Macrophage chemiluminescence induced by interaction with transparent and opaque colonial variants of *Mycobacterium intracellulare*

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Macrophage (MΦ) chemiluminescence (CL) induced by interaction with the two types of colonial variants of *Mycobacterium intracellulare* was studied. A smooth, opaque and dome-shaped (SmD) colonial variant triggered more intense MΦ CL than did a smooth, transparent and flat colonial variant (SmT). MΦ CL-inducing activity of the SmD variant was reduced by heating or by treatments with either Pronase P, some endoglycosidases or Tween 80, thereby indicating that the SmD variant possesses MΦ CL-inducing substance(s) having peptide, sugar and/or lipid-like moieties. Treatment of the SmD variant organism with some endoglycosidases, such as cellulase, pectinase, dextranase or α -amylase decreased its MΦ CL-inducing ability. On the other hand, MΦ CL-inducing activity of the SmT variant was not affected by any of above treatments except that it was slightly increased by Pronase P treatment and reduced by α -amylase and dextranase.

Introduction

Two smooth colonial variants are known in the *Mycobacterium avium*–*intracellulare* complex (MAC) (Schaefer *et al.*, 1970; Woodley & David, 1976); a transparent, flat and irregularly shaped colony (SmT variant) and an opaque and dome-shaped colony (SmD variant). These variants differ from each other in biological properties and in virulence for mice and birds (Saito & Tomioka, 1988, 1990; Schaefer *et al.*, 1970; Woodley & David, 1976). Saito & Tomioka (1990) and Gangadharam & Edwards (1984) found that the SmT variants have higher virulence for mice and conversely much reduced activity in inducing the macrophage (MΦ) respiratory burst which is important for the expression of microbicidal activity of MΦs (Johnston, 1978) (measured in terms of chemiluminescence (CL) and O_2^- production), as compared to the SmD variant. Although recent studies have indicated the importance of nitric oxide for the expression of antimicrobial action against some intracellular parasites, at least in mice (Adams *et al.*, 1990; Flesch & Kaufmann, 1991; Liew *et al.*, 1990; Park & Rikihisa, 1992), it is also known that the low ability of

the SmT variant to induce MΦ respiratory burst is closely related to its resistance to the microbicidal action of host MΦs (Saito & Tomioka, 1990). In this study, we investigated some properties of the MΦ CL-inducing substance(s) of *M. intracellulare* and found that it possesses peptide and/or sugar moieties.

Methods

Organisms. *M. intracellulare* N-257 (serovar 13), N-260 (serovar 16) and N-275 (serovar determination was impossible because of auto-agglutination), identified using the Gen-Probe Rapid Diagnostic System for the MAC (Gen-Probe) were used. From these strains, SmT and SmD variants were isolated on 7H10 agar plates by repeating three to four transfers. An isolated colony was inoculated into 7H9 medium and cultivated for 1 week to obtain a stem culture. The stem culture (0.1 ml) was further transferred to 5 ml 7H9 medium and cultivated for 1 week. The resultant culture (0.1 ml) was used as inoculum for 7H10 agar plates; after 7–10 d the organisms were used for measurement of MΦ CL-inducing ability. The SmT and SmD variants were streaked on 7H10 agar plates, cultivated for 10 d, and checked for purity of each colony morphology.

Mice. C57BL/6 (Bcg^s; MAC-susceptible strain) and C3H/He or CBA/JN (Bcg^f; MAC-resistant strains) mice were used (Goto *et al.*, 1984; Skamene, 1989). The first two mouse strains were purchased from Japan SLC and CBA/JN mice were obtained from Charles River.

Special agents. α -Amylase (Type IX-A, from human saliva), isoamylase (from *Pseudomonas amyloclavata*), cellulase (type II, from *Aspergillus niger*), dextranase (from *Penicillium* sp.), hyaluronidase (type I-S, from bovine testis), laminarinase (from *Penicillium* sp.), pullulanase (from *Enterobacter aerogenes*), and pectinase (from *Rizopus* sp.) were obtained from Sigma. Pronase P was purchased from Kaken Kayaku Co.

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Abbreviations: MΦ, macrophage; CL, chemiluminescence; HBSS, Hanks' balanced salt solution; MAC, *Mycobacterium avium*–*intracellulare* complex.

Physical and chemical treatments of *M. intracellulare*. SmT or SmD variants of *M. intracellulare* N-260 cultured on 7H10 agar plates in a CO₂ incubator (5% CO₂-95% humidified air) at 37 °C for 7–10 d were harvested by gently scraping into distilled water to minimize the contamination of medium constituents, washed once with distilled water by centrifugation (1500 g, 15 min), suspended in distilled water and gently sonicated with a Handy Sonic (Model UR-20P, Tomy Seiko Co.) for 15 s to disperse bacterial clumps. No cell destruction was observed. After centrifugation at 200 g for 5 min to remove remaining bacterial clumps, the bacterial suspension ($1\text{--}2 \times 10^9$ cells ml⁻¹) was subjected to the following treatments: heating at 100 °C, for 15 min, or treatment with 3% (v/v) formalin in 0.1 M-Tris/HCl, pH 7.5, at 37 °C for 2 h; or Pronase P (0.8 mg ml⁻¹ in 0.1 M-Tris/HCl, pH 7.5, containing 8 mM-CaCl₂) at 37 °C for 2 h.

Endoglycosidase treatments were done under the optimum condition for each enzyme (Dorfman, 1955; Fukumoto *et al.*, 1971; Kertesz, 1955; Mercier *et al.*, 1972; Moore & Stone, 1972; Nishizawa *et al.*, 1978; Thoma *et al.*, 1971) as follows: α -amylase (50 U ml⁻¹) in phosphate-buffered saline (PBS: 0.15 M-NaCl, 1.3 mM-KCl, 1.5 mM-KH₂PO₄, 8.1 mM-Na₂HPO₄), pH 7.2, at 40 °C for 2 h; isoamylase (50 U ml⁻¹) in PBS, pH 7.2, with 5 mM-ammonium sulphate at 40 °C for 2 h; cellulase (10 U ml⁻¹) in 0.1 M-sodium acetate, pH 4.0, at 30 °C for 2 h; dextranase (10 U ml⁻¹) in 50 mM-sodium acetate, pH 5.0, at 40 °C for 2 h; hyaluronidase (50 U ml⁻¹) in 20 mM-sodium phosphate, pH 7.0, containing 0.45% NaCl and 0.01% bovine serum albumin at 37 °C for 2 h; laminarinase (0.5 U ml⁻¹) in 50 mM-sodium acetate, pH 5.0, at 40 °C for 2 h; pullulanase (1 U ml⁻¹) in 20 mM-citrate/phosphate with 0.16 M-ammonium sulphate, pH 5.0, at 30 °C for 2 h; pectinase (50 U ml⁻¹) in 0.1 M-sodium acetate, pH 4.0, at 25 °C for 2 h; 1% (w/v) Tween 80 in PBS, pH 7.2, at 37 °C for 6 h. After each treatment, the organisms were washed four times with distilled water by centrifugation at 20000 g for 20 min, resuspended in distilled water, sonicated with a Handy Sonic for 15 s, and centrifuged at 200 g for 5 min before testing for MΦ CL-inducing activity. Counts of bacteria were made after Ziehl-Neelsen staining using 1% (v/v) HCl/ethanol for decolorization. In some cases, counting of c.f.u. was done by plating on 7H10 agar medium.

MΦ CL. This was measured as previously reported (Yamada *et al.*, 1987). Briefly, peritoneal exudate cells of mice (2.5×10^6 cells), harvested 4 d after intraperitoneal injection of zymosan A (1 mg), were incubated at 37 °C for up to 10 min in the following reaction mixture (1 ml in 13 × 32 mm vial): 1 ml phenol red/free Hanks' balanced salt solution (HBSS) containing 10 mM-HEPES, pH 7.4 and 0.1 mM-luminol with or without the addition of the indicated amount of SmT or SmD colonial variant of *M. intracellulare*. Photoemission was measured using an ATP lumiphotometer, Lumicounter ATP-237 (Tokyo Kagaku) at 37 °C for 10 s at 1 or 2 min intervals for up to 10 min.

Since the intensity of MΦ CL induced by MAC organisms varied considerably from experiment to experiment depending on the functional state of harvested MΦs, interexperimental mean values could not be estimated even when the MΦ CL assay was carried out under the same conditions.

Estimation of the number of MΦ-associated organisms. The number of MΦ-associated (attached or phagocytosed) organisms during the incubation for MΦ CL measurement was estimated as follows. The incubation mixture was washed four times with HBSS containing 5% (v/v) foetal bovine serum (FBS) (M. A. Bioproducts) by centrifugation at 50 g for 5 min to remove attached organisms and the resultant cell pellet was suspended in 1 ml 10% FBS/RPMI 1640 medium, placed in multi-well plates (Corning; 24 wells) and incubated at 37 °C in a CO₂ incubator (5% CO₂/95% air) for 30 min. After thorough washing with HBSS, the MΦ culture was subjected to Ziehl-Neelsen staining and counted.

Results

MΦ CL-inducing ability of SmT and SmD colonial variants

Fig. 1 shows CL from MΦs of C3H/He (Bcg^r) and C57BL/6 (Bcg^s) mice triggered with the two colonial variants of *M. intracellulare* strain N-260. In both Bcg^r and Bcg^s MΦs, the SmD variant induced higher MΦ CL, compared to the SmT variant. The intensity of MΦ CL induced by the SmD variant was higher in Bcg^r MΦs than in Bcg^s MΦs. Fig. 1 also shows that MΦ CL induced by the SmD variant was not affected by the addition of SmT variant, indicating that the SmT variant exerted no inhibitory action against MΦ CL. Similar results were obtained for other *M. intracellulare* strains, N-257 and N-275, although the intensity of MΦ CL varied depending on strain of the MAC. Cumulative CL (10⁶ counts per tube) from C3H/He MΦs induced by N-257, N-260 and N-275 strains (added at 10⁹ c.f.u. per tube) during a 10 min incubation was as follows: N-257, SmD (3.3), SmT (1.0); N-260, SmD (21.4), SmT (1.1); N-275, SmD (6.4), SmT (0.9). Similarly, the cumulative CL from C57BL/6 MΦs induced by N-260 and N-275 strains was as follows: N-260, SmD (2.0), SmT (0.6); N-275, SmD (4.6), SmT (0.7).

In a separate experiment with C3H/He MΦs, the number of MΦ-associated organisms of *M. intracellulare* N-260 SmD variant was about 2.2 times greater than that of the SmT variant. During a 10 min incubation for MΦ CL measurement, 5.0 ± 0.1 and 2.3 ± 0.5 organisms of SmD and SmT variants, respectively, were caught by one MΦ, when 5×10^8 organisms were added without opsonin. In this case, the intensity of MΦ CL induced by

Fig. 1. Photoemission from MΦs induced with SmT and SmD variants of *M. intracellulare* strain N-260. (a) CL from MΦs of C3H/He mice; ○, SmT (8.5×10^8 per tube); ●, SmD (6.3×10^8); ▲, SmD (3.1×10^8); □, SmT (4.2×10^8) + SmD (3.1×10^8). (b) CL from MΦs of C57BL/6 mice; ○, SmT (1.7×10^9 per tube); ●, SmD (1.3×10^9); ▲, SmD (6.3×10^8); □, SmT (8.5×10^8) + SmD (6.3×10^8). A representative result obtained in one of four separate experiments is indicated. Reproducibility of this result was confirmed in the other experiments.

Fig. 2. MΦ CL-inducing activities of SmT (○, △) and SmD (●, ▲) variants of *M. intracellulare* strain N-260 grown on 7H10 medium (○, ●) and Ogawa egg medium (△, ▲). MΦ photoemission was measured 10 min after the addition of organisms. MΦs were obtained from C3H/He mice.

Fig. 3. Effects of physical and chemical treatments of the SmD colonial variant of *M. intracellulare* N-260 on its MΦ CL-inducing activity. MΦs were obtained from C3H/He mice. (a) ○, Control (untreated); △, heating (100 °C, 15 min); ●, Pronase P treatment (0.8 mg ml⁻¹, 2 h); ▲, 3% (v/v) formalin treatment (2 h). (b) ○, Control (untreated); ●, 1% (w/v) Tween 80 treatment (2 h). Each plot indicates the mean ± SD ($n = 2$ or 3). The SD bars smaller than the size of each symbol were omitted. The 95% confidence limit of the variation of plots indicated in individual symbols was as follows. (a) ○, $\pm 1.03 \times 10^3$ c.p.s.; △, $\pm 0.09 \times 10^3$ c.p.s.; ●, $\pm 0.13 \times 10^3$ c.p.s.; ▲, $\pm 0.15 \times 10^3$ c.p.s. (b) ○, $\pm 1.14 \times 10^3$ c.p.s.; ●, $\pm 0.27 \times 10^3$ c.p.s.

the SmD variant was about 6.4-fold greater than that induced by the SmT variant: 3.4×10^3 (SmD) versus 5.3×10^2 (SmT) c.p.s. per 10^6 MΦs. Therefore, the difference in the number of attached organisms of the SmD and SmT variants on MΦs is not a main cause for the lowered MΦ CL-triggering activity of the SmT variant compared to that of the SmD variant.

Fig. 2 compares MΦ CL induced by the SmT and SmD variants grown on 7H10 agar medium or Ogawa egg

medium (Ogawa & Saba, 1949). The intensity of MΦ CL was in the order: SmD on 7H10 medium > SmD on Ogawa egg medium $\geq$ SmT on Ogawa egg medium > SmT on 7H10 medium. In the case of MAC organisms grown on Ogawa egg medium, there was only a small difference in the MΦ CL-inducing activity between the SmT and SmD variants. Therefore, the differential MΦ CL-inducing activities of the SmT and SmD variants are not a fixed characteristic but are expressed depending on the nutrient conditions of the bacterial growth.

Characteristics of MΦ CL-inducing substance of the SmD and SmT variant

Fig. 3 shows effects of some physical, chemical and enzymic treatments of the SmD variant of *M. intracellulare* strain N-260 upon its MΦ CL-triggering activity. Heating (100 °C), Pronase P digestion, and Tween 80 treatment caused a significant reduction in MΦ CL-inducing activity ($P < 0.01$; t -test). However, formalin treatment failed to show such an effect. Therefore, the SmD variant possesses a heat-labile component for MΦ CL-triggering, having peptide moieties. Tween 80 treatment might remove hydrophobically attached proteins or carbohydrates on the SmD organisms.

Treatment of the SmD variant with endoglycosidases reduced MΦ CL-inducing activity in the following order (percentage reduction in CL at 5 min due to enzyme treatment is indicated in parentheses; $n = 2$): cellulase (85 ± 1), pectinase (78 ± 4), α -amylase (62 ± 3), dextranase (60 ± 11), isoamylase (39 ± 0.3). In contrast, pullulanase and laminarinase increased MΦ CL-inducing activity of the SmD organisms: (percentage increase) pullulanase (62 ± 2), laminarinase (77 ± 9). Hyaluronidase had no effect. These results suggest roles for some sugar components in the MΦ CL-triggering by MAC SmD variants.

Some sugar residues are known to be involved in the receptors for MΦ lectinophagocytosis (Ofek & Sharon, 1988). Thus, we examined whether or not the SmD variant-mediated triggering of MΦ CL involves a similar interaction to that seen in lectinophagocytosis. However, none of α -methyl glucoside, α -methyl mannoside, galactose, xylose, glucosamine, maltose, lactose, melibiose, raffinose or trehalose inhibited the MΦ CL even at a concentration of 1 mM. Lectinophagocytosis is known to be inhibited by hapten sugars (such as α -methyl mannoside etc.) at concentrations lower than 0.1 mM (Ofek & Sharon, 1988). Thus, it is unlikely that MΦ CL-triggering by the SmD variant involved the interaction between hapten sugars and its receptor, although there remains the possibility that the MAC organisms use different sugars.

Fig. 4 shows effects of some physical, chemical and

Fig. 4. Effects of physical and chemical treatments of the SmT colonial variant of *M. intracellulare* N-260 on its MΦ CL-inducing activity. MΦs were obtained from C3H/He mice. (a) ○, Control (untreated); △, heating (100 °C, 15 min); ●, Pronase P treatment (0.8 mg ml⁻¹, 2 h); ▲, 3% (v/v) formalin treatment (2 h); ■, Untreated SmD variant. (b) ○, Control (untreated); ●, 1% (w/v) Tween 80 treatment (2 h); ■, Untreated SmD variant. Each plot indicates the mean $\pm$ SD ($n = 2$). The SD bars smaller than the size of each symbol have been omitted. The 95% confidence limit of the variation of plots indicated in individual symbols was as follows. (a) ○, $\pm 0.02 \times 10^3$ c.p.s.; △, $\pm 0.06 \times 10^3$ c.p.s.; ●, $\pm 0.30 \times 10^3$ c.p.s.; ▲, $\pm 0.07 \times 10^3$ c.p.s.; ■, $\pm 1.06 \times 10^3$ c.p.s. (b) ○, $\pm 0.17 \times 10^3$ c.p.s.; ●, $\pm 0.06 \times 10^3$ c.p.s.; ■, $\pm 1.14 \times 10^3$ c.p.s.

enzymic treatments of the SmT variant of *M. intracellulare* N-260 strain upon its MΦ CL-triggering activity. MΦ CL-inducing activity of the SmT variant was not significantly affected by heating or Tween 80 treatment but was considerably increased by Pronase P digestion ($P < 0.05$, t -test). Furthermore, treatments with endoglycosidases did not overcome the reduced MΦ CL-inducing activity in the SmT variant (data not shown).

Discussion

The present study indicates the following: first, there was a relatively loose correlation between the intensity of MΦ CL induced by the two MAC colonial variants and the number of MΦ-associated (attached or phagocytosed) organisms. For this reason, it is plausible that the degree of expression of MΦ CL-inducing substance per cell is larger in the SmD variant than the SmT variant. As previously reported (Saito & Tomioka, 1990), the SmT variant could grow continuously in the sites of infection of a MAC-susceptible mouse strain (C57BL/6) and persisted well even in the MAC-resistant strain (C3H/He). In contrast, the SmD variant was rapidly eliminated from the sites of infection in both strains of mice. Therefore, the MΦ CL-inducing ability of the MAC colonial variants is inversely correlated to their virulence in mice. This suggests the importance of the operation of the respiratory burst in host MΦs in host defence

mechanisms against MAC organisms. However, such type of MΦ function seems not to be crucial, since, in the case of MAC SmT variants, there was no positive correlation between their MΦ CL-inducing activities and virulence to mice (Tomioka *et al.*, 1992).

Second, MΦ CL-inducing activity of the SmD variant was reduced by heating, and Pronase P, Tween 80, and some endoglycosidase treatments, indicating that the MΦ CL-inducing substance(s) of the SmD variant may be some kind of hydrophobically attached heat-labile peptides and/or carbohydrates. Presumably, it has certain sugar moieties important for the expression of its MΦ CL-inducing activity. However, the possibility cannot be excluded that the SmD variant possesses more than one component with MΦ CL-triggering activity having different physical and chemical properties. In a separate experiment, peptidoglycolipid fraction prepared by the method of Dhariwal *et al.* (1986) showed no MΦ CL-triggering activity (data not shown).

Significant reduction of MΦ CL-inducing activity of the SmD organisms by treatments with cellulase, pectinase, dextranase or α -amylase may imply some important role of sugar moieties having β -1,4-, α -1,4-, or α -1,6-glucoside bonds or α -1,4-glycoside bond. However, further studies on endoglycosidase-induced changes in sugar moieties on the SmD organisms are necessary.

Third, it is unlikely that specific sugar residues directly participate in the interaction between the SmD MΦ CL-inducing substance and their MΦ receptors, since none of the hapten sugars tested (α -methyl glucoside, α -methyl mannoside, galactose, xylose, glucosamine, maltose, lactose, melibiose, raffinose, trehalose) inhibited MΦ CL-inducing action of the SmD variant. However, the possibility remains that other sugars such as 6-deoxysugars of the SmD organisms play important roles.

The MΦ CL-inducing activity of the SmT variant was lower than that of the SmD variant. Unlike the case of the SmD variant, neither heating, Tween 80 treatment, nor treatments with various endoglycosidases affected the activity of the SmT variant. Moreover, Pronase P treatment, which reduced the MΦ CL-inducing activity of the SmD variant, caused an increase in the activity of the SmT variant. These findings suggest some possible differences in the nature of MΦ CL-inducing substance(s) of the SmT from that of the SmD variant, or a difference in the mode of their MΦ CL-triggering action. However, it is also possible that the SmT organism possesses the MΦ CL-inducing component in a smaller amount than the SmD variant, and that the SmT organism can induce MΦ respiratory burst only when it is ingested into MΦs, causing membrane perturbation of the MΦ cells. Further studies on the detailed properties of the MΦ CL-inducing components are currently under way to elucidate these aspects.

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