Induction of inflammatory cytokines by a soluble moiety prepared from an enzyme lysate of *Actinomyces viscosus* cell walls

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Summary. A carbohydrate-rich and immunobiologically active component, M1Av, was prepared from an M-1 endo-*N*-acetylmuramidase digest of cell walls of *Actinomyces viscosus* ATCC 19246 by CM Sephadex C-25 and Sephadex G-100 chromatography. M1Av stimulated thioglycolate-induced peritoneal macrophages from C3H/HeN and C3H/HeJ mice to release cell-free tumour necrosis factor (TNF) and interleukin (IL)-6, and a cell-associated thymocyte activating factor, probably IL-1. An intravenous (i.v.) injection of M1Av induced increased levels of TNF and IL-6 in the serum of C3H/HeN mice that had been primed with 100 µg of muramyl dipeptide (MDP) i.v. However, M1Av did not induce TNF release in C3H/HeJ mice similarly primed with MDP. Simultaneous administration of M1Av (100 µg, i.v.) and galactosamine (18 mg, intraperitoneally) killed C3H/HeN, but not C3H/HeJ mice. M1Av was shown to be practically free of endotoxin by the *Limulus* test. These findings indicate that the solubilised *A. viscosus* cell-wall carbohydrate moiety induced inflammatory cytokines both *in vitro* and *in vivo*.

Introduction

Actinomyces species are filamentous, gram-positive bacteria, and *Actinomyces viscosus* and *A. naeslundii* are important members of human dental plaque flora that have been implicated in the development of gingivitis and the early phase of periodontitis.^{1–3} Increased levels of serum IgG or IgM specific for *A. viscosus* have been found in human subjects and rats who harboured *A. viscosus* in their oral cavity.^{4,5} Sonic lysates of *A. viscosus* whole cells exhibit mitogenic and adjuvant activities in lymphoid cells and polyclonal B cell activation.^{6–8} Furthermore, these cellular fractions from *A. viscosus* showed cytostatic or cytotoxic effects on lymphocytes and fibroblasts. The specific factors responsible for these activities remain to be elucidated.^{9,10} A water-soluble, carbohydrate-rich moiety was isolated from digests of purified cell walls of *A. viscosus* ATCC 19246 that had been treated with a cell-wall lytic enzyme, M-1 endo-*N*-acetylmuramidase (M1 enzyme); it was designated M1Av.¹¹ This was serologically active, formed a precipitin band with antiserum to *A. viscosus* whole cells and exhibited B cell mitogenicity and polyclonal B cell activation in murine

lymphoid cell cultures.¹² Bacterial components generally activate macrophages to induce cytokines such as interleukin (IL)-1, IL-6, IL-8 and tumour necrosis factor (TNF), that are involved in inflammatory and immunological reactions in the host. These cytokines are produced by various host cells, and diffuse locally or are transported systemically to regulate the functions of other host cells, including the central nervous and endocrine systems. This cytokine network is activated during inflammation, injury, healing and microbial infections.¹³

Therefore, we examined whether M1Av induces inflammatory cytokines in murine macrophage cultures and in mice treated with muramyl dipeptide (MDP).

Materials and methods

Preparation of M1Av

M1Av was prepared from M1 endo-*N*-acetylmuramidase digests of cell walls of *A. viscosus* ATCC 19246 as described previously.¹¹ Briefly, lyophilised cells of *A. viscosus* were disrupted mechanically, treated with pronase and trypsin, followed by differential centrifugation to isolate purified cell walls. The cell walls were degraded with M-1 enzyme and the lysate was chromatographed on a CM Sephadex C-25 column (5.3 × 50 cm) to remove the M1 enzyme. The non-adsorbed material was applied to a Sephadex G-100 column (5.3 × 100 cm). The major peak fraction

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that reacted with rabbit antiserum to *A. viscosus* whole cells was collected, dialysed and lyophilised. This was designated M1Av and its mol. wt was calculated to be 35000–40000.¹² Chemical analysis showed that M1Av consisted of carbohydrate 75%—the major components of which were 6-deoxytalose, rhamnose and galactose—and protein 23%. The endotoxin content in M1Av was c. 14.6 ng/mg based upon the results of the Endospecy Test (Seikagaku Kogyo Co., Tokyo, Japan), which is a colorimetric *Limulus* test.¹⁴

Biochemical standards

A purified lipopolysaccharide (LPS) preparation from *Salmonella abortus-equi* (Novo-Pyrexal)¹⁵ was kindly supplied by C. Galanos (Max-Planck Institut für Immunbiologie, Freiburg, Germany) and was used as a reference standard in most of the experiments. In the Endospecy and lethal toxicity test, LPS of *Escherichia coli* O111:B4, prepared by hot phenol-water extraction (Difco) was used as the reference LPS. MDP was a gift from Daiichi Pharmaceutical Co., Tokyo. Recombinant human IL-6 (rHuIL-6; 5×10^6 U/mg of protein) was generously supplied by Drs T. Kishimoto and T. Hirano, Osaka University Medical School, Suita-Osaka, Japan.

Mice

Both male and female C3H/HeN and C3H/HeJ mice (6–12 weeks old; CLEA Japan Co., Osaka, Japan) were used for in-vivo assays and macrophage preparation. C3H/HeJ mice were also bred and maintained in the animal facility of Osaka University Faculty of Dentistry. Thymocyte activating factor (TAF) was assayed in thymocytes from 3–5-week-old C3H/HeJ mice.

Macrophage culture

Peritoneal exudate macrophages were collected from mice that had received 1.5 ml of Thioglycolate Medium (Nissui Seiyaku Co., Tokyo) intraperitoneally (i.p.); 100- μ l volumes of the cell suspensions (10^6 /ml) in RPMI 1640 medium were distributed into flat-bottomed microculture plates (Corning 25860; Corning Glass Works, Corning, NY, USA). After incubation in an atmosphere of CO₂ 5% in air at 37°C for 2 h, non-adherent cells were removed from the plate. Adherent cells were cultured with test specimens in triplicate in 100 μ l of RPMI 1640 for 24 h. The culture supernates were pooled for cell-free TNF, IL-6 and TAF assays. Adherent cells were washed, and after the addition of 100 μ l of RPMI 1640, were then fractured by freezing at -80°C and thawing at 25°C three times. Cell-associated TAF activity was measured in the fractured cell suspensions.

Cytokine assays

TNF. For TNF assay, 100 μ l of a suspension of L-M cells (ATCC CCL-1.2; American Type Culture Collection, Rockville, MD, USA) 5×10^5 cells/ml in

RPMI 1640 medium with fetal calf serum (FCS; Gibco Laboratories, Grand Island, NY, USA) 10% was placed in each well of a 96-well plate and cultured for 24 h to establish a monolayer. In parallel microculture plates, serial dilutions of test specimens were made in RPMI 1640 media containing actinomycin-D (Sigma) 10 μ g/ml and incubated for 24 h; 25 μ l of this solution were then transferred to plates with the L-M cells giving a final actinomycin-D concentration of 2 μ g/ml. After culture for a further 24 h, viable cells adhering to the cultures plates were fixed with formaldehyde 2% and stained with crystal violet 0.2% solution at 25°C for 20 min. The intensity of the stain was measured with a microplate reader (Titertek Multiskan MC; Flow Laboratories, Inc., McLean, VA) at 595 nm. One unit of activity was defined as the dilution required to produce a 50% decrease in absorbance relative to control cells exposed to actinomycin-D alone.

IL-6 activity was determined by measuring in-vitro growth of an IL-6-dependent cell line MH60. BSF2.¹⁶ Briefly, cell suspensions (1×10^4 /175 μ l) were cultured with 25 μ l of serially diluted test specimens in RPMI 1640 with FCS 10% for 48 h, then pulsed with 0.5 μ Ci (18.5 KBq) of [³H]thymidine (ICN 24070; ICN Radiochemicals, Irvine, CA, USA) during the last 6 h of incubation. Radioactivity incorporated by the cells was measured in a scintillation counter. IL-6 activity was standardised on the basis of a dose response curve obtained with rHuIL-6.

TAF activity was determined by a conventional co-mitogenic assay with thymocytes from 3–5-week-old C3H/HeJ mice. Thymocytes (1.5×10^6 /175 μ l of RPMI 1640 with FCS 10%) were cultured with 25 μ l of test specimens in the presence of a submitogenic dose (final concentration, 1 μ g/ml) of phytohaemagglutinin (PHA: HA16, Wellcome Diagnostics, Dartford) for 72 h. During the last 6 h of culture, the cells were pulsed with 0.5 μ Ci (18.5 KBq) of [³H]thymidine. The incorporated radioactivity was quantified in a scintillation counter. The results were expressed as the stimulation index (SI)—the ratio of the radioactivity in the test culture to that of the control culture (PHA alone).

All biological assays were performed three times in triplicate.

Assays in vivo

Groups of three C3H/HeN or C3H/HeJ mice (6–12 weeks old, male and female) were primed by an intravenous (i.v.) injection of 100 μ g of MDP 4 h before i.v. injection of M1Av 100 μ g or LPS from *S. abortus-equi* (Novo-Pyrexal) 0.1 μ g. Non-primed mice were also given M1Av 100 μ g or LPS 0.1 μ g. Blood was collected 90 min after injection of the test material and serum TNF and IL-6 levels were measured. The lethal toxicity for mice was determined according to the method of Galanos *et al.*¹⁷ Groups of four to six C3H/HeN or C3H/HeJ mice (8–12 weeks old, male and female) were sensitised by an i.p.

Fig. 1. TAF induction by M1Av in C3H/HeN mouse macrophage cultures. Peritoneal macrophages from C3H/HeN mice were incubated with M1Av or *S. abortus-equi* LPS for 24 h. Cell-free (diluted to 1 in 20) and cell-associated (1 in 80) samples were added to cultures of thymocytes ($1.5 \times 10^6/200 \mu\text{l}/\text{culture}$) from C3H/HeJ mice in the presence of PHA $1 \mu\text{g}/\text{ml}$. TAF activity (SI) was measured in triplicate. The control cultures with medium alone and with PHA alone resulted in 92 (SEM 28) and 236 (SEM 45) dpm, respectively. * Indicates statistical differences between the control and test groups as determined by multiple comparisons.

Fig. 2. Cell-associated TAF induction by M1Av and *S. abortus-equi* LPS in peritoneal macrophage cultures from C3H/HeN and C3H/HeJ mice. TAF activity of cell-associated specimens (diluted to 1 in 20) was measured in triplicate as described for fig. 1. The amounts of radioactivity incorporated in control cultures with medium alone and with PHA alone were 310 (SEM 15) and 734 (SEM 65) dpm, respectively. The TAF activity (SI) of non-stimulated macrophage specimens from C3H/HeN and C3H/HeJ mice was 3.12 (SEM 0.22) and 1.17 (SEM 0.15), respectively. * Indicates statistical differences between the control and test groups as determined by multiple comparisons.

injection of galactosamine hydrochloride (Wako Pure Chemicals Co., Osaka, Japan) 18 mg in 0.5 ml of saline. Simultaneously, the mice were challenged by an i.v. injection of M1Av (1, 10 or 100 μg) or LPS from *E. coli* O111:B4 (Difco) (0.01, 0.1, 1 or 10 ng for C3H/HeN mice; 100 μg for C3H/HeJ mice) in 0.2 ml of saline, and death was recorded for up to 3 days.

Statistical analysis

Data were analysed by the multiple comparison method. The p values were corrected by Dunn's method.¹⁸

Results

When peritoneal macrophages from C3H/HeN mice were cultured in the presence of M1Av, cell-free and cell-associated TAF were induced in a dose-dependent manner (fig. 1). In the case of LPS, a peak response was obtained at an LPS concentration of 10 $\mu\text{g}/\text{ml}$, whereas with M1Av, the highest TAF levels were induced at the highest concentration (500 $\mu\text{g}/\text{ml}$) examined. The maximum cell-free and cell-associated TAF levels induced by M1Av were higher than those induced by *S. abortus-equi* LPS (at concentrations of 10 $\mu\text{g}/\text{ml}$). Approximately 50-fold more M1Av than

Fig. 3. TNF induction by M1Av and *S. abortus-equi* LPS in peritoneal macrophage cultures from C3H/HeN and C3H/HeJ mice. The cytotoxic activity of a series of diluted cell-free specimens was examined in actinomycin D-treated L-M cells. TNF activity was expressed as the test specimen dilution required to produce 50% cytotoxic effects on target cells. Results represent means of triplicate assays. The standard error of TNF activity in each triplicate assay was < 10% of the respective mean value. ND, not detected.

Table I. IL-6 induction by M1Av in C3H/HeN macrophage cultures

Stimulant	Concentration (µg/ml)	Mean IL-6 activity, U/ml (SEM)	
		Cell-free	Cell-associated
M1Av	500	3600 (211)*	6.40 (0.07)*
	200	3600 (146)*	6.56 (0.18)*
	100	1120 (36)*	6.40 (0.27)*
	50	576 (28)*	4.48 (0.11)*
	20	144 (7)*	2.72 (0.02)*
	10	44 (2)*	1.36 (0.02)*
LPS†	1	10 (0.1)	0.64 (0.01)
	100	656 (6.6)*	4.48 (0.27)*
	10	576 (9.1)*	4.96 (0.20)*
	1	464 (14.7)*	4.00 (0.11)*
	0.1	560 (18.5)*	4.16 (0.30)*
	0.01	1120 (16.5)*	8.00 (0.09)*
None	0.001	61 (1.4)*	3.52 (0.12)*
	0.0001	9 (0.6)	0.66 (0.04)
		9 (0.3)	0.66 (0.02)

*Significantly different from the respective control by the multiple comparison method.

†LPS from *S. abortus-equi* (Novo-Pyrexal).

Table II. Serum TNF induction by M1Av and *S. abortus-equi* LPS in MDP-primed and non-primed mice

Treatment		TNF activity, mean U/ml (SEM)	
MDP priming	Inducing injection (i.v.)	C3H/HeN	C3H/HeJ
No	M1Av	7 (2)	ND
Yes	M1Av	153 (22)*	ND
No	LPS	23 (5)	ND
Yes	LPS	195 (35)*	ND

ND, not detected.

*Significantly different from the respective control by multiple comparisons.

LPS was required to induce a similar level of TAF in macrophage cultures. M1Av also induced cell-free and cell-associated TAF in macrophage cultures from C3H/HeJ mice. However, *S. abortus-equi* LPS did not induce TAF in C3H/HeJ macrophage cultures (fig. 2). Furthermore, macrophages from C3H/HeJ as well as C3H/HeN mice released significant levels of TNF into the culture supernate after stimulation with M1Av (fig. 3). In C3H/HeN mouse macrophages, the maximum response induced by M1Av (200 µg/ml) was lower than that induced by LPS (1 µg/ml), and the minimum effective concentration of M1Av was 100–1000-fold higher than that of LPS. Macrophages of C3H/HeJ mice were less responsive to M1Av than those of C3H/HeN mice in terms of TNF production. M1Av also induced significantly higher levels of IL-6 in culture supernates than those induced by LPS in macrophage cultures from C3H/HeN mice (table I).

To examine the cytokine-inducing ability of M1Av *in vivo*, M1Av (100 µg) was administered i.v. to C3H/HeN and C3H/HeJ mice, half of which were primed by i.v. injection with MDP (100 µg) 4 h before the challenge with M1Av or LPS. Both M1Av and *S. abortus-equi* LPS induced significantly increased levels of serum TNF in the MDP-primed C3H/HeN mice, whereas these stimulants did not induce it in C3H/HeJ mice irrespective of MDP priming (table II). In MDP-primed C3H/HeN mice elicited with either M1Av or LPS, markedly higher levels of IL-6 were released into the blood (table III).

Administration of a large amount of galactosamine i.v. sensitises mice to lethal toxicity of TNF, and M1Av was compared with *E. coli* LPS for its lethal toxicity in galactosamine-treated mice. M1Av (10 µg) killed all C3H/HeN mice sensitised with galactosamine (table IV). The LD₅₀ of M1Av was 3.16 µg/

Table III. Serum IL-6 induction by M1Av and LPS from *S. abortus-equi* in MDP-primed C3H/HeN mice

Preparations administered	Serum IL-6 activity, mean U/ml (SEM)
M1Av alone	679 (59)
MDP-M1Av	14458 (1291)*
MDP-LPS	13417 (2682)

*Significantly different from the control group administered with M1Av alone by the multiple comparison method.

Table IV. Lethal toxicity of M1Av and *E. coli* O111:B4 LPS in galactosamine-loaded mice

Strain of mouse	Test material	Dose (/mouse)	Dead/ tested	LD50
C3H/HeN	M1Av	10 µg	6/6	3.16 µg
	M1Av	1 µg	0/6	...
	LPS	10 ng	4/4	0.75 ng
	LPS	1 ng	3/4	...
	LPS	0.1 ng	2/4	...
	LPS	0.01 ng	0/4	...
C3H/HeJ	M1Av	100 µg	0/6	> 100 µg
	LPS	100 µg	0/4	> 100 µg

mouse. This indicated that M1Av contained the equivalent of 0.046 ng of LPS from *E. coli* O111:B4 based upon the *Limulus* test mentioned above. In this assay system, the LD50 for LPS from *E. coli* O111:B4 was 0.75 ng/mouse, whereas up to 100 µg of M1Av did not kill galactosamine-loaded C3H/HeJ mice.

To examine the possibility of a synergic effect of M1Av and LPS in inducing TNF, C3H/HeN mice primed with MDP (100 µg) (three/group) were given *S. abortus-equi* LPS (0.1 µg), M1Av (1 µg) or a mixture of the two compounds by i.v. injection. M1Av alone at this dosage did not induce a detectable serum level of TNF. The combination of M1Av and LPS induced higher levels of serum TNF than that induced by LPS alone but this was not significant.

Discussion

In this study we showed that M1Av, the carbohydrate-rich moiety from *A. viscosus* cell-wall-lysate, induced TNF, IL-6 and IL-1 *in vivo* and *in vitro*. We showed previously that M1Av exhibited species-specific antigenicity, powerful B cell mitogenicity, and polyclonal B cell activation. Cell-wall components that exhibited similar biological properties were also found amongst other oral *Actinomyces* strains, including a strain of *A. viscosus* isolated from human

dental plaque and *A. naeslundii* ATCC 12104.^{11,12} Thus, several oral *Actinomyces* spp. may possess carbohydrate antigens that exhibit various immunobiological activities *in vitro* and *in vivo*. Since *Actinomyces* spp. are predominant in the dental plaque from sites with active gingivitis,¹⁻³ their cell-wall carbohydrate antigen may induce excessive amounts of inflammatory cytokines in collaboration with LPS from the gram-negative anaerobic bacteria that appear in dental plaque during the development of gingivitis and periodontitis. The cytokines released locally might in turn induce inflammation in the periodontal tissues, with subsequent localised tissue destruction.

Oral and pyogenic streptococcal species also contain cell-wall carbohydrate antigens, and M1 antigens prepared similarly from purified streptococcal cell walls exhibit B cell mitogenicity and polyclonal B cell activation.¹⁹⁻²¹ Furthermore, Kimura *et al.*^{22,23} reported that the M1 carbohydrate of mutans streptococci, when trinitrophenylated, is a novel T-cell independent type 2 antigen.

M1Av reportedly tags a portion of the peptidoglycan fragment, namely glutamic acid, alanine and ornithine in the molar ratio 2:2:1,¹¹ which is an atypical peptidoglycan peptide, unique to some *Actinomyces* spp.²⁴ Peptidoglycan and MDP exhibit a wide variety of enhanced immunobiological responses.²⁵ However, Nagao *et al.*²⁶ claimed that peptidoglycan and MDP failed to induced IL-1 and TNF in murine macrophage cultures, which is contrary to the positive results with guinea-pig macrophages and human monocyte cultures. Controversy still exists as to the responsiveness of murine macrophages to peptidoglycan and MDP. Therefore, it is possible that a small portion of peptidoglycan residues tagged to the M1Av carbohydrate antigen may affect the responses in mice.

M1Av should have activated macrophages of LPS-resistant C3H-HeJ mice to produce cytokines *in vitro*. However, it failed to induce TNF and IL-6 in MDP-primed C3H/HeJ mice, although positive tests were recorded in C3H/HeN mice. In accordance with these findings, M1Av killed galactosamine-loaded C3H/HeN, but not C3H/HeJ mice. Our calculations from the colorimetric *Limulus* test indicated that contamination by endotoxic substances in M1Av was low, which did not support the notion that the stimulative activities of M1Av were due to contaminating endotoxin. Similar findings have been reported with lipoteichoic acid (LTA-2)²⁷ from *Enterococcus hirae* and an amphiphile^{28,29} from *Mycobacterium bovis* BCG. These preparations induced TNF and IL-6 in macrophage cultures from both C3H/HeJ and C3H/HeN mice. However, they induced cytokines in MDP-primed C3H/HeN, but not C3H/HeJ mice.³⁰ Further studies will be required to elucidate the mechanisms of the different cytokine-inducing responses to non-endotoxic cell-wall substances in C3H/HeN and C3H/HeJ mice.

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