HOST RESPONSE TO INFECTION

Antigenic characterisation of a novel *Streptococcus anginosus* antigen that induces nitric oxide synthesis by murine peritoneal exudate cells

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A novel antigen that induces nitric oxide (NO) synthesis by murine peritoneal exudate cells (PEC) was prepared from a culture supernate of *Streptococcus anginosus* NCTC 10713 in dialysed medium by column chromatography with DEAE-Sephacel followed by size-exclusion high performance liquid chromatography (HPLC). A chemical analysis of the *S. anginosus* antigen (SAA) revealed that it mainly consisted of carbohydrates (rhamnose, *N*-acetylglucosamine, glucose and galactose), smaller quantities of protein and a trace amount of phosphorus. The SAA stimulated PEC from C57BL/6N mice to produce NO and accumulate induced NO synthetase (iNOS) mRNA in a dose-dependent manner, reaching a plateau with 10–30 µg/ml. Furthermore, a reverse transcription-PCR assay revealed that SAA 10 µg/ml could induce mRNA accumulation of tumour necrosis factor- α , interleukin (IL)-1 β and IL-6 as well as iNOS. In contrast, Rantz-Randall antigen (RRA), a carbohydrate antigen prepared from the organisms, could not induce NO synthesis or cause the accumulation of iNOS mRNA, although cytokine production was observed after stimulation. The SAA-induced NO synthesis, but not the cytokine production, was sensitive to heat. Furthermore, an immunoblot analysis of SAA indicated that the 43-kDa protein band reacted with anti-SAA but not anti-RRA antibodies. In immunodiffusion, SAA reacted with both anti-SAA and anti-RRA antibodies, and the precipitin bands formed crossing lines, suggesting that SAA could possess two different antigenic components – one that reacts specifically with anti-SAA antibodies and another that has an identity similar to that of RRA. Taken together, SAA, a novel antigen of *S. anginosus*, was found to induce NO synthesis as well as produce inflammatory cytokines in murine PEC. It is suggested that the protein molecule of SAA may exclusively induce NO synthesis, and its carbohydrate component(s) could have a relationship to cytokine production.

Introduction

Streptococcus anginosus, one of the oral viridans streptococci, is a common commensal organism found in the human oral cavity and on other mucosal surfaces, such as the upper respiratory and gastrointestinal tracts. This organism is generally considered to have a low pathogenicity; however, it can cause serious purulent abscesses in various body sites [1–3] and subacute bacterial endocarditis [1, 4]. Despite its recently increased clinical significance, little is known about the pathogenic mechanisms of *S. anginosus* and

the specific factors for its activities remain to be elucidated.

Several components of the oral streptococcal cell wall including peptidoglycans, lipoteichoic acids, surface proteins and carbohydrate antigens, can trigger macrophages to induce cytokines, such as tumour necrosis factor (TNF)- α , and interleukin (IL)-1 and IL-6, that are involved in inflammatory and immunological reactions in the host [5–9]. Furthermore, some cell wall components from oral viridans streptococci have been shown to induce the production of nitric oxide (NO) from murine macrophages; e.g., serotype polysides (rhamnose glucose polymers, RGPs) from *S. mutans* [10] and lipoteichoic acid (LTA) from *S. sanguis* and *S. mutans* [11]. Although NO has been implicated in macrophage-mediated cytotoxicity against various pathogens and may play a role in persistent or latent

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infections, its overproduction, induced by such bacterial components as lipopolysaccharide (LPS) and LTA, could cause damage to host tissues or induce circulatory failure in gram-positive septic shock, or both [12].

The present study examined the induction of production of NO as well as inflammatory cytokines from murine peritoneal exudate cells (PEC) by a novel antigen purified from the culture supernate of *S. anginosus* (SAA).

Materials and methods

Preparation of SAA and biological standards

S. anginosus NCTC 10713 (Lancefield group G) was cultured in dialysed Todd Hewitt Broth (BBL Microbiology Systems, Cockeysville, MD, USA) at 37°C for 24 h. The supernate was collected by centrifugation and filtered through a 0.45- μ m membrane. The supernate was then applied to a DEAE-Sephacel column (Amersham Pharmacia Biotech, Uppsala, Sweden) and washed with 20 mM Tris-HCl buffer at pH 8.0. The absorbed fractions were eluted with a linear gradient of 0–0.5 M NaCl in the same buffer. The fractions showing NO-inducing activity were combined, and then concentrated by ultrafiltration with a YM-10 membrane (Amicon, Beverly, MA, USA). The concentrated fraction was further subjected to size-exclusion high-performance liquid chromatography (HPLC) with a TSK gel G4000 column (Tosoh, Tokyo, Japan) equilibrated with 20 mM Tris-HCl buffer at pH 8.0. The eluted fraction was designated as SAA. In some experiments, heat-treated (121°C for 20 min) SAA was used.

The Rantz-Randall antigen, a carbohydrate antigen, was prepared from *S. anginosus* NCTC 10713 as described by Hamada *et al.* [13]. Briefly, *S. anginosus* whole cells 50 mg/ml in sterile saline were autoclaved at 121°C for 20 min and the centrifuged supernate (RRA) was collected. The LPS used in this study was prepared by further purification from a commercial preparation of LPS from *Escherichia coli* O128:B12 (Sigma) by the phenol-water extraction method. Purified LTA from *S. sanguis* was purchased from Sigma.

Chemical analysis of SAA

Total hexose was quantified by the phenol-sulphuric acid method [14] and rhamnose, glucose, galactose and hexosamine (*N*-acetylglucosamine and *N*-acetylgalactosamine) by the colorimetric method [15–18]. The protein and phosphorus contents of SAA were measured by the methods of Bradford [19] and Chen *et al.* [20], respectively.

NO and TNF- α production

PEC were collected by lavage from C57BL/6N mice (8

weeks old; CLEA Japan, Osaka, Japan) that had been given 1 ml of Thioglycolate Medium (Nissui, Tokyo, Japan) by intraperitoneal injection as described previously [21]. The PEC were resuspended at 5×10^5 cells/ml in sterile RPMI 1640 medium (Sigma) supplemented with gentamicin (Sigma) 50 μ g/ml and heat-inactivated fetal calf serum (FCS; ICN Biomedicals Japan, Osaka, Japan) 10% v/v, and cultured with SAA or the biological standards in triplicate at 37°C in humidified CO₂ 5% in air. After incubation for 24 h, the NO content of the culture supernates was estimated by an analysis of nitrite (production of rapid oxidation of NO in aqueous solution) with Griess reagent [22]. The TNF- α concentration in the culture supernates was measured with a mouse TNF- α ELISA kit (R&D System, Minneapolis, MN, USA), according to the manufacturer's instructions.

RT-PCR analysis

The expressions of induced NO synthetase (iNOS), TNF- α , IL-1 β and IL-6 specific mRNA in SAA-stimulated PEC were examined by reverse transcriptase (RT)-PCR. RT-PCR was performed essentially as reported previously [9, 23]. Briefly, RNA was purified by the acid guanidinium thiocyanate-phenol-chloroform extraction procedure with TRIzol Reagent (GIBCO-BRL, Gaithersburg, MD, USA). The RNA was reverse transcribed into cDNA in a reverse transcription mixture consisting of a first strand buffer (50 mM Tris-HCl, pH 8.3; 75 mM KCl; 3 mM MgCl₂), 1 mM dNTP, 10 units of RNAase inhibitor (RNasin; Promega, Madison, WI, USA), 4 μ M oligo(dT)_{8–12} and 2 units of Superscript II reverse transcriptase (GIBCO-BRL). After a series of incubations (37°C for 60 min, 99°C for 5 min and then 0°C for 5 min), the RT product was mixed with 1.25 units of Ampli-Taq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA), and 0.15 μ M sense and anti-sense primers of iNOS, TNF- α , IL-1 β and IL-6 genes in a GeneAmp PCR gold buffer (15 mM Tris-HCl, pH 8.0; 50 mM KCl) supplemented with 2 mM MgCl₂ and 50 μ M dNTP. PCR amplification was then performed in sequential cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, followed by an extension step for 7 min after the last cycle with a GeneAmp PCR System 9600 (Applied Biosystems). All samples were amplified for 25 cycles, and the PCR samples were electrophoresed on agarose 1.8% gels (FMC BioProducts, Rockland, ME, USA). Bands in the gel were visualised with ethidium bromide 1 μ g/ml. The primers used in this study are listed in Table 1.

Antigenic characterisation of SAA

Rabbit antisera were prepared by subcutaneous immunisation with SAA and RRA emulsified with Freund's incomplete adjuvant, as described previously [13]. Antigen-specific antibodies were purified from the antisera by Protein A Sepharose (Amersham Pharmacia

Table 1. Primers for PCR

Primers for		Sequence		Amplicon size (bp)
		5'	3'	
iNOS	F	CCCTTCCGAAGTTTCTGGCAGC		494
	R	GGCTGTCAGAGCCTCGTGGCTTTGG		
TNF- α	F	TCTCATCAGTTCTATTGGCCC		212
	R	GGGAGTAGACAAGGTACAAC		
IL-1 β	F	TTGACGGACCCCAAAAGATG		204
	R	AGAAGGTGCTCATGTCCTCA		
IL-6	F	GTTCTCTGGGAAATCGTGGA		208
	R	TGTACTCCAGGTAGCTATGG		

Biotech) column chromatography. These antibodies were used in an immunodiffusion test against SAA and RRA in an agar 1% gel with 0.01 M sodium phosphate buffer (pH 7.2). For further antigenic characterisation of SAA, a Western blot analysis was performed with the antibodies, as described previously [24, 25]. Briefly, SAA (2 μ g of protein) was separated by SDS-PAGE (12.5% gel). The separated proteins were transferred to a PVDF membrane (1 h at 7 V) and immunoblotted with the anti-SAA or anti-RRA antibodies, or both. Immunoreactive proteins were then detected by alkaline phosphatase-conjugated goat anti-rabbit antibody (Promega). The molecular masses of the bands were calculated by means of molecular size standards.

Statistical analysis

Data for NO and TNF- α production were analysed by either a multiple comparison method (ANOVA) or an unpaired *t* test.

Results

Chemical composition of SAA

The culture supernate of *S. anginosus* NCTC 10713 collected by centrifugation and filtration was applied to DEAE-Sephacel and the absorbed fractions were concentrated and applied to size-exclusion HPLC with a TSK gel G4000. Major NO-inducing components were eluted in the earlier fractions (Fig. 1). Therefore, those fractions nos (16–21) concurrent with major hexose and minor protein peaks were pooled and designated as SAA.

Quantitative analyses of SAA and RRA, including hexose (galactose and glucose), de-oxyhexose (rhamnose), hexosamine (*N*-acetylglucosamine and *N*-acetyl-galactosamine), protein and phosphorus showed that SAA consisted mainly of carbohydrates, smaller quantities of protein and a trace amount of phosphorus (Table 2). Rhamnose and *N*-acetylglucosamine were detected in significant amounts in the carbohydrate

Fig. 1. Size-exclusion HPLC profiles of the NO-inducing activity of *S. anginosus* culture supernate. Sample (0.5 ml) was loaded on to a TSK G4000 SW column equilibrated with 20 mM Tris-HCl, pH 8.0, elution was at a flow rate of 0.25 ml/min, and a 2-min fraction was collected. Arrows indicate the elution position of the void volume (Vo) and total column volume (Vt). Symbols indicate content of hexose (○), protein (△) and NO (●).

Table 2. Chemical composition of SAA and RRA ($\mu\text{g/ml}$)

Components	SAA	RRA
Rhamnose	180	200
Galactose	32	24
Glucose	44	28
<i>N</i> -Acetylglucosamine	100	70
<i>N</i> -Acetylgalactosamine	10	8
Phosphorus	1.1	2.4
Protein	44	74

component of SAA. The chemical composition of SAA was similar to that of RRA, although the contents were slightly different.

SAA-induced NO production

When PEC from C57BL/6N mice were cultured in the presence of SAA as well as LPS or LTA, NO synthesis was markedly induced, whereas no significant production was observed after stimulation with RRA (Table 3). SAA induced NO synthesis in a dose-dependent manner (0.1–10 $\mu\text{g/ml}$), reaching a plateau with 10–30 $\mu\text{g/ml}$. Treatment of SAA with polymyxin B did not alter its NO-inducing activity, whereas the activity of LPS was abolished with polymyxin B 10 $\mu\text{g/ml}$. The SAA-induced NO synthesis was abolished by heat (121°C, 20 min) (Fig. 2). Furthermore, RT-PCR analysis indicated that SAA induced the accumulation of iNOS mRNA, whereas this accumulation was not detected after stimulation with RRA (Fig. 3).

Table 3. NO production after stimulation of murine PEC with SAA, RRA, LPS and LTA at 37°C for 24 h

Stimulant	Amount added ($\mu\text{g/ml}$)	Mean (SEM) (mM) NO*
SAA	0.1	3.7 (1.7)
	1.0	17.7 (1.7)
	10	52.6 (2.0)
	30	54.2 (4.8)
	10 + PMB	49.0 (4.2)
RRA	0.1	4.3 (1.7)
	1.0	4.5 (1.3)
	10	7.3 (2.2)
	30	11.7 (3.0)
	10 + PMB	6.0 (1.1)
LPS	0.1	37.5 (6.4)
	1.0	48.2 (7.2)
	10	54.7 (12.0)
	30	59.7 (6.8)
	10 + PMB	8.5 (1.9)
LTA	0.1	4.2 (1.7)
	1.0	24.5 (6.6)
	10	46.0 (2.8)
	30	40.5 (4.1)
	10 + PMB	43.0 (2.4)

*Polymyxin B (PMB) was added to the reaction mixtures at 10 $\mu\text{g/ml}$. Data are the means (SEM) of NO concentration of four different experiments.

SAA-induced cytokine production

To assess SAA-induced cytokine production by PEC, the study examined TNF- α production by ELISA and TNF- α , IL-1 β and IL-6 mRNA expression by means of RT-PCR. As shown in Fig. 4, SAA 10 $\mu\text{g/ml}$ and RRA each stimulated PEC to produce significant amounts of TNF- α in the culture supernate. Moreover, heated SAA also induced the production of TNF- α . RT-PCR revealed that SAA could induce accumulation of TNF- α ,

Fig. 2. Effect of heat on NO-inducing activity of SAA. Murine PEC were incubated at 37°C with SAA, RRA (10 $\mu\text{g/ml}$), heated (121°C for 20 min) SAA and heated RRA. After 24 h, NO in the culture supernates was measured by the Griess method. Data are expressed as the means (SEM) of NO concentration of four different experiments.

Fig. 3. Induction of iNOS, TNF- α , IL-1 β and IL-6 mRNA in murine PEC stimulated with SAA, RRA, heated SAA and heated RRA (each 10 μ g/ml) at 37°C. After 3 h, total RNA was prepared, and β -actin, iNOS, TNF- α , IL-1 β and IL-6 mRNA were amplified by RT-PCR. The PCR products were analysed in an agarose 1.8% gel. Lane 1, medium; 2, SAA; 3, heated SAA; 4, RRA; 5, heated RRA.

IL-1 β and IL-6 mRNA as well as iNOS mRNA (Fig. 3). In contrast, stimulation with RRA and heated SAA resulted in a definitive accumulation of TNF- α , IL-1 β and IL-6, but not iNOS, mRNA, in PEC (Fig. 3).

Antigenic characterisation of SAA

In an immunodiffusion test, anti-SAA antibodies formed a single precipitin band with SAA but not

with RRA (Fig. 5A). On the other hand, anti-RRA antibodies formed a line of identity with both RRA and SAA (Fig. 5B). SAA formed crossing lines in reactions with anti-SAA and anti-RRA antibodies (Fig. 5C), indicating that it might possess two different antigenic components – one that reacts specifically with anti-SAA antibodies and the other with anti-RRA antibodies.

To characterise the antigenic component that reacted specifically with anti-SAA antibodies, an immunoblot analysis with anti-SAA antibodies was performed. Although both SAA and RRA contain several protein components, the 43 kDa-protein band (p43) in SAA reacted with anti-SAA antibodies (Fig. 6), although not with anti-RRA antibodies (data not shown). Furthermore, RRA showed no obvious band in the immunoblot analysis with anti-SAA antibodies.

Discussion

SAA, a novel antigen purified from the culture supernate of *S. anginosus* NCTC 10713, induced NO synthesis in PEC from C57BL/6N mice. Chemical analysis revealed that SAA consists mainly of carbohydrates, such as rhamnose and *N*-acetylglucosamine, and smaller quantities of protein. Its composition was essentially similar to that of RRA (Table 2), as well as to that of the carbohydrate antigen of the serotype k/Lancefield group G extracted by autoclaving whole cells of *S. anginosus* K-214-2K [26]. These results strongly suggest that the composition of SAA could be closely related to serotype carbohydrate antigen(s), but not to that of LTA or RGP.

When PEC were cultured in the presence of SAA, NO

Fig. 4. TNF- α production from C57BL/6 murine PEC stimulated with SAA, RRA, heated SAA and heated RRA (each 10 μ g/ml) at 37°C for 24 h. TNF- α content in the culture supernates was measured by ELISA. Data are expressed as the mean (SEM) TNF- α concentration of four different experiments. The TNF- α content of the non-stimulated control was < 50 pg/ml.

Fig. 5. Immunodiffusion: antigens and antibodies were allowed to react in an agar 1% gel in 0.01 M sodium phosphate buffer (pH 7.2). **a**, anti-SAA antibodies; **b**, anti-RRA antibodies; **S**, SAA (5 µg/ml); **R**, RRA (5 µg/ml).

Fig. 6. SDS-PAGE (polyacrylamide 12.5% gel) of SAA and RRA processed by Coomassie staining (**A**) and developed by immunoblotting (**B**). Lanes **a** and **c**, SAA (2 µg); **b** and **d**, RRA (2 µg).

synthesis was induced (Table 3). RT-PCR also revealed that SAA could induce accumulation of iNOS mRNA (Fig. 3). In contrast, RRA could not induce NO synthesis or accumulation of iNOS mRNA, although production and mRNA accumulation of TNF- α , IL-1 β and IL-6 were observed, which was the same as with SAA (Fig. 3). An immunodiffusion assay indicated that SAA could possess two different antigenic components – one that reacts specifically with anti-SAA antibodies, and another that has an identity similar to that of RRA (Fig. 5). Moreover, the 43-kDa protein band reacted with anti-SAA antibodies, but not with anti-RRA antibodies, in an immunoblot analysis (Fig. 6). Therefore, it is most likely that the protein molecule (p43) of SAA in reaction with anti-SAA antibodies could exclusively induce NO synthesis, and that the carbo-

hydrate component(s) closely related to the serotype carbohydrate antigen(s) of *S. anginosus* could induce cytokine production. In fact, SAA-induced NO synthesis was abolished by heat, which is a condition needed for the preparation of RRA as well as the carbohydrate antigen of the serotype k/Lancefield group G (Fig. 3). This finding makes it unlikely that SAA-induced NO synthesis is related to contamination by RGPs (rhamnose glucose polymers) or LTA, or both, in SAA, as these streptococcal antigens are heat-stable. Furthermore, putative contamination of LPS was also ruled out by the finding that treatment of SAA with polymyxin B did not alter its NO-inducing activity (Table 3).

SAA stimulated murine PEC to induce NO production regardless of the mouse strains used, as with LTA and RGPs (data not shown). However, the precise mechanism of NO production from PEC after stimulation with bacterial components, including LTA and RGPs, has not been fully addressed. English *et al.* [11] indicated that streptococcal LTA provoked NO production from murine macrophages only in the presence of recombinant interferon (IFN)- γ , while Drapier *et al.* [27] reported that IFN- γ and TNF- α are synergically to induce nitrite synthesis in murine macrophages after stimulation with bacterial antigens. Furthermore, Mond *et al.* [28] suggested that T-independent type 2 antigens, such as carbohydrate antigens, could activate immunocompetent cells by cross-linking multiple cell surface molecules, thereby inducing signal transduction events that result in cellular activation and differentiation. In fact, a previous report found that a serotype carbohydrate antigen from *S. mutans*, a T-independent type 2 antigen, induced murine B cell responses [6] and that a cell wall carbohydrate antigen from *Actinomyces viscosus* induced cytokine production from murine macrophages [21]. Thus, it is possible that the carbohydrate molecule (or protein molecule, or both) in SAA could stimulate the lymphoid cell populations in PEC, resulting in the production of cytokine(s), which is required for NO production from the macrophages in PEC by stimulation with the protein molecule in SAA. This hypothesis is supported by the observation that SAA-induced NO synthesis, but not cytokine production, was sensitive to heat.

Taken together, the present results clearly indicate that SAA, a novel cell surface antigen of *S. anginosus*, has a potential to interact with host cells to induce NO synthesis as well as produce inflammatory cytokines in murine PEC. These findings may facilitate further understanding of the aetiological mechanisms in some oral and systemic inflammatory diseases caused by *S. anginosus* infection, although further studies are clearly required.

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