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The macrophage response to bacteria. Modulation of macrophage functional activity by peptidoglycan from *Moraxella (Branhamella) catarrhalis*

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SUMMARY

Moraxella (Branhamella) catarrhalis organisms have been shown to be particularly efficient in inducing in a pure population of bone marrow-derived mononuclear phagocytes secretory and cellular activities. In the present study, the ability of peptidoglycan from this Gram-negative organism to trigger a macrophage response was compared with that elicited by peptidoglycan from *Staphylococcus aureus* and *Bacillus subtilis*. The results show that the three peptidoglycans were similarly active in triggering the secretion of tumour necrosis factor and tumouricidal activity but differed considerably in their ability to induce the generation of nitrite in macrophages; in this respect, peptidoglycan from *M. catarrhalis* was particularly potent. The impressive capacity of *M. catarrhalis* peptidoglycan to induce in low concentration the secretion of tumour necrosis factor and nitrite and tumouricidal activity may, in addition to its lipopolysaccharide, contribute to the extraordinary potential of this organism to trigger the functional activities of macrophages.

Keywords tumour necrosis factor nitrite tumouricidal activity polysaccharides muramyl dipeptide

INTRODUCTION

Moraxella (Branhamella) catarrhalis, a Gram-negative diplococcus, has long been regarded as an occasional non-pathogenic constituent of the upper respiratory tract [1]. However, during the past decade, it was increasingly found to be associated with respiratory tract infections such as acute otitis media, sinusitis and bronchitis [2–5].

Recent work from this laboratory has shown that different bacterial species and/or different strains within the same species differed considerably in their ability to induce in a pure population of bone marrow-derived mononuclear phagocytes (BMMϕ) the secretion of nitric oxide and/or nitrite (NO/NO_2^-), and tumouricidal activity. Gram-negative bacteria, lipopolysaccharide (LPS) and lipid A were potent in triggering the secretion of NO/NO_2^- but the great majority were poor in inducing tumouricidal activity in macrophages; on the other hand, Gram-positive bacteria were active in triggering tumouricidal activity without concomitant induction of NO/NO_2^- [6–9]. As the only exception of the examined Gram-negative species, *M. catarrhalis* organisms in low concentration were able to trigger in resting macrophages both the secretion of NO/NO_2^- and tumouricidal activity [7]. To arrive at a better understanding of the extraordinary macrophage-activating potential of this organism, the ability of peptidoglycan isolated from *M. catarrhalis* to induce in macrophages a secretory and cellular response was compared in the present study with that of peptidoglycans from other species and with some other bacterial products.

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MATERIALS AND METHODS

Materials

Purified pneumococcal polysaccharide, types 1 to 5, 8, 14, 20, 22, 34 and 43, were purchased from American Type Culture Collection (ATCC; Rockville, MD) [10]; bacterial oligosaccharides 76/01 through 76/04 were from BioCarb Chemicals (Lund, Sweden) [11,12]. N^G-monomethyl-L-arginine (NMMA) was from Bachem (Bubendorf, Switzerland); LPS from *Escherichia coli* O128:B12 and polymyxin B were from Sigma Chemical Co. (St Louis, MO) and the Endospecy reagent set from Seikagaku Kogyo (Tokyo, Japan); recombinant rat interferon-gamma (IFN-γ) was a generous gift from Dr P. H. van der Meide, muramyl dipeptide from Ciba-Geigy (Basel, Switzerland).

Bacteria

The bacteria included in this study were selected from a single colony on solid brain-heart infusion (Difco Laboratories, Detroit, MI) or on Columbia agar base (BBL, Cockeysville, MA) supplemented with 7% sheep blood and grown in liquid brain-heart infusion (Difco) or on blood agar plates [7]. After harvesting by centrifugation, bacteria were washed twice in

Table 1. Ability of whole organisms and/or peptidoglycan from *Moraxella* (*Branhamella*) *catarrhalis*, *Staphylococcus aureus*, and *Bacillus subtilis* to trigger the secretion of nitrite and/or tumouricidal activity in bone marrow-derived mononuclear phagocytes (BMMϕ)

Bacterial species and fraction	Nitrite secretion elicited by*		Tumouricidal activity elicited by†	
	1 μg/ml	10 μg/ml	1 μg/ml	10 μg/ml
M. catarrhalis				
Whole cells	66 (±12)	89 (±21)	46 (±7)	61 (±11)
Isolated PG	58 (±8)	85 (±10)	47 (±9)	66 (±11)
S. aureus				
Whole cells	33 (±9)	54 (±8)	2 (±2)	72 (±4)
Isolated PG	36 (±10)	95 (±10)	74 (±7)	72 (±6)
B. subtilis				
Whole cells	20 (±4)	47 (±8)	0 (±2)	40 (±9)
Isolated PG	15 (±9)	43 (±10)	51 (±5)	69 (±4)

* Nitrite secretion (μM/10^6 BMMϕ/24 h); values are means (±s.d.) from three to five experiments, each performed in triplicate.

† BMMϕ were first cultured for 24 h in medium supplemented with one of the agents, the medium then replaced by medium supplemented with the same agent and prelabelled P-815 mastocytoma cells; after 36 h incubation, the net percentage of ^{14}C-thymidine release was determined. Values are means from five to six experiments, each performed in triplicate.

PG, Peptidoglycan.

PBS, and the number of colony forming units (cfu) and wet weight determined; wet weight was taken as a quantitative measure of heat-killed (120 min 60°C) bacteria.

Isolation of bacterial cell wall peptidoglycan

The isolation procedure essentially followed the methods described by Peterson *et al.* [13] and Wilkinson *et al.* [14]. *M. catarrhalis* [7], *Bacillus subtilis* [7], and *Staphylococcus aureus* strains BB255 and BB255-1 (BB 330) *fem A*- known to have lowered peptidoglycan-associated glycine [15,16], were grown in one litre of LB (10 g tryptone, 5 g yeast extract, 5 g NaCl) in a 2-*l* flask to an A_{580nm} of 1·0 at 37°C and 200 rev/min, harvested by centrifugation (13 000 *g*, 4°C, 5 min) and washed in cold distilled water (whole cell fraction). The resuspended cells were placed in a Bead Beater™ (Biospec Products, Bartlesville, OK) blending unit with 50 ml of 0·1 mm cold glass beads. In the apparatus packed in ice, the cells were broken in five consecutive 2 min intervals. From the filtrate obtained, the crude cell walls were harvested by centrifugation, washed, resuspended, and boiled for 15 min at 100°C. Purified cell walls were produced by resuspending the boiled crude cell walls in 0·05 M Tris HCl, pH 7·5, containing 5 mM $MgCl_2$, and adding DNase and RNase to a final concentration of 5 μg/ml each and incubating with gentle shaking (75 rev/min) at 37°C for 120 min. Afterwards trypsin was added to a final concentration of 200 μg/ml, and incubation was continued for an additional 4 h as above. Purified cell walls were then harvested and washed. Peptidoglycan from *S. aureus* and *B. subtilis* was isolated by resuspending purified cell walls in 10% (w/v) TCA and stirring slowly at 4°C for 24 h before harvesting and washing.

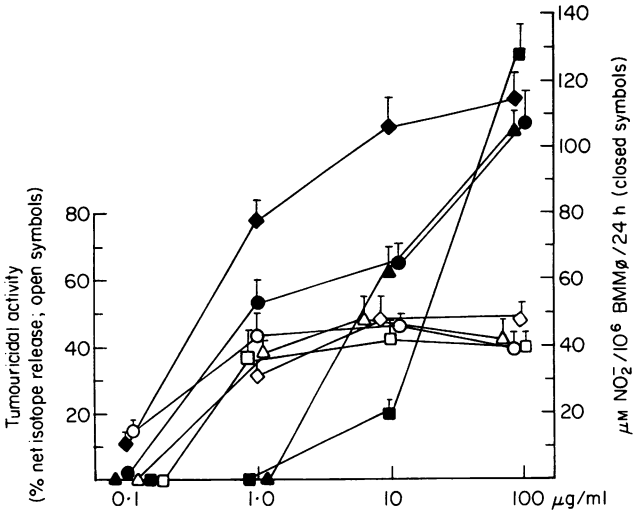

Fig. 1. Dose-dependent induction of NO_2^- secretion and tumouricidal activity in bone marrow-derived mononuclear phagocytes (BMMϕ) by bacterial peptidoglycans. Values for NO_2^- secretion (μM/10^6 BMMϕ/24 h; closed symbols) are means (±s.d.) from three to five experiments. Conditions for assay of tumouricidal activity (open symbols) were as described in Table 1; values are means (±s.d.) from four to five experiments, each performed in triplicate. ○, *Moraxella* (*Branhamella*) *catarrhalis* whole cells; ◇, *M. catarrhalis* peptidoglycan; △, *Staphylococcus aureus* peptidoglycan; □, *Bacillus subtilis* peptidoglycan.

Bone marrow-derived mononuclear phagocytes (BMMϕ)

Bone marrow cells from femurs of inbred male DA rats were cultured in Iscove's modified Dulbecco medium (IMDM) conditioned with supernatant (final concentration 10%) from strain L clone 292 cells as described [6,7]. On day 6 after initiation of the cultures, BMMϕ remaining adherent after repeated washing were cultured for various time intervals (standard 24 h) in medium alone (resting BMMϕ; negative control) or in medium supplemented with one of the agents under test before their functional activities were compared using BMMϕ derived from the same pool.

Assessment of the functional activities of BMMϕ

Reductive capacity of BMMϕ. Resting BMMϕ were seeded in 96-well microplates (5×10^4 or 10^5/well), incubated for the time interval indicated at 37°C in medium (control) or in medium supplemented with one of the agents under test before the reductive capacity of the cells was determined in a MTT tetrazolium assay [17] by measuring absorbance at 570 nm in a Dynatech MR 700 microplate reader.

Tumour necrosis factor activity. Serial dilutions of supernatants harvested from 5×10^5 BMMϕ/ml that had been cultured for various time intervals in medium supplemented with one of the agents, were checked in the absence of actinomycin D for their cytolytic activity using the tumour-necrosis factor-alpha (TNF-α)-sensitive WEHI-164/13 cells as targets [18]. As a reference, a stock solution of murine TNF-α (specific activity $1{\cdot}2 \times 10^7$ U/ml protein; kindly provided by Dr G. R. Adolf) was titered on target cells; after 24 h the cells remaining viable were stained with MTT tetrazolium. The lytic activity (U/ml) of the macrophage supernatants was determined as for the standard TNF assay. Conventional sheep anti-mouse rTNF-α (neutralizing activity *ca* 100 000 NU/ml) neutralized the

Table 2. Ability of polymyxin B and NMMA to affect induction of NO_2^- secretion in bone marrow-derived mononuclear phagocytes (BMMϕ) by peptidoglycans from *Bacillus subtilis* and *Moraxella* (*Branhamella*) *catarrhalis*

Culture conditions	BMMϕ alone (control)	BMMϕ + PG *B. subtilis*		BMMϕ + PG *M. catarrhalis*		BMMϕ + LPS (μg/ml)		
		1 μg/ml	10 μg/ml	1 μg/ml	10 μg/ml	10^{-3}	10^{-2}	0·1
Medium (control)	0	0	45±6	51±7	78±4	73±4	91±8	95±6
Polymyxin B 10 μg/ml	0	0	38±7	30±8*	34±6*	0*	0*	0*
NMMA								
10^{-5} M	0	0	12±1*	0*	44±5*	45±4*	58±7*	68±8*
10^{-4} M	0	0	0*	0*	0*	0*	24±6*	31±5*

* Statistically significantly different from the corresponding control ($P < 0{\cdot}001$; Mann–Whitney *U*-test).

BMMϕ were incubated for 24 h before NO_2^- was determined in supernatants; values (μM NO_2^-/10^6 BMMϕ/24 h) are means (±s.d.) from six to ten determinations, each performed in triplicate.

PG, Peptidoglycan; LPS *E. coli* 0128:B12.

activity of rat TNF-α to a similar extent as that of murine TNF-α [18]. Repeated tests have shown that the lytic activity manifested by supernatants against WEHI-164/13 cells was markedly diminished or even abolished by anti-TNF-α (1000 NU/ml); in contrast, tumouricidal activity expressed by activated macrophages against TNF-α-resistant P-815 mastocytoma cells was in no case affected by the antiserum (data not shown).

NO_2^- determination. After culturing BMMϕ (10^5 or 10^6 cells/well, 96-well microplates) for the time interval indicated in medium or in medium supplemented with one of the agents, 100 μl cell-free sample aliquots and/or sodium nitrite standard dilutions were mixed with 50 μl Griess reagent (0·1% naphthylethylene diamine dihydrochloride, 1% sulphonylamide, 3% H_3PO_4) and incubated for 10 min at room temperature before NO_2^- concentration was determined by measuring absorbance at 550 nm in a Dynatech MR 700 microplate reader [18].

Assay of tumouricidal activity. Resting day 6 BMMϕ were incubated for 24 h in medium or in medium supplemented with one of the agents being tested. The medium was then replaced by new medium supplemented with the same agent and prelabelled tumour targets, and tumouricidal activity was determined in a 36 h ^{14}C-thymidine release assay [18]. Labelled P-815 murine mastocytoma cells and DA rat dimethylbenz(a)anthracene-(D-12; 6) and methylcholanthrene-induced (M-1, M-3; 6) tumour cells were interacted for 36 h with BMMϕ (initial effector: target cell ratio 2·5:1 and 5:1) before radioactivity in cell-free supernatants was measured in order to calculate the percentage of specific isotope release [19].

RESULTS

Ability of bacterial peptidoglycan to induce the generation of nitrite and tumouricidal activity

Peptidoglycan from *M. catarrhalis*, *S. aureus* and *B. subtilis* exhibited considerable macrophage-activating ability (Table 1). Comparison of the capacity of whole cells and isolated peptidoglycans to induce tumouricidal activity has shown, however, that the preparations from *M. catarrhalis* were similarly active, while in low concentration whole cells from Gram-positive organisms were clearly weaker than the corresponding peptidoglycan. BMMϕ that had been incubated with one of the peptidoglycans expressed tumouricidal activity against all four tumour target cell lines examined; however, only the results obtained with the particularly susceptible P-815 mastocytoma cells are reported here. At a concentration of 1 μg/ml, each peptidoglycan triggered considerable cytolytic activity against these TNF-α-resistant target cells (Fig. 1). Peptidoglycan from the isogenic BB255-1 *fem A S. aureus* strain, with lowered peptidoglycan-associated glycine content [16], was as active as peptidoglycan from the parent strain (not shown). The three peptidoglycans were also similarly active in inducing the secretion of TNF-α and in enhancing the reductive capacity of macrophages (data not shown). On the other hand, these compounds differed considerably in their ability to elicit the generation of NO/NO_2^- in BMMϕ. For induction of a comparable concentration of nitrite, approximately 1 μg/ml of peptidoglycan from *M. catarrhalis*, 25 μg/ml of peptidoglycan from *S. aureus*, and 50 μg/ml of peptidoglycan from *B. subtilis* were required (Fig. 1). To assess whether the marked ability of the *M. catarrhalis* peptidoglycan to trigger the secretion of NO/NO_2^- might be due to contamination with LPS, two additional tests were performed. Endotoxin determination with the sensitive Endospecy reagent set revealed a comparable, low endotoxin level in each of the peptidoglycan preparations (not shown). Moreover, stimulation of nitrite secretion by peptidoglycans from Gram-negative *M. catarrhalis* and Gram-positive *B. subtilis* organisms was, similarly, only partially affected by polymyxin B in a concentration that fully abolished the NO_2^- secretory response of BMMϕ to LPS (Table 2), suggesting that the effect of *M. catarrhalis* peptidoglycan was not due to contaminating LPS. On the other hand, the L-arginine analogue, NMMA, clearly diminished nitrite secretion triggered in BMMϕ by peptidoglycan and LPS (Table 2), underlining the dependence of nitrite production on L-arginine.

Ability of muramyl dipeptide and microbial polysaccharides and oligosaccharides to trigger a secretory and cellular response in macrophages

Muramyl dipeptide. Muramyl dipeptide (MDP) enhanced TNF-α and, to a minor extent, NO_2^- secretion without affecting the reductive capacity or tumouricidal activity of macrophages (Table 3).

Pneumococcal polysaccharides. The 11 pneumococcal polysaccharide types examined were poor in inducing macrophage

Table 3. Effects of pneumococcal polysaccharides, *Salmonella* oligosaccharides, muramyl dipeptide, heat-killed bacteria, IFN-γ on macrophage activity

Macrophage activity	Agent with which BMMϕ had been incubated													
	Resting BMMϕ (control)	Pneumococcal polysaccharides (10–50 μg/ml) type				*Salmonella* oligosaccharides (25–50 μg/ml)				MDP (1–10 μg/ml)	CP (25–50 μg/ml)	EF (25–50 μg/ml)	MC (2·5–5·0 μg/ml)	IFN-γ (1–2·5 U/ml)
		2	3	8	20	01	02	03	04					
Reductive capacity (% in 24 h)	100	90	100	90	90	100	100	100	100	100	120–130*	110	120–130*	110
TNF secretion (range in U/ml in 4 h SN)	1·8–7·2	1·8–7·2	14·4*–28·8	1·8–7·2	28·8*–230	230*	1·8–7·2	7·2*–14·4	14·4*–28·8	28·8*–230	230*	28·8*–230	230*	14·4*–28·8
NO_2^- secretion (μM/10^6 BMMϕ h)	0	0	0	0	15–40*	15–40*	0	0	0	2–15*	15–40*	2–15*	40*	2–15*
Tumouricidal activity (24/36 h; net % thymidine release)	0	0	0	0	0	0	0	0	0	0	60*	60*	60*	40–60*

Values represent the range from five to seven experiments, each performed in triplicate.

* Statistically significantly different from controls ($P < 0{\cdot}001$; Mann–Whitney *U*-test).

CP, *Corynebacterium parvum*; EF, *Enterococcus faecalis*; MC, *Moraxella catarrhalis*; MDP, muramyl dipeptide; SN, supernatant.

responses. Type 20 polysaccharide was the only agent that triggered the secretion of considerable amounts of TNF-α and NO_2^- and also diminished the reductive capacity of macrophages. Type 2 and type 8 pneumococcal polysaccharide somewhat diminished the reductive capacity while type 3 polysaccharide elicited some TNF-α activity. The pneumococcal polysaccharides did not induce tumouricidal activity in BMMϕ (Table 3).

Oligosaccharides from Salmonella *species.* Of the *Salmonella* oligosaccharides [11,12], compound 01 clearly enhanced the secretion of TNF-α and nitrite while compounds 03 and 04 somewhat stimulated the generation of TNF-α. None of the oligosaccharides was able to affect the reductive capacity or to trigger tumouricidal activity in BMMϕ (Table 3).

Other agents. Corynebacterium parvum, E. faecalis, and/or *M. catarrhalis* organisms and IFN-γ, included as a reference, all enhanced each of the macrophage parameters, although to a different degree (Table 3).

DISCUSSION

Earlier work has shown that pure lymphocyte-free BMMϕ that exhibit only limited spontaneous functional activity, are able to respond to interaction with various heat-killed bacteria with an enhancement of their secretory and cellular capacities, indicating that these organisms are recognized as foreign [6,19,20]. The macrophage response could vary considerably depending on the type of bacteria and/or bacterial product. Gram-negative bacteria, LPS and lipid A were potent in inducing the secretion of NO/NO_2^- but mostly exhibited only limited ability to generate TNF-α-independent tumouricidal activity; on the other hand, the majority of Gram-positive organisms triggered marked tumouricidal activity but were poor in eliciting the production of NO/NO_2^- [7,9]. As a striking exception from this scheme, Gram-negative *M. catarrhalis* organisms proved to be efficient in inducing both the secretion of NO/NO_2^- and tumouricidal activity. In an attempt to identify the structural component responsible for the unusual capacities of this organism, the macrophage-activating potential of its isolated peptidoglycan was analysed in the present study. Bacterial peptidoglycans and their derivatives are known to mediate various immunomodulatory activities [21,22]. Such compounds have in particular been reported to exert an adjuvant effect on humoral and cell-mediated immune responses [23–25], to activate macrophages [26,27], and to enhance resistance to tumours [22,26].

From the comparison of whole organisms and isolated peptidoglycan, it became clear that peptidoglycan from *M. catarrhalis* and Gram-positive bacteria expressed considerable ability to trigger the secretion of NO/NO_2^- and/or TNF-α-independent tumouricidal activity in BMMϕ (Table 1). As the concentration of peptidoglycan was distinctly lower in whole organisms than in the peptidoglycan fraction, it appears likely that in addition to peptidoglycan, other structures may also contribute to the macrophage stimulatory potential of intact bacteria. Interestingly, the peptidoglycan from *M. catarrhalis* and the Gram-positive organisms exhibited a similar potential to trigger tumouricidal activity but differed in their ability to induce the secretion of nitrite in BMMϕ; for induction of nitrite secretion equivalent to that by peptidoglycan from *M. catarrhalis*, 25–50 times higher concentrations of peptidoglycan from Gram-positive organisms were required (Fig. 1). To assess whether this difference could be ascribed to LPS, two different trials were made. Both attempts, comparison of LPS levels of the dissolved peptidoglycan samples and the ability of polymyxin B to affect induction of nitrite secretion by peptidoglycan, suggested that the activity of peptidoglycan from *M. catarrhalis* was not due to contaminating LPS (Table 2).

For the assessment of the role of peptidoglycan from *M. catarrhalis* as a macrophage-activating agent, the quantitative aspects are of central importance. It is generally agreed that although the materials in the two groups of bacteria are largely the same, Gram-positive bacteria have between 20 and 40 layers of peptidoglycan whereas Gram-negative bacteria have a much thinner cell wall [28,29]. Under identical culture conditions and isolation procedures, the two Gram-positive and the Gram-negative bacterial species examined in the present study to our astonishment yielded similar amounts of peptidoglycan, suggesting that *M. catarrhalis* organisms may have a more than single layered peptidoglycan architecture [30,31]. Further studies are required to affirm such a concept. It is, however, conceivable that the extraordinary macrophage-activating potential of *M. catarrhalis* organisms may be essential for the low pathogenicity of this species.

MDP, the water soluble minimal structure of bacterial cell wall peptidoglycan required for adjuvancy, has been shown to induce various functions in macrophages [32,33]. In the concentrations tested, MDP somewhat enhanced secretion of TNF-α, slightly promoted production of nitrite but was unable to affect reductive and tumouricidal activities of BMMϕ (Table 3); it was therefore not able to duplicate the pronounced effects of peptidoglycan. Pneumococcal polysaccharides and *Salmonella* oligosaccharides had only limited effects on the macrophage functions and none of these compounds was able to induce tumouricidal activity. That each compound elicited a different pattern of the macrophage response suggests that the macrophage parameters are induced via different pathways and are therefore not directly linked (Fig. 1, Table 3).

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