Biologically active extracellular products of oral viridans streptococci and the aetiology of Kawasaki disease

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Summary. A bacteriological study of isolates from the oral cavity of patients with Kawasaki disease (KD), age-matched non-KD patients and healthy children, showed that over half the KD and control isolates had gram-positive, catalase-negative cocci. About 50% of these organisms were identified as viridans streptococci by means of an API Strep 20 kit. Further identification by fluorometric DNA-DNA hybridisation demonstrated that the predominant species were *S. oralis* and *S. mitis*, each of which accounted for 25% of the isolates of viridans streptococci; 40% of viridans strains were unidentifiable; and *S. sanguis* and *S. parasanguis* were minor components. Studies *in vivo* showed that insertion of culture supernates of most of the viridans streptococci increased capillary permeability and induced redness with swelling and occasional bleeding in rabbit skin. One-third of *S. mitis* strains and one-fifth of the unidentified strains caused aggregation of human blood platelets, whereas *S. oralis* and other strains had no such effect. The distribution of extracellular lipoteichoic acids and glucan produced in the presence of sucrose was also examined. There were no significant differences in the recovery rate of viridans streptococci forming these biologically active extracellular products between KD and control groups.

Introduction

The epidemiological features, clinical symptoms and laboratory findings of Kawasaki disease (KD)—acute febrile, mucocutaneous, lymph node syndrome—have suggested that a possible cause is an infectious organism¹ resident in the oral cavity or throat whose toxic and biologically active products are capable of causing immunological disorders and inflammatory reactions.^{2–4}

However, several attempts to identify a specific microbe that would satisfy Koch's postulates, particularly one recoverable exclusively from KD patients, have so far failed. So we speculated that the causative agent of KD is either an organism that cannot be isolated by conventional techniques or an agent that requires other factors to produce clinical symptoms and signs as well as pathological changes.

As the first step in a study based on the hypothesis that oral or throat bacteria capable of producing biologically active factors are involved in the pathogenesis of KD, we attempted to isolate a significant

group of bacteria from KD patients and age-matched control children that could produce immunobiologically active extracellular factors.

Materials and methods

Patients

KD patients were diagnosed according to the clinical criteria proposed by the study group on KD, the Ministry of Welfare, Japan,⁵ at the Japan Red Cross Medical Centre (Tokyo), 1983, 1990 and 1991, the National Cardiovascular Centre (Osaka) and the Second Hospital, Nippon Medical School (Kawasaki), 1990 and 1991. The age of the KD patients ranged from 3 months to 6 years. Age-matched controls (23) were either patients with non-KD febrile diseases (17) including pharyngitis, viral pneumonia, viral meningitis, exanthema subitum, salmonella enteritis and acute bronchitis, or healthy children (6).

Collection of specimens

Clinical specimens were collected with sterile swabs

taken from the throat and tooth surfaces of 89 patients with KD during the acute phases, 2–5 days after onset, and before intravenous gammaglobulin therapy, from 17 children with other febrile diseases and from six healthy children.

Isolation procedures and classification of clinical isolates

Swabs were transported promptly from the clinic to the laboratory in CO₂-enriched anaerobic transport medium (Kenki Porter, Clinical Supply, Gifu, Japan). Microbes on the swabs were transferred to GAM semi-solid medium (Gifu Anaerobic Medium, Nissui Corp., Tokyo, Japan) and cultured at 37°C for 24–48 h. Bacteria were streaked on pairs of Columbia Sheep Blood Agar plates (bioMérieux, Marcy L'Etoile, France). One of each pair was cultured aerobically and the others were incubated anaerobically in jars made anaerobic with the Gas Pak system (Becton Dickinson Microbiology System, Cockeysville, MD, USA) at 37°C for 24 h. Representative single colonies on the blood-agar plates in Gas Pak jars were selected, streaked again on blood agar plates and cultured aerobically at 37°C for 24 h. The fact that virtually 100% recovery was achieved by this method indicated that all bacteria grown in the Gas Pak can grow aerobically. The strains obtained in pure culture were inoculated in GDO (Groups of Difficult Organisms) medium (Nissui Corp.) devised to store groups of "difficult organisms" and stored at –20°C until use.

The isolates were cultured aerobically on blood-agar plates at 37°C for 18 h, and the bacteria that had grown were stained by Gram's method. Gram-positive cocci were then checked by the catalase test,⁶ and divided into catalase-positive and -negative groups. Gram-negative rods were divided into oxidase-positive and -negative groups. Bacterial strains isolated from KD patients and control children were thus broadly divided into the following genera and groups. (1) *Micrococcus* and *Staphylococcus* (gram-positive, catalase-positive cocci); (2) *Streptococcus*, *Enterococcus* and *Lactococcus* (gram-positive, catalase-negative cocci); (3) *Bacillus* and *Corynebacterium* (gram-positive bacilli); (4) *Neisseriaceae* (gram-negative cocci); (5) Non-fermenting gram-negative bacteria (gram-negative, oxidase-positive bacilli); (6) *Enterobacteriaceae* (gram-negative, oxidase-negative bacilli).

We concentrated on group 2 described above, which accounted for more than half of all isolates from both KD and control children (table I): 98 and 27 strains derived from clinical specimens from 22 patients with KD and 11 with other febrile diseases, respectively, collected at the Department of Pediatrics, Japan Red Cross Medical Center (Tokyo) in 1990–1991 were identified according to the manufacturer's manual for the API Strep 20 kit (bioMérieux, LaBalme les Grottes, France) and the Analytical Profile Index.

Identification of viridans group streptococci by fluorometric DNA-DNA hybridisation

Gram-positive, catalase-negative α -haemolytic cocci classified into three groups, *S. sanguis* II, *S. sanguis* II or *S. pneumoniae* and *S. mitis* (see table II) were identified by fluorometric hybridisation in microdilution wells.^{7–9} Reference strains *S. mitis* ATCC903, ATCC6249 and ATCC15911, *S. oralis* ATCC9811 and *S. parasanguis* ATCC15909 were also identified by this method. The type strains were as follows: *S. dysgalactiae* NCFB2023, *S. suis* NCTC10234, *S. pneumoniae* NCTC7465, *S. oralis* NCTC11427, *S. mitis* NCTC12261 (neotype), *S. sanguis* ATCC10556, *S. parasanguis* ATCC15912, *S. gordonii* NCTC7865, *S. salivarius* ATCC7073, *S. intermedius* ATCC27335 and *Escherichia coli* K-12. A 100- μ l volume of heat-denatured reference DNA (10 μ g/ml) in phosphate-buffered saline (PBS), pH 7.2, containing 0.1 M MgCl₂ was incubated for 1 h at 37°C in a microdilution well (MicroFluor "B" plate, Dynatech Laboratories Inc., Alexandria, VA, USA). The solution was removed and the plate was dried at 45°C for several hours to immobilise the DNA. A 200- μ l volume of pre-hybridisation solution containing 200 μ g of denatured salmon sperm DNA (Sigma) was added to each well and incubated at 45°C for 1 h. The pre-hybridisation solution was discarded and replaced with 100 μ l of hybridisation solution containing 10–20 ng of biotinylated DNA of clinical isolates/well. Hybridisation proceeded for 2 h at 45°C. The wells were washed three times with PBS containing Triton X100 0.1% (PBS-T). One hundred μ l of 0.3 U/ml of streptavidin-conjugated β -D-galactosidase (Zymed Laboratories Inc., San Francisco, CA, USA) dissolved in PBS-T containing bovine serum albumin 2% (PBS-BSA-T) were added to the wells and incubated at 37°C for 15 min. The plate was washed with PBS-T and a 50 μ l volume of 3×10^{-4} M methylumbelliferyl- β -D-galactopyranoside (Koch-light Ltd, Haverhill, Suffolk) in PBS-BSA was added to each plate. After incubation at 30°C for 30 min, the intensity of developing fluorescence was measured with a Micro Fluor reader (Dynatech Laboratories Inc.) at a wavelength of 360 nm for excitation and at 450 nm for emission. The intensity of fluorescence in the well containing salmon sperm DNA was taken at zero, and in the well that emitted the strongest fluorescence this was taken as 100%. *E. coli* DNA served as a negative control. When the second highest intensity was < 70% and the ratio of the maximum:control value > 5, a test strain was identified as being of the same species as the type strain that gave the greatest intensity.

Preparation of culture supernate concentrate

Test strains of viridans streptococci were cultured in 5 ml of a chemically defined medium (CDM)¹⁰ at 37°C for 18 h in a CO₂ 5% incubator. Each growth was transferred to a flask containing 500 ml of CDM and

Table I. Identification of bacterial isolates from 89 KD patients and 23 control children consisting of 17 non-KD patients and 6 healthy children

Identification	Percentage of total isolates	
	KD (256)*	control (85)
<i>Micrococcus</i> spp.	17.3	20.4
<i>Staphylococcus</i> spp.		
<i>Streptococcus</i> spp.	60.9	55.1
<i>Enterococcus</i> spp.		
<i>Lactococcus</i> spp.		
<i>Bacillus</i> spp.	6.2	8.2
<i>Corynebacterium</i> spp.		
Neisseriaceae	0	0
Non-fermenting gram-negative bacteria	14.9	10.2
Enterobacteriaceae	0.6	6.1

* Number of isolates.

Table II. Identification of gram-positive, catalase-negative cocci isolated from 22 KD patients and from 11 control children with an API Strep 20 kit

Identification with API Strep 20 (code no.)	Number (%) of isolates from	
	KD	control
<i>S. sanguis</i> II		
(0240040)	1	0
(0260440)	0	1
(0260441)	7	4
(0261440)	1	0
(0262441)	1	0
(0270441)	2	0
(0270451)	0	1
<i>S. sanguis</i> II or <i>S. pneumoniae</i>		
(0240440)	1	0
(0240441)	2	0
(0240450)	2	1
(0250441)	1	2
Subtotal	18 (18.4)	9 (33.3)
<i>S. mitis</i>		
(0040400)	8	1
(0040401)	5	1
(0042403)	1	0
(0050400)	3	0
(0050401)	5	0
(0050461)	0	1
(0051400)	1	0
(0060400)	1	0
(0060401)	3	4
(0070400)	1	0
(0070401)	5	0
(0070420)	1	0
Subtotal	34 (34.7)	7 (25.9)
Others*	46 (46.9)	11 (40.9)
Total	98 (100)	27 (100)

* *S. sanguis* I, *S. salivarius*, *S. bovis*, *S. canis*, *S. equisimilis*, *S. pneumoniae* 1 or 2, *E. faecalis*, *E. faecium*, *E. avium*, *L. cremoris*, *L. lactis*, *A. viridans*, *G. morbillorum* etc.

incubated at 37°C for 18 h. The culture supernate obtained by centrifugation at 10000 *g* for 30 min at 4°C was filtered through a 0.45 µm membrane filter

(Millipore Co., Bedford, MA., USA), placed in dialysis tubing with mol. mass cut-off of 12000–14000 (Sanko Pure Chemical Co., Tokyo), and concentrated by dialysis against Aquacide II, a sodium salt of carboxymethyl cellulose of high viscosity, mol. wt 500000 (Calbiochem Corp., LaJolla, CA, USA). The dialysis tube contents were washed out with 5 ml of 0.15 M PBS, pH 7.2, and adjusted to pH 7.2 with NaHCO₃ 0.1% if necessary. The culture supernate concentrated about 100-fold was sterilised by filtration through a 0.45-µm membrane filter and the protein content was measured by the Lowry method.¹¹ The culture supernate concentrate thus obtained was used in experiments as the test extracellular products.

Vascular permeability and erythrogenic effects on rabbit skin

Into the depilated dorsal skin of albino, female domestic rabbits weighing c. 2.5 kg (Nippon Biosupply Centre, Tokyo) 0.1 ml of test specimens, containing protein 0.8–1.0 mg/ml in 0.15 M PBS, was injected intradermally at intervals. Skins were then examined for redness, swelling and bleeding. After the final intradermal injection, the rabbits were given an intravenous injection of aqueous Evans blue 1.0%. After 30 min, the rabbits were killed, the skin was dissected from the abdominal wall and turned inside out to look for any blue colour due to dye leakage (an indication of increased capillary permeability), and bleeding of the test skin sites.¹² A piece of skin was fixed with formalin, embedded in paraffin and stained with haematoxylin and eosin (H and E) by conventional procedures. The intensity of blue dye and redness with swelling, with or without bleeding was scored macroscopically as –, + or ++.

Blood platelet aggregation

Human peripheral blood was obtained from only one adult female donor (YT) throughout the study. Nine volumes (either 4.5 or 9.0 ml) of peripheral blood were drawn into a syringe containing one volume (0.5 or 1.0 ml) sodium citrate 3.8%. Platelet-rich plasma (PRP) was obtained by centrifugation of the citrated blood at 170 *g* for 10 min at 20°C. Aggregation of a mixture consisting of 200 µl of PRP and 20 µl of a test or a reference specimen was determined at 37°C with a two-channel aggregometer (Model PAT-2A, Nikko Bioscience Ltd, Tokyo) attached to a tracer (Model T-228, Nikko Bioscience Ltd). Adenosine 5'-diphosphate (ADP, Sigma) in saline 0.85% (10⁻³ M) served as the reference aggregation agent. The aggregation activity was measured 7 min after the addition of a test or reference specimen to the PRP. The potency of ADP in terms of increased light transmission of the reaction mixture was taken as 100%, and that of each test specimen was expressed as a percentage of that of ADP. Values > 10 < 50% were scored +, and those > 50%, ++; values < 10% were discarded.

Haemolytic activity

A mixture of 50- μ l volumes of a test specimen and a sheep red blood cell (SRBC) 2% suspension in 0.01 M PBS, pH 7.2, was examined for haemolysis on a microtitration plate (Nunc, Roskilde, Denmark) after incubation overnight at room temperature. Complete and partial haemolysis were scored as ++ and +, respectively. Marginal haemolysis was designated $\pm$.

Detection of lipoteichoic acids

An antiserum to lipoteichoic acid (LTA) was prepared by immunising albino female rabbits (Nippon Biosupply Centre) weighing *c.* 3.0 kg with lyophilised *Enterococcus hirae* (*E. faecalis*, ATCC9790) whole cells (a gift from Dr K. Kato, School of Dentistry, Okayama University, Okayama, Japan). This was done as follows. Rabbits received foot-pad injections four times at 10-day intervals with 10 mg of whole cells, of *E. hirae* suspended in 0.01 M PBS, pH 7.2, and emulsified in Freund's complete adjuvant (Difco). An anti-LTA serum was obtained 10 days after the fourth injection, and semi-quantified at the anti-LTA antibody level by a passive haemagglutination test (PHA).¹³ Two-fold dilutions (25 μ l) of the anti-LTA serum were dispensed into the wells of U-bottomed, 96-well microtitration plates (Nunc), followed by 25 μ l of an SRBC suspension (2% in PBS, pH 7.2) sensitised with *E. faecalis* LTA (Sigma). The haemagglutinating titre of one representative rabbit immune serum was 1 in 5120.

A mixture of equal amounts of the test specimen and a 1 in 320 dilution of the above rabbit antiserum was incubated for 2 h at 37°C and overnight at 4°C. A supernate of the mixture was incubated with an equal volume of a 2% suspension of SRBC sensitised with LTA for 3 h at room temperature, to determine the haemagglutination inhibition effects of the test specimens. The minimum inhibitory doses of reference strain *E. hirae* LTA-2 (diacyl-[phosphatidylkojibiosyl] glycerol) and a polyglycerophosphate¹⁴ (a gift from Dr K. Kato) were 20 and 500 μ g/ml, respectively. The LTA content of the test specimens, with and without LTA, was graded semi-quantitatively into complete (++), partial (+) and no inhibition (-).

Glucan production

Test strains were cultured in Brain-Heart Infusion Broth (Nissui Corp.) at 37°C for 18 h, and one drop of the growth was inoculated into 4 ml of Tryptic Soy Broth (Difco) supplemented with sucrose 5%.¹⁵ After incubation for 48 h at 37°C, the supernate was separated by centrifugation at 1500 *g* for 20 min and mixed with an equal volume of absolute ethanol. The resulting turbidity and precipitation due to soluble glucan in the culture supernate were compared with the non-inoculated control medium which was similarly treated. Glucan production was expressed as either positive (+) or negative (-).

Antibiotic susceptibility of test and reference viridans streptococci

Disks (Tridisk[®], Eiken Kagaku Co. Ltd, Tokyo) containing various antimicrobial agents were applied to the surface of Mueller-Hinton S Disk Agar (Eiken Kagaku Co. Ltd) plates which were inoculated with 0.1 ml of a culture containing *c.* 10⁸ cfu/ml from KD patients or control subjects and of reference type strains. After incubation for 20 h at 37°C, the plates were examined for inhibition of growth around the disks, according to the manufacturer's instructions. When the inhibitory zone was > 1 mm, the streptococci were taken to be sensitive. Antibiotics tested were: cephalixin (5, 10 and 30 μ g/disk), cefaclor (5, 10 and 30 μ g), ampicillin (2, 5 and 20 μ g); amoxicillin (2, 5 and 20 μ g); minocycline (2, 5 and 30 μ g); and erythromycin (0.5, 2 and 10 μ g).

Results

Bacterial isolates from KD patients and control children

Table I shows that gram-positive, catalase-negative cocci (tentatively classified as *Streptococcus* spp.,

Table III. Re-evaluation of *S. sanguis* II and *S. mitis* identified with API Strep 20 kit by relative DNA homology values with DNA of type strains*

API Strep 20 kit		DNA-DNA hybridisation†	
Strain and source	Number of strains (%)	Strain and source	Number of strains (%)
KD:			
<i>S. sanguis</i> II‡	18 (100)	<i>S. oralis</i>	8 (44.4)
		<i>S. mitis</i>	3 (37.5)
		<i>S. parasanguis</i>	1 (5.5)
		<i>S. sanguis</i>	1 (5.5)
		Unknown strains§	5 (27.7)
Control:			
<i>S. sanguis</i> II	9 (100)	<i>S. oralis</i>	4 (44.4)
		<i>S. mitis</i>	1 (11.1)
		<i>S. sanguis</i>	1 (11.1)
		Unknown strains	3 (33.3)
KD:			
<i>S. mitis</i>	34 (100)	<i>S. oralis</i>	5 (14.7)
		<i>S. mitis</i>	9 (26.4)
		<i>S. parasanguis</i>	3 (8.8)
		<i>S. sanguis</i>	1 (2.9)
		Unknown strains	16 (47.0)
Control			
<i>S. mitis</i>	7 (100)	<i>S. oralis</i>	0 (0)
		<i>S. mitis</i>	4 (57.1)
		Unknown strains	3 (42.0)

* *S. dysgalactiae* NCFB 2023, *S. suis* NTCC 10232, *S. pneumoniae* NCTC 7465, *S. oralis* NCTC 11427, *S. mitis* NCTC 12261 (neotype), *S. sanguis* ATCC 10556, *S. parasanguis* ATCC 15912, *S. gordonii* NCTC 7865, *S. salivarius* ATCC 7072 and *S. intermedius* ATCC 27335.

† See *Materials and methods*.

‡ Strains of *S. sanguis* II or *S. pneumoniae* were included in *S. sanguis* II.

§ Strains not identified by homology with DNA of the above type strains.

Table IV. The biological activities of extracellular products of viridans streptococci (52 KD, 16 control, three reference and four type strains)

Species identified by DNA-DNA hybridisation	Strain no.		Increased capillary permeability* (30 min; rabbit)	Redness and swelling (24 h; rabbit)	Platelet aggregation† (%) (human)	Haemolysis (sheep)	Production of LTA	Production of soluble glucan	Species (API 20 strep)
KD:									
<i>S. oralis</i>	No-2	(T)‡	+	+	—	—	++	+	<i>S. sanguis</i> II
	No-5	(P)§	+	+	—	—	—	+	<i>S. sanguis</i> II
	No-7	(P)	+	+	—	—	—	+	<i>S. sanguis</i> II
	No-9	(T)	+	+	—	—	—	+	<i>S. sanguis</i> II
	No-12	(T)	—	—	—	—	—	+	<i>S. sanguis</i> II
	No-14	(T)	—	—	—	—	—	+	<i>S. sanguis</i> II
	No-18	(T)	+	+	—	±	—	+	<i>S. sanguis</i> II
	No-20	(P)	—	—	—	—	—	—	<i>S. Sanguis</i> II
	No-69	(T)	+	+	—	—	—	+	<i>S. mitis</i>
	No-70	(T)	+	+	—	—	—	+	<i>S. mitis</i>
	No-71	(P)	+	+	—	—	—	—	<i>S. mitis</i>
	No-86	(P)	++	++	—	—	++	—	<i>S. mitis</i>
	No-100	(T)	+	+	—	—	—	—	<i>S. mitis</i>
Subtotal	13		10	10	0	0	2	9	
<i>S. mitis</i>	Nm-1	(P)	+	+	++	++	nd	—	<i>S. sanguis</i> II
	Nm-3	(T)	+	+	—	±	++	—	<i>S. sanguis</i> II
	Nm-6	(T)	—	—	—	—	—	—	<i>S. sanguis</i> II
	Nm-65	(T)	+	+	++	++	nd	—	<i>S. mitis</i>
	Nm-66	(P)	+	+	—	—	—	—	<i>S. mitis</i>
	Nm-76	(P)	+	+	++	++	nd	—	<i>S. mitis</i>
	Nm-81	(P)	++	+	—	±	—	—	<i>S. mitis</i>
	Nm-82	(T)	+	+	—	±	++	—	<i>S. mitis</i>
	Nm-85	(T)	+	++	—	—	—	—	<i>S. mitis</i>
	Nm-88	(P)	+	+	—	—	++	—	<i>S. mitis</i>
	Nm-98	(T)	+	+	—	—	+	—	<i>S. mitis</i>
	Nm-99	(P)	+	++	—	—	—	—	<i>S. mitis</i>
Subtotal	12		11	11	3 (25)	3	4	0	
KD:									
<i>S. sanguis</i>	Ns-19	(T)	—	—	—	—	—	—	<i>S. sanguis</i> II
	Ns-96	(T)	+	+	—	—	—	+	<i>S. mitis</i>
Subtotal	2		1	1	0	0	0	1	
<i>S. parasanguis</i>	Np-10	(P)	+	+	—	—	—	—	<i>S. sanguis</i> II
	Np-72	(P)	+	+	—	—	++	—	<i>S. mitis</i>
	Np-75	(P)	+	+	—	—	—	—	<i>S. mitis</i>
	Np-79	(P)	+	++	—	—	—	—	<i>S. mitis</i>
Subtotal	4		4	4	0	0	1	0	
Unknown strains									
	Nu-4	(T)	—	—	—	—	+	—	<i>S. sanguis</i> II
	Nu-8	(T)	+	+	—	—	+	—	<i>S. sanguis</i> II
	Nu-15	(T)	+	+	—	—	+	—	<i>S. sanguis</i> II
	Nu-16	(T)	+	+++	++	++	nd	—	<i>S. sanguis</i> II
	Nu-17	(T)	+	+	—	—	—	—	<i>S. sanguis</i> II
	Nu-67	(P)	+	++	++	++	nd	—	<i>S. mitis</i>
	Nu-68	(T)	+	+	—	—	—	+	<i>S. mitis</i>
	Nu-73	(P)	+	+	++	++	nd	—	<i>S. mitis</i>
	Nu-78	(P)	+	+	—	++	nd	—	<i>S. mitis</i>
	Nu-80	(T)	+	+	++	++	nd	—	<i>S. mitis</i>
	Nu-84	(T)	+	+	—	±	+	—	<i>S. mitis</i>
	Nu-87	(T)	+	+	—	—	++	—	<i>S. mitis</i>
	Nu-89	(T)	+	+	—	++	nd	—	<i>S. mitis</i>
	Nu-90	(P)	+	+	—	—	++	—	<i>S. mitis</i>
	Nu-91	(P)	+	+	—	—	++	—	<i>S. mitis</i>
	Nu-92	(P)	+	+	—	—	—	—	<i>S. mitis</i>
	Nu-93	(T)	+	+	—	±	+	—	<i>S. mitis</i>
	Nu-94	(P)	+	++	—	—	—	—	<i>S. mitis</i>
	Nu-95	(P)	+	+	—	++	nd	—	<i>S. mitis</i>
	Nu-97	(P)	+	+	—	—	—	—	<i>S. mitis</i>
	Nu-101	(T)	+	+	—	—	—	—	<i>S. mitis</i>
Subtotal	21		20	20	4 (19)	7	8	1	
Total (%)	52 (100)		46 (88.5)	46 (88.5)	7 (13.5)	10 (19.2)	15/42 (35.7)	11 (21.2)	

Table IV continued

Species identified by DNA-DNA hybridisation	Strain no.	Increased capillary permeability* (30 min; rabbit)	Redness and swelling (24 h; rabbit)	Platelet aggregation† (%) (human)	Haemolysis (sheep)	Production of LTA	Production of soluble glucan	Species (API 20 strep)
Control:								
<i>S. oralis</i>	No-21 (T)	+	+	—	—	++	—	<i>S. sanguis</i> II
	No-22 (P)	+	+	—	—	++	—	<i>S. sanguis</i> II
	No-23 (T)	+	+	—	—	—	—	<i>S. sanguis</i> II
	No-27 (T)	+	+	—	—	—	+	<i>S. sanguis</i> II
Subtotal	4	4	4	0	0	2	1	
<i>S. mitis</i>	Nm-28 (P)	+	+	—	—	++	—	<i>S. sanguis</i> II
	Nm-103 (P)	+	+	+	—	—	—	<i>S. mitis</i>
	Nm-105 (T)	+	++	—	—	—	—	<i>S. mitis</i>
	Nm-108 (T)	++	+++	+	++	nd	—	<i>S. mitis</i>
	Nm-109 (T)	+	++	—	—	—	—	<i>S. mitis</i>
Subtotal	5	5	5	2 (40)	1	1	0	
<i>S. sanguis</i>	Ns-25 (T)	+	+	—	—	—	—	<i>S. sanguis</i> II
Subtotal	1	1	1	0	0	0	0	
Unknown strains								
	Nu-24 (T)	++	+++	++	++	nd	—	<i>S. sanguis</i> II
	Nu-26 (P)	+	+	—	++	nd	—	<i>S. sanguis</i> II
	Nu-29 (T)	+	+	—	—	—	+	<i>S. sanguis</i> II
	Nu-104 (T)	+	++	—	—	—	—	<i>S. mitis</i>
	Nu-107 (T)	+	++	—	—	+	—	<i>S. mitis</i>
	Nu-110 (P)	+	++	++	++	nd	—	<i>S. mitis</i>
Subtotal	6	6	6	2 (33)	3	1	1	
Total	16 (100)	16 (100)	16 (100)	4 (25.0)	4 (25.0)	4/12 (33.6)	2 (12.5)	
Reference strains								
<i>S. parasanguis</i> (<i>S. mitis</i> ATCC 903)		+	+	—	—	+	—	
<i>S. oralis</i> (<i>S. mitis</i> ATCC 15911)		+	+	—	—	+	—	
<i>S. oralis</i> (<i>S. oralis</i> ATCC9811)		+	+	—	—	—	+	
Type strains								
<i>S. oralis</i> (NCTC 11427)		+	+	—	—	—	—	
<i>S. mitis</i> (NCTC 12261, neotype)		+	+	—	—	+	—	
<i>S. sanguis</i> (ATCC 10556)		+	—	—	—	—	+	
<i>S. gordonii</i> (NCTC 7865)		+	—	—	—	—	+	
Total	7 (100)	7 (100)	4 (57)	0	0	3 (43)	3 (43)	

nd, haemolysis of LTA-sensitised sheep erythrocytes could not be used to determine LTA production

* Leakage of Evans blue.

† Scored by OD₆₈₀ reduction (%): — < 10%, + 10–50%, ++ > 50%.

‡ Isolated from tooth surface.

§ Isolated from pharynx.

Enterococcus spp. and *Lactococcus* spp.) accounted for 61 and 55% of all bacterial strains (256 and 85 isolates) from the tooth surface and throat swabs of 89 KD patients and 23 controls.

Identification of gram-positive, catalase-negative cocci

Ninety-eight strains of gram-positive, catalase-negative cocci isolated from 22 KD patients and 27 strains from 11 control children were identified according to their biochemical activities with an API Strep 20 kit (table II); 18% of strains from KD patients and 33% of those from control individuals were assigned to *S. sanguis* II (including *S. sanguis* II or *S. pneumoniae* group) and 35 and 26% of the isolates from KD and control children were identified as *S. mitis*, respectively. (Hereafter these streptococci will be collectively referred to as viridans streptococci.) The remainder belonged to miscellaneous species (table II).

Identification of viridans streptococci by DNA-DNA hybridisation

Strains biochemically classified as *S. sanguis* II and *S. mitis* were further identified by fluorometric DNA-DNA hybridisation (table III). Nearly half (12 of 27 strains) of the *S. sanguis* II group, irrespective of source, were identified as *S. oralis*, and then *S. mitis*, *S. sanguis* and *S. parasanguis*, in that order. One-third of biochemical *S. sanguis* II strains were unidentifiable. The predominant species (13 of 41 strains) in the biochemical *S. mitis* group was identified genetically as *S. mitis*, followed by *S. oralis*, but nearly half the biochemical *S. mitis* strains could not be identified genetically. Thus, 13 (25%) and 12 (23%) strains of 52 isolates from KD patients were identified as *S. oralis* and *S. mitis*, respectively. Among those from the control children, four (25%) and five (31%) of 16 were *S. oralis* and *S. mitis*, respectively. Viridans

Fig. 1. Histopathological changes in rabbit skin 30 min after intradermal injection of a culture supernate concentrate of *S. mitis* strain Nm-65. H and E stain; $\times 280$.

Fig. 2. Histopathological changes in rabbit skin 24 h after intradermal injection of a culture supernate concentrate of *S. oralis* strain No-23. H and E stain; $\times 280$.

streptococci used as reference strains, namely *S. mitis* ATCC903, *S. mitis* ATCC6249 and *S. mitis* ATCC15911 were identified as *S. parasanguis*, *S. oralis* and *S. parasanguis*, respectively, and both *S. oralis* ATCC9811 and *S. parasanguis* ATCC15909 were identified as *S. oralis*.

Biological activities of extracellular products of isolates of viridans streptococci from KD patients and control children

Induction of increased capillary permeability and vasculitis in rabbit skin. As shown in table IV, culture supernate concentrates of 88% of KD strains caused increased capillary permeability without bleeding for between 5 min and 3 h after intradermal injection. The culture supernate concentrates of all 16 strains from control subjects and those of seven reference strains also caused an increase in capillary permeability. The enhanced capillary permeability in the rabbit skin by test and reference viridans streptococci was followed by redness, swelling and sometimes bleeding after a few hours and up to 48 h; this lasted for 4 days in some animals. The two type strains, *S. sanguis* ATCC10556

Fig. 3. Aggregation of human blood platelets by a culture supernate concentrate of viridans streptococci, fresh clinical isolates and type strains. Aggregation of a reaction mixture consisting of 200 μ l of PRP and 20 μ l (containing 20 μ g of protein) of a test specimen was determined with an aggregometer; 10^{-8} M ADP was the reference aggregation agent.

and *S. gordonii* NCTC7865, did not produce these signs.

Histological examination of the test skin sites showing the early reaction revealed increased dilatation of the veins and capillaries in the dermis, but no infiltration of inflammatory cells (fig. 1). The lesions in the later stages of the reaction were characterised by extravasation of many neutrophils along dilated veins, where the endothelial cells appeared to be hypertrophic. Perivascular fibroblasts increased in number and their cytoplasm was hypertrophic (fig. 2). Massive infiltration of neutrophils occasionally resulted in foci of abscess-like changes (not shown).

Human blood platelet aggregation and SRBC haemolysis. Table IV shows that (25%) three of 12 *S. mitis*

Table V. Comparison of drug susceptibility among viridans group streptococci isolated from KD patients and control children with those of reference and type strains

Strains	Number tested	Number of strains resistant to																	
		CEX*			CCL			ABPC			AMPC			MINO			EM		
		30	10	5†	30	10	5	20	5	2	20	5	2	30	5	2	10	2	0.5
KD																			
<i>S. oralis</i>	13	7	7	12	5	8	9	0	0	5	0	0	1	0	5	8	5	6	9
<i>S. mitis</i>	12	6	6	9	3	6	6	0	0	5	0	0	2	0	1	4	1	2	6
<i>S. sanguis</i>	2	0	2	2	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
<i>S. parasanguis</i>	4	2	2	2	0	2	2	0	0	0	0	0	1	0	1	2	0	1	2
Unknown strains	21	10	13	15	6	9	10	0	1	7	0	0	4	0	2	11	8	9	12
Control:																			
<i>S. oralis</i>	4	2	2	2	2	2	2	0	0	2	0	0	0	0	2	2	2	2	2
<i>S. mitis</i>	5	3	3	3	2	3	3	0	1	2	0	0	2	0	1	1	4	4	4
<i>S. sanguis</i>	1	1	1	1	0	0	1	0	0	0	0	0	0	0	1	1	1	1	1
Unknown strains	6	3	4	5	2	3	3	0	1	2	0	1	2	0	1	4	2	2	3
Reference strains																			
<i>S. parasanguis</i> (<i>S. mitis</i> ATCC903)		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>S. oralis</i> (<i>S. mitis</i> ATCC15911)		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>S. oralis</i> (<i>S. oralis</i> ATCC9811)		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Type strains																			
<i>S. oralis</i> NCTC 11427		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>S. mitis</i> NCTC 12261		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>S. sanguis</i> ATCC 10556		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>S. gordonii</i> NCTC 7865		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* CEX, cephalixin; CCL, cefaclor; ABPC, ampicillin; AMPC, amoxycillin; MINO, minocycline; EM, erythromycin.

† Drug content (μ g disk).

strains from KD and (40%) two of five *S. mitis* strains from control aggregated YT blood platelets, whereas none of 17 *S. oralis* strains from KD and control subjects caused significant aggregation (fig. 3). Culture supernate concentrates of seven test strains of *S. sanguis* and *S. parasanguis* were inactive in this assay, but six (22%) of 27 strains whose species remained unknown, were powerfully active. Seven reference strains did not form extracellular products capable of causing platelet aggregation.

Table IV demonstrates a significant parallel between the ability of culture supernate concentrates to aggregate human blood platelets and to haemolyse SRBC although six of 27 concentrates of unidentified viridans streptococci strongly haemolysed SRBC but did not cause blood platelet aggregation.

Detection of LTA in the test specimens. Since LTA was detected by the PHA inhibition test with LTA-sensitized SRBC, culture supernate concentrates showing haemolytic activity against SRBC could not be assayed for the presence of LTA. Within this limitation, LTA was detected in the culture supernate concentrates of four *S. oralis*, five *S. mitis* and nine unidentified strains among a total of 42 KD and 12 control strains that showed no haemolysis, indicating that 35% of viridans streptococci tested produced extracellular LTA. LTA was detected in the culture supernates of (43%) three of seven reference and type strains (table IV).

Glucan production. Production of extracellular, sol-

uble glucan in the medium supplemented with sucrose was almost confined to *S. oralis* strains, (69%) nine of 13 KD strains and (25%) one of four control strains. Only exceptional strains of viridans streptococci other than *S. oralis* produced extracellular glucan. Three of seven reference or type strains gave positive results in this test (table IV).

Drug susceptibility of test viridans streptococci and type or reference viridans strains

All 68 fresh isolates of viridans streptococci, irrespective of species or source, showed greater resistance to six test antimicrobial drugs compared with serially subcultured reference strains. This was particularly true with cephalosporins such as cephalixin and cefaclor with erythromycin. Acquisition of resistance to other drugs, particularly the broad-spectrum semi-synthetic β -lactam agents such as ampicillin and amoxycillin, was minimal (table V).

Discussion

This study showed that a major group of bacteria isolated from the oral cavity of KD patients and non-KD control children included *S. sanguis* II (*S. sanguis* II or *S. pneumoniae*) and *S. mitis*, identified by biochemical tests, although the taxonomy of these viridans streptococci has not yet been finally estab-

lished.¹⁶⁻¹⁸ Initially, we tried to identify the viridans streptococci by fluorometric DNA-DNA hybridisation,⁷ with 10 type strains as reference strains. When the homology value between the test and the type strain was > 70%, a test strain was identified as being of the same species as the respective type strain. The isolates homologous with several type strains were unidentified. No KD-specific bacteria were identified by DNA hybridisation studies. The novel finding in the present study was that during growth many viridans streptococci released biologically active, extracellular products that were responsible for increased capillary permeability, erythrogenic activity, human blood platelet aggregation and SRBC haemolysis, as well as production of LTA and glucan. However, there were no significant differences in the isolation rates of the viridans streptococci producing biologically active extracellular products between KD patients, age-matched non-KD patients and healthy children.

The ability to aggregate human blood platelets was much more restricted to the culture supernate concentrates of a limited number of viridans streptococci than other effects such as changes in rabbit skin. However, there were no significant differences between KD and control isolates in the isolation rates of viridans streptococci whose culture supernates caused blood platelet aggregation. A preliminary study demonstrated that there are significant individual differences in the susceptibility of platelets to aggregation factor in the culture supernate amongst members of one *S. mitis* strain (strain Nm-65).

Human platelet aggregation and SRBC haemolytic activity correlated well. Nearly all the culture supernates that caused platelet aggregation haemolysed SRBC, although the reverse was not necessarily true. Nevertheless, our attempt to purify a platelet aggregation principle revealed that a fraction causing strong platelet aggregation activity was distinct from a concentrate that haemolysed SRBC, by DEAE column chromatography (unpublished data).

Studies on biologically active, extracellular products of viridans streptococci are limited, but there are several studies on viridans streptococci associated with subacute bacterial endocarditis (SBE). Proteases have been identified in culture supernate of *S. mitis*, *S. sanguis*, *S. mutans*, *S. salivarius* and *S. MG intermedius* isolated from confirmed cases of SBE.^{19, 20} Extracellular neuraminidase is produced by a *S. sanguis* strain associated with SBE,²¹ and an immunoglobulin A1 degrading enzyme has been reported as an extracellular product of oral streptococci such as *S. sanguis*, *S. mitis* and *S. oralis*.²² The interaction between human platelets and *S. sanguis* whole cells was studied by Herzberg *et al.*,²³⁻²⁵ to elucidate the pathological mechanisms in SBE. They observed that the interaction was mediated by two or more antigens in the fibrillar surface appendages and showed that one antigen designated as the class I component bound with the platelets, that the other antigen (class II component) triggered platelet aggregation and that

the class II component shared a cross-reactive immunodeterminant with collagen.^{26, 27} Consequently, the extracellular platelet aggregation factor reported here may be of bacterial cell-surface origin. However, a recent study showed that YT platelets thoroughly washed with ACD (citric acid-0.8% w/v sodium citrate-2.2% w/v glucose 2.2% w/v), to remove blood plasma components, and resuspended in ACD-0.01 M PBS (pH 8.5) were aggregated by a test extracellular aggregation factor (unpublished data). As Sullam *et al.*²⁸ reported that plasma components were needed for platelet aggregation by whole *S. sanguis* and *S. salivarius* cells, the mechanism of platelet aggregation by the culture supernate concentrates described here may differ from that of whole bacterial cells.

Because of the reported immunobiological activity of *E. faecalis* (*E. hirae*) LTA,¹⁴ the distribution of extracellular LTA in viridans streptococci was examined. Extracellular LTA may be LTA which was originally associated with the cell walls before being released extracellularly in a manner similar to the extracellular glycoprotein antigen of some *S. sanguis* species.²⁹

Regarding the correlation between the human platelet aggregation and the presence of extracellular LTA, the fact that the LTA-containing culture supernates of a few test strains did not show any aggregation excludes the possibility that the platelet aggregation is due to extracellular LTA. Indeed, a report that the LTA of group A streptococci bound to human blood platelets but did not cause platelet aggregation,³⁰ suggests the opposite. The extracellular LTA of some viridans streptococci bound to the platelets and may have interfered with platelet aggregation.

A recent study by Furusho *et al.*³¹ showed that an insoluble glucan produced by *S. sanguis*, particularly in the presence of detergent, stimulated interleukin-1 α production in human monocyte cultures, and prompted us to investigate the production of extracellular (soluble) glucan by viridans streptococci. Most genetically identified *S. oralis* but none of the *S. mitis* strains produced glucan extracellularly. No correlation was found between glucan production, platelet aggregation, haemolytic activity and the existence of LTA.

Viridans streptococci freshly isolated from KD patients and control children in general showed greater resistance to the antibiotics commonly prescribed for paediatric patients, especially to the cephalosporins and erythromycin, than the type culture strains.

We also demonstrated that erythrogenic culture supernates of two representative strains of *S. oralis* and *S. mitis* were capable of inducing serum tumour necrosis factor- α (TNF- α), interleukin-6 (IL-6) and gamma interferon (INF- γ) by administration into appropriately primed mice (Takada *et al.*, unpublished observations). The concentrate also induced TNF- α , IL-6 and a thymocyte activating factor (TAF, essentially IL-1) in murine peritoneal macrophages and

human monocyte cultures. Partially purified "toxin" fractions (F-1 and F-2) were obtained from the culture supernate of one *S. mitis* strain. The erythrogenic F-1 fraction induced high levels of serum TNF- α , IL-6 and IFN- γ in mice, and stimulated human peripheral whole blood to produce the above cytokines. This fraction also induced TAF and IL-6 activity in human umbilical vascular endothelial cells (HUVEC) and gingival fibroblast cultures. On the other hand fraction F-2, which increases capillary permeability but is not erythrogenic to rabbit skin, functions as a class II histocompatibility antigen-restricted T-cell stimulator to induce interleukin-2 (IL-2), as a super-antigen on human lymphoid cells (Fujikawa *et al.*, unpublished observations). Furukawa *et al.* further showed that fraction F-1, but not F-2, caused increased expression of intercellular adhesion molecule-1 and endothelium-lymphocyte adhesion molecule-1 on HUVEC,

indicating vascular inflammation (unpublished observations).

These findings lead us to speculate that some oral viridans streptococci can reproduce many of the pathological and clinical features and laboratory findings of KD through production of immunobiologically active extracellular products. Nevertheless, the fact that there were no differences in the recovery rates of these viridans streptococci indicates the involvement of secondary factors in the production of clinical signs and symptoms of KD, if viridans streptococci are designated as the primary causative agents. Studies on the elucidation of possible secondary factors are currently in progress.

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