD-infected rats at all inocula except at 10⁶ in the spleen. Diamonds in A, C, and E, GS-5; circles in B, D, and F, NHS1DD; bar, mean for GS-5 or NHS1DD.

wild-type *S. mutans* and the isogenic mutant strain are capable of inducing typical infective endocarditis with septic vegetations and valvular inflammation. Immunostaining was carried out subsequently on these sections to detect proinflammatory

cytokines, IL-1 β , IL-6, and TNF- α , and representative sections are shown in Fig. 4. A normal aortic valve is avascular and walled off by a thin layer of endothelial cells under hematoxylin and eosin staining (Fig. 4A). Twenty-four hours after GS-5

TABLE 1. Comparative virulence of wild-type and mutant strains at different inocula^a

Strain	Inoculum (CFU)	No. of animals	Vegetation wt (mg)	Bacteria in vegetation (log ₁₀ CFU/g)	Bacteria in spleen (log ₁₀ CFU/g)
GS-5	10 ⁵	3	<1	<1	<1
	10 ⁶	3	1.0 ± 1.0	<1	3.6 ± 1.2*
	10 ⁷	8	2.8 ± 1.0	5.0 ± 1.0	3.3 ± 0.2
	10 ⁸	6	3.6 ± 2.1	6.0 ± 1.3	4.0 ± 0.5
	10 ⁹	9	4.7 ± 2.1	6.6 ± 0.3	3.2 ± 0.3
NHS1DD	10 ⁵	4	0.5 ± 0.7	<1	<1
	10 ⁶	5	1.0 ± 1.1	<1	1.8 ± 0.0*
	10 ⁷	8	3.0 ± 0.8	4.3 ± 0.4	3.3 ± 0.2
	10 ⁸	6	3.2 ± 0.6	5.8 ± 0.9	3.7 ± 0.5
	10 ⁹	7	4.5 ± 2.6	6.1 ± 0.3	3.6 ± 0.4
Control		4	0.2 ± 0.2	ND	ND

^a Values are means ± standard deviation. ND, not detected. *, $P < 0.01$.

inoculation, only IL-6, not IL-1 β or TNF- α , was detected readily, primarily on the endothelial linings of the catheter-injured aortic valves (Fig. 4D). In contrast, no IL-6-secreting cells were detected in the experimental groups infected by NHS1DD on the valvular or surrounding regions in serial sections, even though well-formed vegetations were present (Fig. 4C). None of the three cytokines tested could be detected in the sections taken from uninfected control animals carrying catheter injuries for the same period of time as the infected group (Fig. 4B). Analogous results were also found in the spleens, and the GS-5 infection resulted in a constant increase of IL-6-secreting neutrophils or macrophages, greater than for NHS1DD infection (representative sections are shown in Fig. 5), even though the number of disseminated bacteria in the

FIG. 4. Detection of IL-6 in situ in frozen sections of noninfected valves or valves infected with different strains. (A) Normal aortic valve (hematoxylin and eosin, 100 $\times$). (B) Immunostaining for IL-6 secretion in catheter-injured valve, uninfected. (C) NHS1DD-infected vegetations and perivalvular regions. (D) GS-5-infected aortic valve. (ABC method; B to D, 100 $\times$). Positive IL-6 secretion in endothelial linings.

FIG. 5. Detection of IL-6 in situ in frozen section of spleens infected with GS-5 (A) or NHS1DD (B). Fewer IL-6-secreting neutrophils or macrophages were detected in disseminated NHS1DD infection after prolonged staining, as indicated by the higher background (B). (ABC method, 100 $\times$).

spleens from animals infected with either strain was about the same ($\approx 10^3$ CFU/g spleen). Analogous results of differential IL-6 induction found both in aortic valves and spleens were obtained from four different animals in each group tested. Considering the descriptive and qualitative nature of the immunostaining, the tissues of the valvular and surrounding regions were dissected from another group of experimental animals and controls at various intervals for semiquantitative assay by Western blot analysis.

Preferential induction of IL-6 in situ by GTFs during acute inflammation. Conclusive evidence for the role of GTFs in IL-6 induction was derived from the results shown in Fig. 6. First, significantly higher amounts of IL-6 were detected ubiquitously 24 h later in animals infected by GS-5 compared to NHS1DD (less than 5% of the density of the bands in GS-5, left and middle panels, top column). Depending on the animal, the level of IL-6 or TNF- α could be variable. In addition, the level of IL-6 increased up to 56% of the GS-5 in band density without any effect on TNF- α in animals inoculated with NHR1DD, which expresses GtfB only (middle panel, top column).

FIG. 6. Western blot analysis of infected valves and surrounding tissues. Aortic valves and surrounding tissues from three different animals were dissected under a stereoscope and prepared as described in Materials and Methods. β -Actin was used as an internal control to normalize the total amount of proteins loaded in each lane. IV, catheter-injured valve; NV, normal valve.

The bacterial density recovered from the vegetation after infection with NHR1DD at a dose of 10^9 CFU was comparable to that found in the NHS1DD or parental GS-5 strain. During a later stage of inflammation, after 72 h, both IL-6 and TNF- α persisted at nearly constant levels in various animals tested, but the level of IL-6 was still slightly higher (2- to 3.5-fold higher) in animals infected with GS-5 than in animals with NHS1DD infection (right panel). Analogous results of preferential IL-6 induction found in aortic valves were obtained from four different animals in each group tested. In all animals tested, no IL-1 β (middle lower column of each panel) could be detected 24 or 72 h after infection. These results suggested that, during acute inflammation in experimental endocarditis, GTFs might be the major modulins in *S. mutans* that preferentially induce IL-6 production from endothelial cells in the perivalvular regions.

DISCUSSION

In this study, a left-sided rat model of endocarditis was adopted and bacteria in aortic valve vegetations were examined after 24 h, a period of time sufficient for developing vegetation and bacterial colonization (36). Using this model, we demonstrated for the first time the infectivity and inflammatory response caused by *S. mutans*, a member of the viridans streptococci. The ID₉₅ for inducing aortic endocarditis with concomitant disseminated infection in the spleen by a laboratory strain, GS-5, or clinical isolates, NTU5526 and NTU4312, were similar, $\approx 10^7$ CFU/rat. The infectivity of *S. mutans* in experimental endocarditis was lower than that of other members of the viridans streptococci, *S. mitior*, *S. intermedius* (ID₉₀ = 10^5 CFU), or *S. sanguis* (ID₉₀ = 10^6 CFU) in Wistar rats (17). But the infectivity, in terms of ID₉₅, was similar to that of *Pseudomonas aeruginosa*, a gram-negative microorganism (46). Although the incidence of endocarditis was lower than for *S. sanguis* or *S. mitior*, *S. mutans* is the best-characterized species among the viridans streptococci in terms of its detailed genomic information (1), as well as the characteristics of its *gtf* genes. For these reasons, *S. mutans* was chosen as the representative species for this analysis of the potential roles of GTFs in colonization and inflammation.

Exopolysaccharides, glucans, or fructans, synthesized through GTFs or fructosyltransferase, require sucrose as a substrate, a sugar found infrequently in human blood. In a rat experimental endocarditis model, Munro and Macrina (31) demonstrated that existing glucans and fructans, synthesized by incubating the *S. mutans* V403 strain in sucrose media prior to inoculation in vivo, could increase the incidence of endocarditis. But the comparative virulence (ID or bacterial densities in vegetation) between the wild-type and a GTF- plus fructosyltransferase-defective isogenic mutant strain was not addressed, and a possible role for the GTFs in the colonization process was not excluded.

Several species of viridans streptococci contain GTF homologs sharing $\approx 50\%$ sequence homology with the GTFs of *S. mutans*. GtfG, a GTF of *S. gordonii*, could function as an adhesin and interact directly with endothelial cells in vitro (43). These findings prompted us to test the role of GTFs as adhesins in mediating the bacterial colonization in vivo. The results of this study suggested that GTFs B, C, and D did not

affect the infectivity or the bacterial density in vegetations. The effect of the GTFs occurred, on the other hand, through triggering inflammatory responses, surrounding and underlying the infected valves. Our results suggested GTF alone, in the absence of the glucan, did not contribute to the infectivity but rather affected the inflammatory responses.

Bacteria produce a wide range of virulence factors which cause host tissue pathology, and, depending on their interaction with the host cells, these diverse factors have been grouped into various families: adhesins, aggressins, impedins, invasins, and modulins. Modulins comprise an array of cytokine-inducing molecules, so named because the action of cytokines is to modulate eukaryotic cell behavior (20). For example, upregulation of proinflammatory mediators such as TNF- α , interleukin-1, and IL-6 leads to an increase in the expression of cell adhesion molecules and chemokines that in turn attract macrophages and neutrophils that are capable of eliminating microorganisms during the first line of defense. Protein I/II, an extracellular protein from *S. mutans* and other oral streptococci, is a potent modulin that triggers in vitro the production and release of inflammatory mediators such as IL-6 and IL-8 from fibroblast-like synoviocytes, cells that are critically involved in joint inflammation associated with rheumatoid arthritis (32). Two other components from *S. mutans*, protein SR, an I/II-related antigen, and rhamnose-glucose polymer, a serotype-specific antigen, also have been shown in vitro to be modulins that activate monocytes through specific receptors (38, 39). But the possible roles of protein I/II and polysaccharides have not yet been investigated in vivo.

Our previous results suggested that GTFs act as modulins when added to circulating monocytes in vitro and also in vivo, producing IL-6 preferentially and not TNF- α (10, 12). Serum levels of IL-6 (10) were elevated two hours after intravenous challenge with the GS-5 strain and peaked at 6 h (4 to 10 ng/ml). A significantly lower amount of IL-6 was detected in all animals challenged with NHS1DD (<4 ng/ml after 6 h). The results of this study provide additional evidence that GTFs may exert their IL-6-stimulating potential during systemic infection inside the spleen and surrounding vegetations. This activity was most pronounced during the initial stage of inflammation, immediately after the formation of vegetations (within 24 h). The identity of the IL-6-producing cells might be the endothelial cells around the vegetations, as shown in Fig. 4D.

An interesting pathological finding derived from previous studies of experimental endocarditis in the rat and rabbit was that the infecting microorganism, either *S. mutans* (31) or *Enterococcus faecalis* (28), was walled off inside the vegetations, surrounded by a platelet/fibrin layer, and bordered by an influx of immune cells. The bacteria were rarely found in the surrounding valves or underlying endocardium, at which sites the typical inflammatory responses were observed most frequently. The results of immunostaining in this study suggested the possibility that, rather than the whole bacterium, secreted proteins or other modulators such as GTFs might gain access to the surrounding endothelium or penetrate deep inside the underlying endocardium, where the interaction would take place with the endothelium directly or with the recruited monocytes.

In our rat model of endocarditis, the vegetations were well established 24 h after inoculation and the inflammation was

considered to be at an acute stage of infection. Accordingly, two proinflammatory cytokines, IL-6 and TNF- α , were elevated during this initial stage of infection and in the following 72 h period could be detected by immunohistochemistry and Western blotting. The major difference between the wild-type GS-5 and the *gtf*-defective mutant was the production of IL-6 24 h after infection. The reduced level of IL-6 was identified not only in the endocardium (Fig. 4) but also in the spleen (Fig. 5). In parallel experiments, the reduced levels of IL-6 could be partially increased from less than 5% for the NHS1DD strain up to 56% of the parental GS-5 level for the strain NHR1DD, which expresses GtfB only. The decreased levels of IL-6 were related directly to the lack or decreased levels of GTFs, because the NHS1DD and NHR1DD mutants could be isolated from vegetations at densities indistinguishable from that of the parental GS-5 strain. These results provided direct evidence to support our hypothesis that the GTFs might be major components in *S. mutans*, and possibly also in other GTF-containing viridans streptococci, that are responsible for inducing IL-6 in situ at the infected valves, particularly during the acute stage of the experimental model of endocarditis.

In this model, IL-6 was also present 72 h after infection at a level slightly higher in the wild type than *gtf*-defective NHS1DD strain. The origin of the IL-6 during the later stage is more complicated than that detected earlier after infection, because IL-6 could be stimulated through the paracrine activation of other cytokines released from monocytes instead of through direct interaction of bacterial modulins or pathogen-associated molecular patterns, such as GTFs, with the pattern recognition receptors present on endothelial cells or other cells in the innate immune response. Either route resulted in the preferential induction of IL-6, as found in this study. These results indicated that IL-6 might be involved not only in the acute stage, but also in the chronic stage of endocarditis. Interestingly, clinical surveys demonstrated that the serum levels of IL-6, but not of other cytokines, were significantly higher at all stages of endocarditis (2, 33).

Inflammatory cytokines have not been documented previously in situ in experimental endocarditis or clinical specimens. However, the possible role of IL-6 in the cardiac innate immune response has been investigated in viral myocarditis caused by encephalomyocarditis virus (EMCV). In an EMCV-infected mouse model, treatment with recombinant human IL-6, started immediately after virus inoculation, improved the survival rates, although recombinant human IL-6 treatment given later (3 days) in the course of infection was not effective (23). In a continuous study, the same group developed an IL-6 transgenic mouse model, which overexpressed the human IL-6 gene under the control of the murine major histocompatibility complex class I (H-2L^d) promoter. In that model, human IL-6 mRNA was detected in several organs, including the heart, and the serum IL-6 concentration was maintained at a high level (288.1 ± 228.8 pg/ml).

After EMCV inoculation, constitutive activation of IL-6 promotes myocardial damage characterized by the necrosis of myocytes and infiltration of neutrophils. In addition, the EMCV titers in the infected hearts of IL-6 transgenic mice were significantly higher than in wild-type mice on day 3, suggesting impaired elimination of virus in the IL-6 transgenic mice. Taken together, these results suggested that IL-6 had a bene-

ficial effect during the early inflammatory stage of the cardiac immune response. However, persistent activation of IL-6 might promote myocardial damage (14, 23, 34).

By analogy with the EMCV animal model, we found that IL-6 also plays an important role in the cardiac immune response against bacterial infection. Distinct from a general phenomenon, the coordinate induction and early release of TNF- α , IL-6, and IL-1 β was not observed, but IL-6 was induced preferentially earlier in time than the other two cytokines in the cardiac immune response. Accordingly, the serum concentration of TNF- α was comparatively lower than that of IL-6 after inoculation of either EMCV (23) or *S. mutans* (12) in either the animal model or clinical investigations on patients suffering endocarditis (2, 33). The results of this study, in conjunction with the elevated level of IL-6 in patients' sera during all stages of infective endocarditis, suggested the persistence of IL-6 during the chronic stage of inflammation of the endocardium.

Inflammation, a complex defense mechanism, is characterized by leukocyte migration from the vasculature to damaged tissues to destroy the injurious agents. Acute inflammation is of limited benefit, particularly during infectious challenge, whereas chronic inflammation is a persistent phenomenon which can progress to inflammatory disease. The induction and production of proinflammatory cytokines play essential roles in regulating the migration and transition of leukocytes. One hallmark of acute inflammation is that the leukocyte infiltrate is initially mostly neutrophilic but, after 24 to 48 h, monocytic cells such as macrophages and lymphocytes predominate (29).

IL-6 and its soluble receptor have been proposed recently as a key mediator regulating the leukocyte recruitment transition from neutrophil to mononuclear-cell infiltrate (24). The recruitment of leukocytes is dependent on both the expression of adhesion molecules and the specificity of chemokines produced at the inflammatory site. Transition from neutrophil to monocyte accumulation might be linked partly to the kinetics of chemokine production, particularly IL-8 and monocyte chemoattractant protein-1 (MCP-1), the most important chemokines for the recruitment of PMN cells and monocytes, respectively. Although controversy still exists regarding the combinatorial role of IL-6 and its soluble receptor in the stimulation of IL-8 production from endothelial cells, independent results from various groups indicated that the IL-6 complex favors the transition from neutrophil to monocyte in inflammation in vivo.

In inflammatory knockout mouse models, the neutrophilic infiltrate is more dominant in IL-6 knockout mice than in wild-type animals (45), and these are the only cells present in the infiltrate (22). The transition from neutrophils to monocytes was hypothesized to be beneficial in inflammation by limiting the toxic effects of the contents of neutrophils on the surrounding tissues in a time- and space-limited fashion. Therefore, during acute inflammation, such as in the case of EMCV-induced myocarditis, IL-6 was considered to be rather protective, and exogenous IL-6 administered at the time of viral inoculation improved the survival rate. But constitutive and sustained activation of IL-6, as in the IL-6 transgenic mouse, promotes myocardial damage. It would be interesting to investigate whether similar situations might also occur in bacterial endocarditis.

Using an established model, we have attempted to investi-

gate the protective role of IL-6 in endocarditis by observing differences in the survival rates caused by *S. mutans* GS-5 vs. NHS1DD. Preliminary data including four mice in each group suggested that the absence of IL-6 during the early stage of infection, as found with NHS1DD, indeed led to decreased survival rates, as all infected rats died within 7 days, whereas the GS-5-infected rats survived two weeks and uninfected catheterized rats survived over two weeks. But, distinct from the viral myocarditis model, the experimental endocarditis, if left untreated, is a more complex situation with many uncontrollable factors that might lead to a difference in survival rate. Therefore, the interpretation of these data should be more cautious and they may be considered inconclusive at present. Nevertheless, our data do suggest that, during the acute stage of inflammation, GTFs are potent and major modulins responsible for the induction of IL-6, probably released from both endothelial lining of the valvular regions and monocytes entrapped inside vegetations.

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