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Modulation of interleukin-12 synthesis by DNA lacking the CpG motif and present in a mycobacterial cell wall complex

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Abstract A mycobacterial cell wall complex prepared from the non-pathogenic microorganism *Mycobacterium phlei*, where mycobacterial DNA is preserved and complexed to cell wall fragments, possesses anticancer and immunomodulatory activity. DNA from a number of prokaryotes has been found to modulate the immune system and to induce cytokine synthesis. We have therefore determined whether the DNA associated with this complex has the ability to induce the synthesis of interleukin-12 (IL-12), a potent anticancer cytokine. Mycobacterial DNA complexed with cell wall fragments or DNA purified from *M. phlei* induced IL-12 synthesis by murine and human monocytes and macrophages in vitro, and was capable of inducing IL-12 synthesis in vivo in mice following i.p. administration. Neutralization of DNA with cationic liposomes or digestion with DNase I significantly decreased the ability of the cell wall complex to induce IL-12. CpG methylation of DNA extracted from these cell walls or from *M. phlei* did not affect the induction of IL-12 synthesis by monocytes and macrophages. In contrast, CpG methylation of DNA from *Escherichia coli* abolished its ability to induce IL-12 synthesis. These results demonstrate that unmethylated CpG motifs present in *M. phlei* DNA are not a prerequisite for the induction of IL-12 synthesis. The size of the mycobacterial DNA, in the range of 5 bp to genomic DNA, did not influence its capacity to induce IL-12. Our results emphasize that *M. phlei* DNA associated with the cell wall complex makes a significant contribution to the overall immunomodulatory and anticancer activity of this mycobacterial cell wall preparation and that these activities are not correlated with the presence of CpG motifs.

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Introduction

Cell wall preparations from gram-positive bacteria possess antitumor activity against a wide range of cancer types such as melanoma and lung, gastric and bladder cancer [29, 43, 44]. A number of studies suggest that the antitumor activity of cell wall preparations from *Mycobacterium bovis* (BCG) and *Nocardia rubra* is mediated by the activation of immune effector cells [31, 37, 45]. The immunomodulatory activity of such cell wall preparations has been attributed to the ability of chemical entities, such as peptidoglycan or muramyl peptides, to stimulate immune effector cells [6, 33, 40]. *Mycobacterium phlei* is a gram-positive microorganism that is not a recognized pathogen for fish, amphibia, birds and mammals. This ubiquitous mycobacterium is found in soil, on plants and in drinking water [30]. A cell wall extract derived from *M. phlei* has been shown to possess potent antitumor activity against mammary tumors in dogs, sarcoma in horses, bladder tumors in mice and adenocarcinoma of the prostate in rats [5, 26]. In humans, this mycobacterial cell wall extract has been shown to have significant activity against carcinoma of the bladder following intravesicle administration [27]. We have recently isolated a cell wall complex from *M. phlei* where mycobacterial DNA in the form of short oligonucleotides is preserved and complexed to the cell wall. This partially delipidated and deproteinized macromolecular complex is composed primarily of carbohydrates, peptides and lipids and complexed DNA and has been found to inhibit the proliferation of bladder cancer cells directly by inducing apoptosis [13].

DNA from a diverse range of organisms (bacteria, yeast, nematodes, molluscs and insects) is capable of modulating the immune system [38]. Interleukin-12 (IL-12) is an inducible cytokine that is synthesized predominately by B cells and macrophages, and possesses

potent antitumor activity following systemic or local administration in mice bearing a variety of malignancies [3, 28]. IL-12 appears to exert its antitumor activity by stimulating natural killer (NK) cells, T cells, enhancing cytokine expression and blocking angiogenesis [41]. In the present study the effect of DNA associated with the cell wall complex on IL-12 synthesis has been evaluated using immune effector cells.

We have found that mycobacterial DNA associated with the cell wall complex, as well as DNA purified from *M. phlei*, is a potent inducer of IL-12 synthesis by monocytes and macrophages in vitro, and is capable of inducing IL-12 synthesis in vivo in mice following i.p. administration. Although neutralization of the DNA by cationic liposomes or digestion by DNase I decreased the ability of the mycobacterial cell-wall–DNA complex to induce the production of IL-12 by monocytes and macrophages, the methylation status of cytosine in putative CpG motifs was not critical. Furthermore, the size of this mycobacterial DNA, in the range of 5 bp to genomic DNA, did not influence its capacity to modulate IL-12 synthesis. Our results demonstrate that the presence of complexed DNA significantly contributes to the overall immunomodulatory activity of this cell wall composition, and that this activity does not correlate with the presence of CpG motifs.

Materials and methods

Cells

Murine macrophages were obtained from female CD1 mice 4 days after the i.p. injection of 1.5 ml sterile Brewer's thioglycollate broth (Difco, Detroit, Mich.). The peritoneal exudate (more than 85% macrophages) was harvested, washed by centrifugation in Hanks' balanced salt solution and plated in six-well flat-bottom microplates at 1.0×10^6 cells/ml in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum (FCS), 20 mM HEPES and 50 μg/ml gentamycin sulfate (all from Gibco Life Science, Burlington, Ontario). The cells were allowed to adhere for 18 h at 37 °C in an atmosphere of 5% CO_2, after which non-adherent cells were removed by gentle washing with warm medium. Human monocytic THP-1 and pro-myelocytic HL-60 cells were obtained from the ATCC (Rockville, Md.). THP-1 and HL-60 cells were cultured in RPMI-1640 medium supplemented with 10% FCS, 2 mM L-glutamine and 20 mM HEPES (all from Gibco BRL) at 37 °C in an atmosphere of 5% CO_2.

Determination of endotoxin level

The endotoxin content of the mycobacterial cell wall complex, cell wall DNA, *M. phlei* DNA, *E. coli* DNA, CpG methylase and DNase I was determined by a colorimetric *Limulus* amebocyte lysate assay using the conditions established by the manufacturer (QCL-1000, BioWhittaker, Walkersville, Md.). The sensitivity of the *Limulus* amebocyte lysate in this assay is 5.5 pg endotoxin/ml.

Purification of DNA

DNA was purified from mycobacterial cell wall complex (Bioniche Life Sciences Inc., London, Ontario) or from *E. coli* (strain XL-1 Blue, Stratagene, La Jolla, Calif.) by phenol extraction and ethanol precipitation [13]. Frozen *M. phlei* (strain 110; Bioniche Life Sciences Inc.) was thawed and suspended in 50 mM TRIS/HCl, 5 mM EDTA, pH 8.0. Lysozyme (Sigma-Aldrich Canada, Oakville, Ontario) was added to a final concentration of 1 mg/ml, and the mixture was incubated for 90 min at 37 °C. Proteinase K (Gibco Life Sciences) and sodium dodecyl sulfate were added to final concentrations of 0.1 mg/ml and 1% respectively, and the incubation was continued for 10 min at 65 °C. The mixture was then phenol-extracted and the DNA precipitated by ethanol as described for the mycobacterial cell wall complex. DNA was analysed by electrophoresis in 0.5% agarose gels containing 0.5 μg/ml ethidium bromide (3 h, 100 V). The molecular mass distribution of the DNA was analyzed after photo-scanning the gel using 1D software (Advance American Biotechnology, Fullerton, Calif.). DNA from herring sperm and calf thymus were purchased from Sigma-Aldrich Canada. The immunomodulatory CpG oligonucleotides GCTAGACGTTAGCGT [19] and ATCGACTCTCGAGCGTTCTC [18] were prepared by conventional solid-phase synthesis (Hukabel Scientific Ltd., Montréal, Québec).

In vitro cytokine and nitric oxide (NO) determination

Cells (murine macrophages, THP-1 or HL-60 cells) were seeded at 1×10^6 cells/ml in six-well flat-bottomed microplates and treated with different concentrations of mycobacterial cell wall complex, DNA extracted from mycobacterial cell wall complex, DNA from *M. phlei*, DNA from *E. coli*, lipopolysaccharide (LPS; *E. coli* 011:B4; Sigma-Aldrich), mycolic acid from *M. tuberculosis* (Sigma-Aldrich), synthetic *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP; Sigma-Aldrich) or cell wall skeleton from *M. phlei* (Ribi Immunochem Research, Hamilton, Mont.) for 48 h. Human IL-12 and murine IL-6, IL-12 and tumor necrosis factor α (TNFα) synthesis were measured after 48 h by means of a commercial enzyme-linked immunosorbent assay (ELISA; all from Biosource, Camarillo, Calif.). The IL-12 ELISA kit detects both the p70 form and p40 subunit. NO_2^-, the stable end-product of NO synthesis, was measured after 48 h incubation by mixing 50 μl supernatant with 50 μl Griess reagent as previously described [12]. The relative potency obtained with mycobacterial cell wall complex, cell wall skeleton and DNA extracted from cell wall complex or from *M. phlei* was determined using Pharm/PCS (version 4) software (Microcomputer Specialists, Philadelphia, Pa.).

In vivo IL-12 determination

Groups of four female CD1 mice were injected i.p. with 3.3 mg/kg body weight of mycobacterial cell wall complex or mycobacterial cell wall complex treated with DNase I. Serum IL-12 was determined by ELISA (BioSource) 0, 3, 6, 8 and 24 h after injection.

DNase I treatment

Mycobacterial cell wall complex (1 μg) was incubated with 1 U RNase-free DNase I (Gibco Life Science) for 1 h at 25 °C in 20 mM TRIS/HCl (pH 8.4), 2 mM $MgCl_2$ and 50 mM KCl. DNase I was inactivated by the addition of EDTA (final concentration 2.5 mM) followed by a 10-min incubation at 65 °C. In a number of experiments an additional cycle of DNase I digestion was performed for 1 h at 37 °C or the incubation time was extended to 24 h.

Charge-neutralization of DNA by DPPC/DOTAP liposomes

Liposomes were prepared under sterile conditions as previously described using depyrogenized glassware (180 °C, 4 h) [23]. Briefly, dipalmitoylglycerophosphocholine (DPPC) and the cationic lipid dioleoyltrimethylammonium propane (DOTAP: both from Avanti Polar Lipids, Alabaster, Ala.) were dissolved at a 1:1 molar ratio in chloroform and evaporated to dryness at 60 °C in a round-bottomed flask by rotary evaporation in vacuo. Liposomes were prepared by adding the required volume of sterile pyrogen-free NaCl

(0.85% w/v) followed by vigorous agitation. Liposomes were mixed with mycobacterial cell wall complex, *M. phlei* DNA or lipopolysaccharide (LPS) at ratios of 1:1, 5:1, 10:1 and 30:1 (w/w), and incubated for 30 min at room temperature. The neutralization of DNA in the mycobacterial cell wall complex by cationic liposomes, as determined by charge neutralization, was measured by Doppler electrophoretic light scattering using a Coulter DELSA 440 SX apparatus (Coulter Corp., Miami, Fla.) and expressed as the zeta (ζ) potential [12].

DNA CpG methylation

M. phlei DNA, *E. coli* DNA or DNA extracted from the cell wall complex was treated with 2.5 U CpG *Sss*I methylase (New England Biolabs, Mississauga, Ontario)/μg DNA in 10 mM TRIS/HCl (pH 7.9), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol and 160 μM *S*-adenosylmethionine for 1 h at 37 °C. The resistance of methylated *M. phlei* DNA to cleavage with the restriction enzyme *Bst*UI (New England Biolabs: 1 h at 37 °C using the manufacturers recommended buffer: enzyme sequence specificity CG↓CG) was determined by electrophoresis in 0.7% agarose gels containing 0.5 μg/ml ethidium bromide (3 h, 100 V).

DNA fragmentation

DNA extracted from *M. phlei* was sonicated using an ultrasonic processor (model W-38; Heat Systems-Ultrasonics Inc., Farmingdale, N.Y.) for 30 s or 15 min at 0 °C, or was cleaved by the restriction endonuclease *Bst*UI using the conditions described by the supplier. Analysis of DNA molecular mass was performed by electrophoresis in 2.0% agarose gels as described above.

Results

Endotoxin content in mycobacterial cell wall complex, purified DNA and enzymes

Endotoxin (LPS), a cell-wall component of gram-negative bacteria, has the ability to induce the synthesis of a number of cytokines by immune effector cells. The endotoxin content of mycobacterial cell wall complex, purified DNA, CpG methylase (from *E. coli*) and DNase I (bovine pancreas) was evaluated since murine macrophages and human monocytic cells are very sensitive to endotoxins [8]. Mycobacterial cell wall, DNA purified from the cell wall and from *M. phlei*, and DNase I enzyme contained less than 5.5 pg endotoxin/ml (detection limit of the colorimetric *Limulus* amebocyte lysate assay), while the DNA from *E. coli* contained approximately 3.0 ng endotoxin/μg DNA and the CpG methylase contained approximately 1.0 ng endotoxin/U enzyme. These concentrations of endotoxin are significantly less than those required for immune stimulation since, in our assay using murine macrophages and human monocytic THP-1 cells, LPS does not induce the synthesis of IL-6, IL-12 or TNFα at concentrations below 10 ng/ml (data not shown).

Mycobacterial DNA induces IL-12 synthesis by monocytes and macrophages

We have observed that mycobacterial cell wall complex prepared from *M. phlei* contains approximately 50 μg mycobacterial DNA/mg cell wall. We have purified this DNA by phenol extraction and ethanol precipitation, and have analyzed it by electrophoresis in 0.5% agarose. We found that the mycobacterial cell wall complex contains DNA molecules ranging from high-molecular-mass genomic DNA to oligonucleotides of low molecular mass (approx. 5 bp), with a large proportion (85%) of the DNA molecules being approximately 10–250 bp in length. In comparison, DNA extracted from *M. phlei* has a larger proportion of genomic DNA. To evaluate whether mycobacterial DNA can induce IL-12 synthesis, we have incubated DNA extracted from mycobacterial cell wall complex or from *M. phlei* with monocytes and macrophages. We have found that DNA isolated from the cell wall complex or from *M. phlei* has the ability to induce high levels of IL-12 synthesis by murine macrophages and human monocytic THP-1 cells and low levels by human pro-myelocytic HL-60 cells (Fig. 1A, B). Since the mycobacterial cell wall complex contains approximately 5% mycobacterial DNA, the potencies obtained with the cell wall complex relative to mycobacterial DNA (1) were ~51 for murine macrophages, ~24 for THP-1 cells and ~23 for HL-60 cells. DNA of eukaryotic origin (calf thymus and herring sperm) did not induce IL-12 synthesis (Fig. 1A, B). DNA isolated from mycobacterial cell wall complex or from *M. phlei* also has the capacity to induce the synthesis of IL-12 in vivo (Fig. 1C), but to a lesser extent than the cell wall complex. Other immunomodulatory cytokines such as TNFα and IL-6 were also produced by murine macrophages and human THP-1 cells in response to treatment with *M. phlei* DNA (Fig. 1D). NO was produced only by murine macrophages in response to *M. phlei* DNA (Fig. 1D). Murine B or T lymphocytes isolated from splenocytes did not synthesize cytokines (IL-2, IL-4, IL-6, IL-10, IL-12 and TNFα) in response to mycobacterial DNA (data not shown).

The immunostimulatory effect of a crude membrane extract prepared from *Babesia bovis* has been shown to be abrogated by treatment with DNase I [2]. Treatment of the mycobacterial cell wall complex with DNase I, which digests both single- and double-stranded DNA to oligodeoxyribonucleotides, resulted in a 60% reduction in the synthesis of IL-12 by THP-1 cells and a 92% reduction of IL-12 synthesis by HL-60 cells (Fig. 2A). The i.p. administration of mycobacterial cell wall complex treated with DNase I to CD1 mice resulted in a 55% lower level of IL-12 than in untreated mycobacterial cell wall complex (Fig. 2B). The synthesis of IL-12 in THP-1 cells was also induced by a *M. phlei* cell wall skeleton preparation but to a lesser extent than by mycobacterial cell wall complex (Fig. 2C). The cell wall complex was significantly more potent than cell wall skeleton in inducing IL-12 synthesis (relative potency 50, 95% confidence limits 47–53, $P < 0.05$ versus cell wall skeleton). The lower activity of the cell wall skeleton in comparison to mycobacterial cell wall complex appeared to correlate with the quantity of associated DNA, the cell wall skeleton having approximately ten times less DNA (0.5 μg DNA/mg cell skeleton) than the mycobacterial cell wall complex.

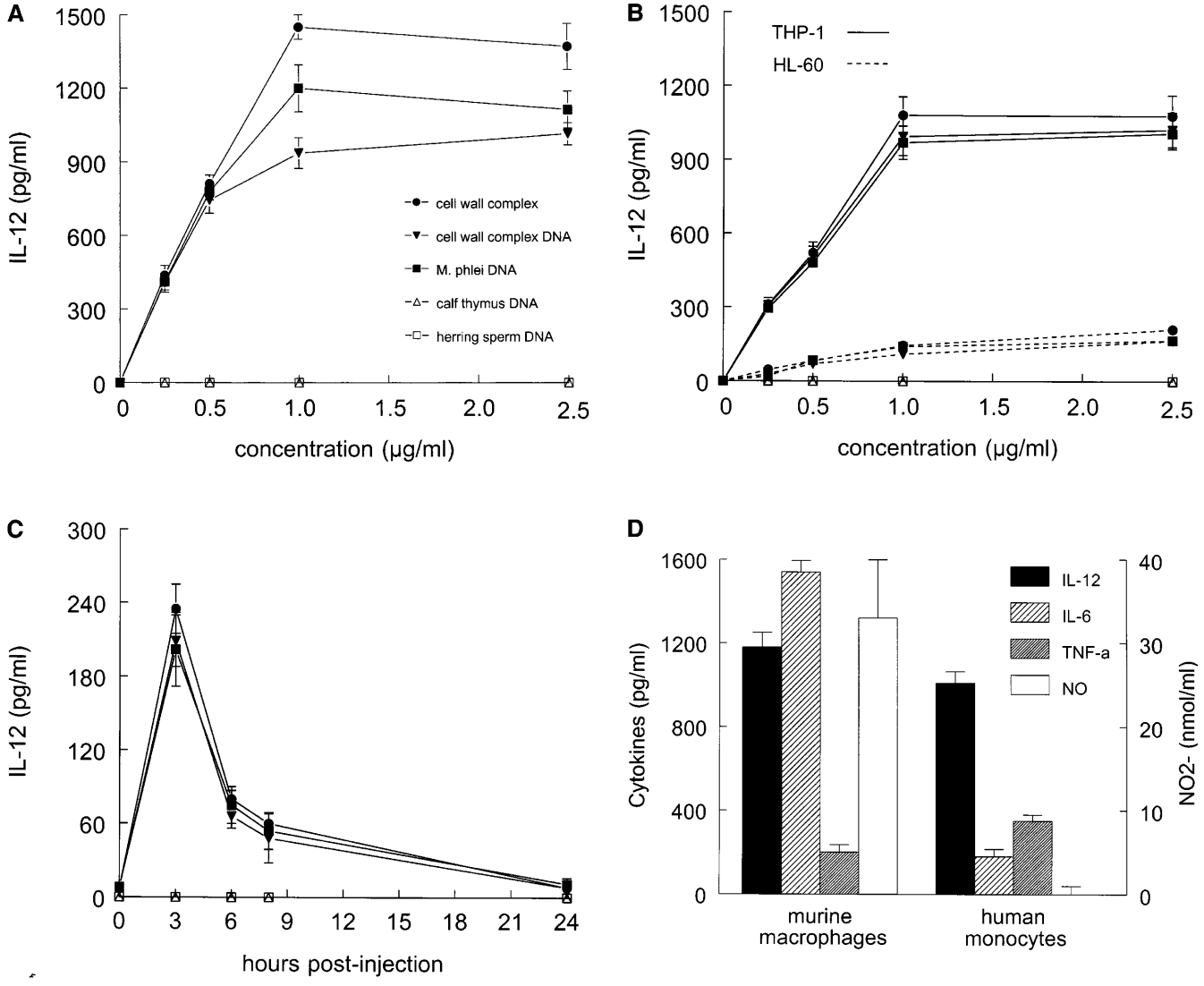

Fig. 1A–D Induction of cytokines and NO by mycobacterial DNA. Murine macrophages (**A**), THP-1 monocytes (**B**) or HL-60 pro-monocytic cells (**B**) were incubated at 1×10^6 cells/ml with mycobacterial cell wall complex, *M. phlei* DNA, DNA purified from the mycobacterial cell wall complex, herring sperm DNA or calf thymus DNA for 48 h. Interleukin-12 (*IL-12*) levels in the supernatant were measured after 48 h by enzyme-linked immunosorbent assay (ELISA). **C** Groups of four CD1 mice were injected i.p. with 3.3 mg/kg body weight of mycobacterial cell wall complex, *M. phlei* DNA, DNA purified from the mycobacterial cell wall, herring sperm DNA or calf thymus DNA. Serum IL-12 levels were determined 0, 3, 6, 8 and 24 h after injection, by ELISA. **D** Murine macrophages and human monocyte (THP-1 cells) were incubated with 1 µg/ml *M. phlei* DNA for 48 h. IL-6, tumor necrosis factor α (*TNFα*) and NO_2^-, one of the end-products of NO synthesis, were measured in the supernatant after 48 h as described in Materials and methods. Data for **A, B** and **D** are expressed as the mean ± SD of three independent experiments while data from **C** are expressed as the mean ± SD of four mice per group

DNA molecular size does not modify IL-12 induction

To determine whether the size of the mycobacterial DNA was important for the induction of IL-12 synthesis, we prepared fragments of different size by sonication or by digestion of *M. phlei* DNA with the restriction endonuclease *Bst*UI, which recognizes the sequence CG↓CG. The digestion of *M. phlei* genomic DNA (69% CG content [42]), with *Bst*UI gave a large number of differently sized fragments ranging from approximately 5 bp to 12 000 bp, while sonication gave defined populations of less than 100 bp (30 s sonication) or less than 20 bp (15 min sonication). We found no significant difference in the quantity of IL-12 produced by 1×10^6 THP-1 monocytes in response to either 1.0 µg/ml DNA of less than 20 bp (969 ± 67 ng/ml, mean ± SD of three experiments), DNA less than 100 bp (999 ± 70 ng/ml) or *Bst*UI-derived fragments (1002 ± 80 ng/ml) in comparison to intact *M. phlei* DNA (995 ± 77 ng/ml).

CpG methylation status does not modify IL-12 induction

It has been reported that the ability of bacterial DNA (for example from *E. coli*) to induce cytokine synthesis is

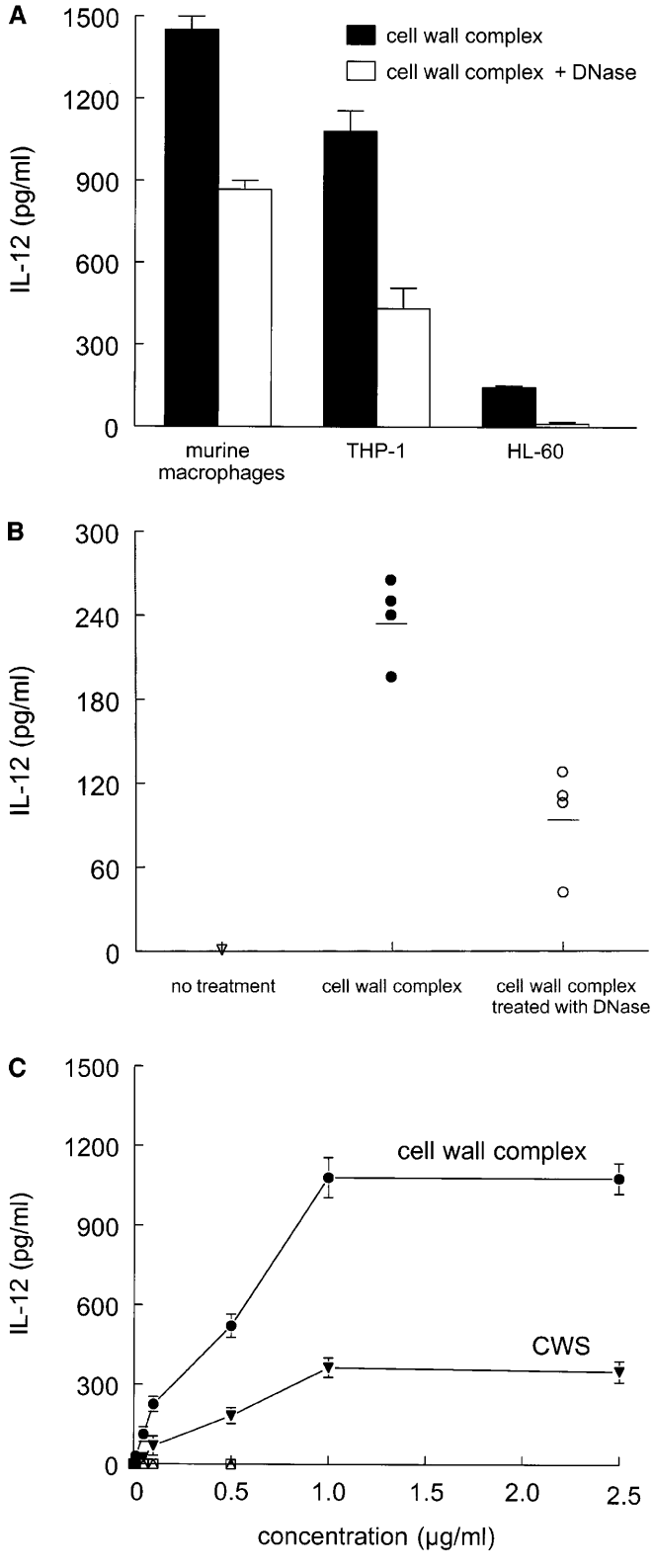

Fig. 2A–C DNase treatment of mycobacterial cell wall complex reduces its ability to induce IL-12. **A** THP-1 cells or HL-60 cells at 1×10^6 cells/ml were incubated with 1 μg mycobacterial cell wall complex or with 1 μg mycobacterial cell wall complex treated with DNase I for 48 h. **B** Groups of four CD1 mice were injected i.p. with 3.3 mg/kg mycobacterial cell wall complex or with 3.3 mg/kg mycobacterial cell wall complex treated with DNase I. **C** 1×10^6 THP-1 cells/ml were incubated with the mycobacterial cell wall complex or cell wall skeleton (*CWS*) for 48 h. Mycobacterial cell wall complex contains approximately 5 μg/ml DNA while cell wall skeleton contains approximately 0.5 μg/ml (data not shown). IL-12 levels in the supernatants of treated cells were measured after 48 h (**a**, **c**) while IL-12 serum levels (**b**) were determined 3 h after injection by the appropriate ELISA. Mice injected with mycobacterial cell wall complex and inactivated DNase show no reduction of IL-12 produced (data not shown). Data are expressed as the mean ± SD of three independent experiments except for the experiment done in mice (data expressed as the actual values and median from a typical experiment)

associated with the presence of unmethylated CpG dinucleotide motifs [16, 38]. Contrary to the inhibition of cytokine synthesis seen following the methylation of cytosine in bacterial DNA [19], treatment of DNA extracted from *M. phlei* with CpG methylase did not modify its ability to induce the synthesis of IL-12 (Fig. 3A). Similar results were obtained with DNA purified from the mycobacterial cell wall complex (data not shown). In contrast, methylation of DNA isolated from *E. coli* abolished its IL-12-synthesis-inducing activity (Fig. 3A). As shown in Fig. 3B, the methylation of cytosine residues in *M. phlei* DNA appeared to be complete since methylated *M. phlei* DNA was protected against digestion by the restriction enzyme *Bst*UI.

We have determined the ability of two CpG-motif-containing oligonucleotides with a natural phosphodiester backbone (GCTAGACGTTAGCGT and ATCGACTCTCGAGCGTTCTC) to induce IL-12 synthesis by THP-1 cells in comparison to mycobacterial DNA. These CpG motif-containing oligonucleotides have been reported to induce significant levels of IL-12 only after the substitution of the nonbridging oxygen atom on the phosphate group with a sulfur atom (phosphorothioate modification) [18, 19]. We have found that natural CpG-motif-containing phosphodiester oligonucleotides were only active at concentrations of at least 25.0 μg/ml with THP-1 cells, while mycobacterial DNA showed optimal activity at 1.0 μg/ml.

IL-12 synthesis is not due to the presence of MDP or mycolic acid

The inability of DNase treatment to abolish completely the activity of the cell wall complex in vitro with THP-1 cells and in vivo (Fig. 2A, B) suggested that other components of the mycobacterial cell wall complex could be responsible, in part, for the induction of IL-12 synthesis. MDP or mycolic acid present in this mycobacterial cell wall complex preparation are potential candidates for the induction of IL-12. MDP is known to induce a wide variety of monocytes and macrophage cytokines [33]. The incubation of MDP with THP-1 and HL-60 cells in the concentration range 0.1–10.0 μg/ml for 48 h did not induce the synthesis of IL-12. Similar

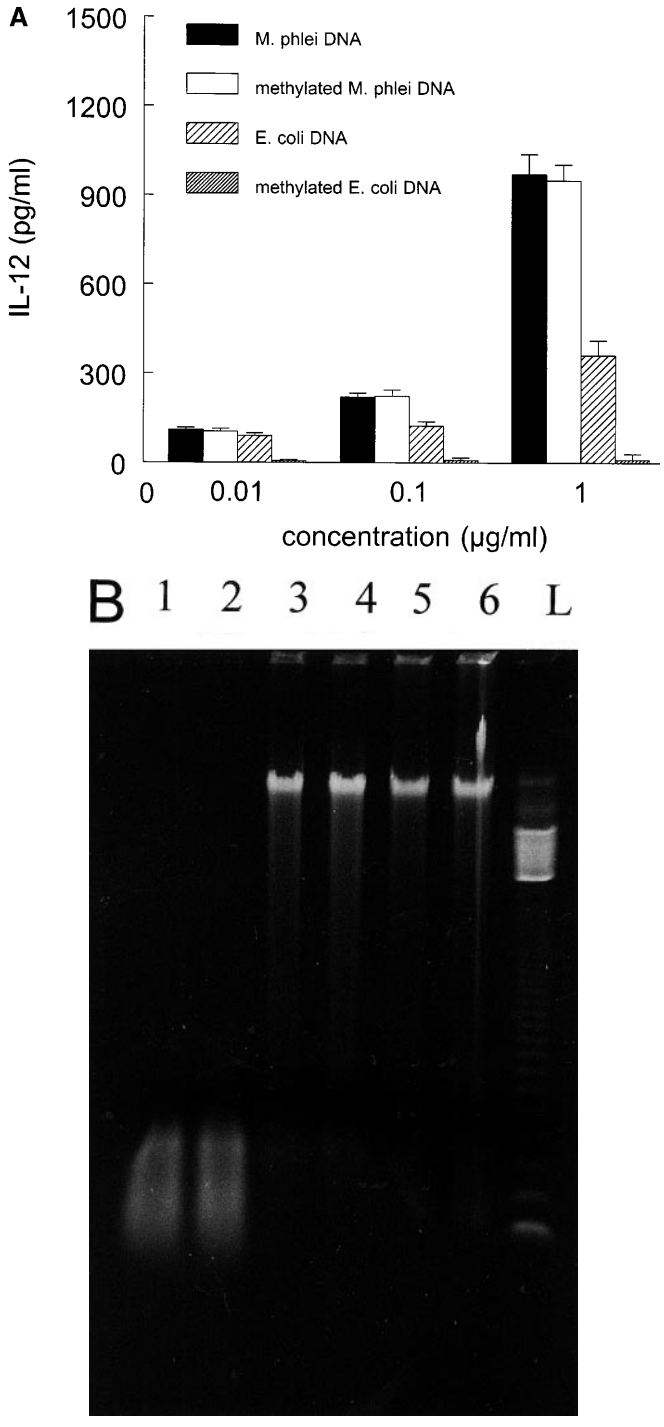

Fig. 3A, B DNA methylation does not modify IL-12 synthesis by *M. phlei* DNA. **A** *M. phlei* DNA and *E. coli* DNA (0.01–1 µg) was treated with 2.5 U CpG *Sss*I methylase/µg DNA and *S*-adenosylmethionine for 1 h at 37 °C as described in Materials and methods. Untreated or methylated *M. phlei* DNA or *E. coli* DNA was incubated with 1×10^6 THP-1 cells/ml for 48 h. IL-12 in the supernatant was measured after 48 h by ELISA. Data are expressed as the mean ± SD of three independent experiments. **B** The efficacy of cytosine residue methylation in *M. phlei* DNA was revealed by electrophoresis in 0.7% agarose gel containing 0.5 µg/ml ethidium bromide (3 h, 100 V). *Lanes*: *1*, *2*, *M. phlei* DNA incubated with *Bst*UI restriction enzyme; *3*, *4*, methylated *M. phlei* DNA incubated with *Bst*UI restriction enzyme; *5*, *6*, *M. phlei* DNA; *L* 123-bp marker (Gibco Life Sciences)

results have also been reported by Cleveland et al. [6]. Mycolic acid does not have the ability to induce the synthesis of IL-12 by either THP-1 or HL-60 cells in the concentration range 0.1–10.0 µg/ml.

Charge-neutralization of DNA by cationic liposomes

Since DNase treatment did not completely inhibit the ability of the mycobacterial cell wall complex to induce IL-12 synthesis, we determined whether this could be achieved by the neutralization of DNA by a cationic lipid. Non-transfecting liposomes formulated with the phospholipid DPPC and the cationic lipid DOTAP can be used to neutralize anionic DNA molecules without toxicity [10]. The titration of the cell wall complex by DPPC/DOTAP liposomes resulted in a gradual inhibition of the ability of the complex to induce IL-12 synthesis (Fig. 4). This correlated with a change in ζ potential from negative to positive, indicative of DNA neutralization (Table 1). In contrast, the gradual addition of DPPC/DOTAP liposomes to LPS did not modify its ability to induce IL-12 synthesis (Fig. 4). The reduction in IL-12 synthesis was almost 100% following neutralization by DPPC/DOTAP liposomes (Table 1 and Fig. 4). Identical results were obtained with purified DNA from *M. phlei* (Fig. 4). These results support the hypothesis that mycobacterial DNA is, in fact, primarily

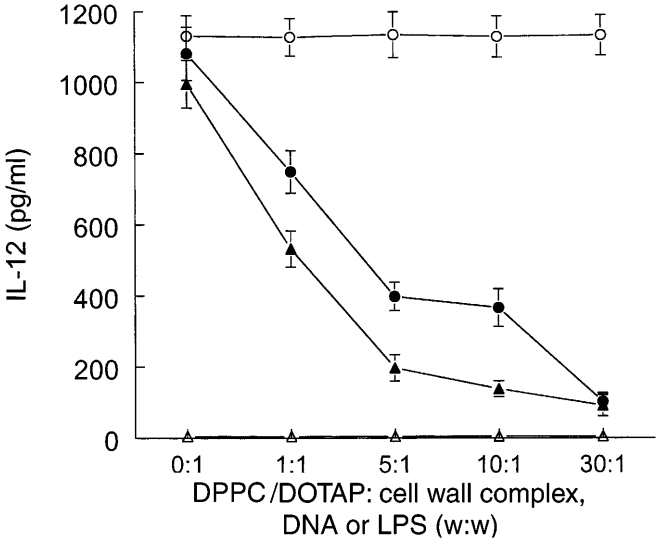

Fig. 4 Neutralization of DNA by DPPC/DOTAP inhibits the synthesis of IL-12. Cationic DPPC/DOTAP liposomes were incubated with 1 µg mycobacterial cell wall complex, *M. phlei* DNA or lipopolysaccharide (*LPS*) at ratios of 1:1, 5:1, 10:1 and 30:1 (w:w) for 30 min at room temperature. 1×10^6 THP-1 cells/ml were then incubated with the different mixtures for 48 h. IL-12 in the supernatant was then measured after 48 h by ELISA. Data are expressed as the mean ± SD of three independent experiments. -●- cell wall complex + $Pam_2GroPcho$/DOTAP; -▲- *M. Phlei* DNA + DPPC/DOTAP; -○- LPS + DPPC/DOTAP; -△- DPPC/DOTAP

Table 1 Charge neutralization of mycobacterial DNA associated with the mycobacterial cell wall complex (*MCC*) by cationic liposomes. Unilamellar DPPC/DOTAP liposomes (1:1 molar ratio) were mixed with mycobacterial cell wall complex in the quantities indicated and incubated for 30 min at room temperature. The neutralization of DNA associated with the cell wall complex was evaluated by the measurement of liposome zeta (ζ) potential [10]. ζ potential was determined in NaCl (0.85% w/v) at 25 °C by Doppler electrophoretic light scattering. The results are expressed as the mean ± SD from three independent experiments

MCC (mg)	DPPC/DOTAP (mg)	ζ Potential (mV)
1.0	–	−9.1 ± 5.1
1.0	1.0	−6.5 ± 3.1
1.0	5.0	+18.3 ± 4.4
1.0	10.0	+28.5 ± 7.1
1.0	30.0	+31.8 ± 9.0
–	1.0	+32.1 ± 8.5

responsible for the induction of IL-12 by the mycobacterial cell wall complex.

Discussion

This study has shown that the presence of DNA significantly contributes to the overall immunomodulatory activity of a mycobacterial cell wall complex. The results also demonstrate that CpG motifs do not contribute to this activity. Treatment of the mycobacterial cell wall complex with DNase I significantly reduced, and DNA neutralization by cationic liposomes abolished, the ability of the mycobacterial cell wall complex to induce the synthesis of IL-12. These results indicate that although a significant proportion of the DNA in mycobacterial cell wall complex does not appear to be accessible to enzyme degradation, it is accessible to neutralization by the small cationic lipid DOTAP. DNA extracted from the mycobacterial cell wall complex retains the ability to induce IL-12, but to a lesser extent than when complexed with the cell wall.

Several groups have recently reported that bacterial DNA acts on the immune system. DNA from *E. coli*, *Micrococcus lysodeikticus* and *M. bovis* strain BCG can induce the synthesis of a number of cytokines by immune cells [16, 39]. The stimulation of the immune system by bacterial DNA has been reported to be due to the presence of unmethylated CpG dinucleotides flanked by two 5′ purines and two 3′ pyrimidines (CpG motifs) [20]. Bacterial DNA or synthetic oligodeoxynucleotides containing unmethylated CpG motifs induce the synthesis of a number of cytokines by macrophages and B lymphocytes [18, 35], increase the activity of NK cells [1] as well as cytotoxic T lymphocytes [22], and enhance T helper-1 responses [32]. Furthermore, DNA from a variety of other organisms (insects, yeasts, nematodes and molluscs) has been shown to stimulate B cell proliferation [38]. In all these cases, the immunomodulatory activity of DNA is correlated with the presence of unmethylated CpG motifs. Methylation of DNA with CpG methylase inhibits a number of these activities [19, 32, 35]. For example, the ability of CpG-containing oligonucleotides to induce the synthesis of IL-6, IL-12 and interferon γ (IFNγ) by spleen cells is abolished following methylation [19]. The immunomodulatory activity of *M. phlei* DNA could potentially be due to the presence of CpG motifs since its genome content is 69% CG [42]. However, the successful methylation of mycobacterial DNA by CpG methylase failed to abolish its immunomodulatory activity, demonstrating that the hypomethylation status of cytosine present in *M. phlei* DNA is not an important factor. These results are strongly indicative of immune stimulation by motifs other than CpG.

The ability of *M. phlei* DNA lacking the CpG motif to stimulate IL-12 production by monocytes and macrophage raises the possibility that the effect of DNA on the immune system is pluralistic, and may differ according to the composition of the DNA sequence. For example, Klinman et al. [18] have observed that although a number of oligonucleotides containing CpG motifs induce the synthesis of IL-6 and IL-12 by B cells, IL-6 and IFNγ by $CD4^+$ T cells and IFNγ by NK cells, they are not able to induce the synthesis of these cytokines by monocytes and macrophages. Other CpG motifs have been shown to activate NK cells specifically [1] or to act only on macrophages in inducing the production of TNFα, IL-6 and IL-12 [35]. We have found that *M. phlei* DNA associated with the mycobacterial cell wall complex acts differentially on monocytic THP-1 cells and on pro-myelocytic HL-60 cells, inducing the synthesis of a large quantity of IL-12 by THP-1 cells and a smaller quantity by HL-60 cells, while showing no activity towards B and T lymphocytes. Treatment with DNase I completely abolished the activity of the mycobacterial cell wall complex towards HL-60 cells but not towards THP-1 cells. The difference between THP-1 and HL-60 cells in their ability to respond to non-CpG mycobacterial DNA cannot be attributed to their capacity to synthesis IL-12, since both these cell lines produce high levels of IL-12 in response to LPS or to lipoteichoic acid (data not shown). Furthermore, we have found that DNA associated with the mycobacterial cell wall complex has the ability to induce apoptosis of bladder cancer cells [27] and that this activity was clearly dissociable from its capacity to induce IL-12 synthesis. Experiments are currently underway to determine the nucleotide motifs responsible for cytokine induction as opposed to apoptosis induction.

We have shown, by sonication or by digestion with the restriction enzyme *Bst*UI, that the size of the DNA fragments does not influence their ability to induce the synthesis of IL-12 by monocytes and macrophages. Very small fragments (less than 20 bp) or genomic DNA extracted from *M. phlei* both possess the ability to induce IL-12 synthesis. Messina et al. [23] have also found that DNA fragment size is not important for the stimulation of B lymphocytes proliferation. They have

demonstrated that *E. coli* DNA (more than 20 000 bp) is able to stimulate B proliferation to the same extent as fragmented DNA in the size ranges 50–300 bp and 125–600 bp Oligonucleotides as small as 5 or 6 bases have been shown to act on the immune system in a manner similar to bacterial genomic DNA [15, 21]. We are actively investigating the potential for such CpG-lacking oligonucleotides to act as immunostimulants (manuscript in preparation).

DNA extracted from the mycobacterial cell wall complex or from *M. phlei* was less effective than DNA associated with the cell wall complex in inducing IL-12. Peptidoglycan associated with the cell wall may, in fact, act as a carrier for DNA. Peptidoglycan, which possesses a positive charge due to the presence of charged amino acids [4], forms stable complexes with anionic nucleic acids over a long period of time (unpublished observations). Peptidoglycan preparations have been shown to induce cytokines and to possess anticancer activity [7, 9, 14, 34]. The presence of nucleic acids in these preparations has not, however, been determined. Experiments are currently underway to quantify DNA levels in a number of peptidoglycan preparations and to determine its role in stimulating immune responses. Our results strongly suggest that peptidoglycan derived from *M. phlei* is, in fact, inert and is unable to induce the synthesis of cytokines by monocytes and macrophages, since charge neutralization of DNA by cationic liposomes abolishes the immunomodulatory activity of the mycobacterial cell wall complex. Furthermore, it appears that *M. phlei* peptidoglycan present in the mycobacterial cell wall complex has the ability partially to protect complexed DNA from degradation by DNase I. Approximately 50% of the *M. phlei* DNA complexed to the cell wall was protected from digestion by DNase I even after an additional cycle of DNase I digestion for 1 h at 37 °C or the extension of the incubation time to 24 h (unpublished observation). This observation could explain the incomplete abolition of IL-12 synthesis following DNase I treatment. Therefore, this raises the possibility that *M. phlei* peptidoglycan, or other contaminating cell wall materials, could also protect CpG motifs from DNase digestion or methylation. We are currently determining if DNA remaining with the cell wall following DNase treatment is enriched in CpG motifs.

Stabilisation of the DNA backbone and protection against nuclease digestion have also been associated with an increase in nucleic acid immunomodulatory activity in other model systems. Kataoka et al. [17] have shown that IFN synthesis induced by DNA in the guinea-pig increased when the DNA was complexed with poly-L-lysine and solubilized by the addition of carboxymethylcellulose. Furthermore, it has been shown that the substitution of the phosphodiester linkage by its phosphorothioate analog in known immunomodulatory oligonucleotides increases their ability to induce cytokine synthesis and to activate B cells [15, 20]. For example, Klinman et al. [18] have shown that phosphorothioate CpG oligonucleotides are approximately 30-fold more efficient than phosphodiester CpG oligonucleotides in their abiltity to induce the synthesis of IL-6, IL-12 and IFNγ by murine spleen cells. However, phosphorothioate oligonucleotides have significant in vivo toxicity associated with a non-specific stimulation of the immune system [24]. Rodents appears to be acutely sensitive to immune stimulation induced by phosphorothioate oligonucleotides [25]. Previous studies have shown that phosphorothioate modification of CpG-containing oligonucleotides appears to be a prerequisite for efficient cytokine induction [18]. This study has confirmed the relative ineffectiveness of phosphodiester-CpG-containing oligonucleotides as cytokine inducers. In contrast, *M. phlei* DNA in the form of short oligonucleotides (fewer than 20 bp) with a natural phosphodiester backbone is able to induce significant cytokine synthesis. When complexed with the cell wall, the relative potency of mycobacterial DNA [43] was increased 23- to 51-fold according to the cell type used. Cell wall may therefore function in much as the same way as poly-L-lysine or as a phosphorothioate by increasing the resistance of DNA to nuclease digestion. However, the addition of exogenous *M. phlei* DNA or purified DNA from mycobacterial cell wall complex to mycobacterial cell wall preparations treated with DNase does not reconstitute the ability of the mycobacterial cell wall complex to induce high levels of IL-12 synthesis by monocytes and macrophages (data not shown). These results are consistent with a structure where short sequestered fragments of complexed DNA remain anchored to a potential peptidoglycan binding site following DNase digestion, thus preventing the binding of exogenous DNA.

We have found in this study that two different compositions of *M. phlei* cell wall compositions mycobacterial cell wall complex and cell wall skeleton, differ significantly in their capacity to induce IL-12 synthesis by monocytes and macrophages. The lower activity of the cell wall skeleton in comparison to the mycobacterial cell wall complex preparation correlates with the quantity of associated DNA, cell wall skeleton having ten times less DNA than the mycobacterial cell wall complex. Experiments are underway to determine whether the ability of other cell wall compositions known to have immunomodulatory and/or anticancer activity is correlated with the presence of DNA.

In summary, this study has found that the presence of DNA complexed with *M. phlei* cell wall significantly contributes to its overall immunomodulatory activity. This activity does not correlate with the presence of CpG motifs. The complex between mycobacterial cell wall and DNA may, in addition to its ability to induce apoptosis directly in tumor cells [27], possess indirect activity by stimulating monocytes and macrophages to synthesize IL-12, a potent anticancer cytokine. The combination of these activities may be an important factor in the overall anticancer activity of such cell wall compositions. Finally, our results emphasize that DNA

extracted from mycobacterial cell wall complex or from *M. phlei* may have potential as an immunoadjuvant for cancer therapy by modulating the synthesis of IL-12.

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