Biological activity of a peptidoglycan extracted from *Leptospira interrogans*: *in vitro* studies

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Peptidoglycan (PG) has been isolated from some species of spirochaetes, including *Leptospira interrogans*. Although leptospiral PG has been chemically characterized, no study has been carried out on its potential biological activity. Since PG of *Treponema* and *Borrelia* is biologically active both *in vivo* and *in vitro*, we investigated the capacity of a leptospiral PG preparation to induce relevant biological effects. PG extracted from *L. interrogans* strain Teramo was mitogenic at 0.1 µg ml⁻¹ for human peripheral blood mononuclear cells (PBMC) since it increased the PBMC fraction positive for Ki-67, an antigen expressed by human proliferating cells; at 4 µg ml⁻¹, PG was able to induce complement consumption and to stimulate leucocyte phagocytosis and the metabolic burst of resting as well as phagocytosing leucocytes. These findings indicate that *Leptospira* PG may play a role in modulating the immunocompetent cell functions and suggest that PG can contribute to the host response during *Leptospira* infection.

Introduction

The presence of cell wall peptidoglycans in the *Spirochaetales* has been demonstrated for some genera and species, e.g. *Borrelia duttoni*, *Borrelia burgdorferi* (Ginger, 1963; Beck *et al.*, 1990), *Spirochaeta stenostrepta*, *Spirochaeta litoralis* (Joseph *et al.*, 1973), Reiter *Treponema*, *Treponema pallidum* (Pillot & Reiter, 1965; Umemoto, 1981) and *Leptospira* (Ginger, 1963; Azuma, 1975). Chemical analysis revealed that the structure of spirochaete peptidoglycans was of the A₂-type, according to the classification of Schleifer & Kandler (1972), and that the diamino acid involved in cross-linking was ornithine in *Spirochaeta*, *Borrelia* and *Treponema*, or diaminopimelic acid for strains representative of the genera *Leptospira* (*L. interrogans* and *L. biflexa*) and *Leptonema* (Yanagihara *et al.*, 1984). Peptidoglycans and muramyl peptide derivatives have diverse and complex biological effects in mammals, including stimulation of cells involved in the immune response, activation of the

complement cascade, adjuvant effect, induction of inflammation, activation of monocytes and neuropharmacological activity (Kotani *et al.*, 1986; Stewart-Tull, 1980). Some of these biological activities have been observed in ornithine A-type PG preparations of *B. burgdorferi* (Beck *et al.*, 1990) and *T. pallidum* (Umemoto *et al.*, 1981). However, no study has been carried out on the biological effects induced by *Leptospira* PG which, in contrast to the other spirochaetes, is pimelic A₂-type (Yanagihara *et al.*, 1984).

Recently, we found that whole cell preparations of *Leptospira* strains were able to elicit a significant proadhesive effect in human endothelial cells (HEC) towards neutrophils, which may represent the first step in vasculitis (A. Dobrina and others, unpublished data). The leptospiral component responsible for this effect was demonstrated to reside in a PG preparation, whereas no activity was found in a *Leptospira* LPS preparation. Since LPS of Gram-negative bacteria is known to induce the proadhesive activity on HEC besides a variety of other biological effects (Schleimer & Rutledge, 1986), we questioned whether peptidoglycans were responsible for further biological activities induced in the host by pathogenic leptospires. Therefore we investigated the capacity of leptospiral PG to trigger relevant biological effects, such as complement activation, stimulation of the

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Abbreviations: HEC, human epithelial cells; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cells; PG, peptidoglycan; PMN, polymorphonuclear leucocytes.

neutrophil and monocyte phagocytosis and metabolic burst, and lymphocyte mitogenicity.

Methods

Leptospira strain and cultivation. The *Leptospira interrogans* strain used in this study was Teramo, which we classified (unpublished data) as serovar *copenhageni*, serogroup Icterohaemorrhagiae, from the *Leptospira* Reference Collection of our Institute. Leptospirae were grown in liquid EMJH medium (Johnson *et al.*, 1973), up to a density of about 10^8 cells ml⁻¹.

Peptidoglycan extraction. PG was extracted according to the procedure of Umemoto *et al.* (1981). Briefly, 5 litres of leptospire culture were harvested by centrifugation at 10000 *g* for 30 min, washed twice in 0.01 M-sodium phosphate buffer, pH 7.2, containing 0.85% (w/v) NaCl (PBS), and resuspended in 100 ml 1% (w/v) SDS in distilled water. The suspension was gently shaken at 37 °C for 18 h and then centrifuged at 110000 *g* for 60 min. After a second treatment with 1% (w/v) SDS the pellet was washed four times with 6 M-urea, resuspended in 200 ml distilled water and centrifuged at 1500 *g* for 10 min. The supernatant was collected and centrifuged at 110000 *g* and the resulting pellet was suspended in 1.46 ml 0.01 M-Tris/HCl buffer, pH 7.4, containing 1.46 mg trypsin; the reaction mixture was incubated at 37 °C for 18 h, washed with distilled water by centrifugation at 110000 *g* for 90 min and then digested with 1.46 mg pronase (Sigma) in 1.46 ml 0.01 M-Tris/HCl, pH 7.4. After digestion at 37 °C for 18 h the pellet was recovered by centrifugation and then lyophilized. The amount of PG extracted was 0.02% (w/w) of the dry weight of intact spirochaetes.

Amino acid analysis of the sample was carried out by gas chromatography, following the method of MacKenzie & Tenashuk (1979). Ornithine was used as an internal standard. Assays were performed on a HP 5890 gas chromatograph supplemented with an HP Ultra 1 capillary column. The analysis revealed a relative molar ratio of the most abundant amino acids, alanine, glutamic acid, glycine and meso-aminopimelic acid, of 0.8, 0.6, 0.66 and 0.62, respectively. Serine, arginine, asparagine, leucine, valine were also detected, but accounted for a very small percentage of the amino acids. This molecular composition was indicative of the presence of a *L. interrogans* PG, as already described (Yanagihara *et al.*, 1984). A 10 µg sample of PG was checked for the presence of LPS by SDS-PAGE and silver staining according to the method of Tsai & Frasch (1982), which detects less than 5 ng of rough LPS; no contamination by LPS was found. Moreover, the sample did not give any clotting activity in the Limulus test (Limusate; Haemachem Inc.), which is sensitive to endotoxin in the order of picograms ml⁻¹ up to a dilution of 10 nanograms ml⁻¹.

Haemolytic assay for complement activation by PG. The method described by Harrison & Lachmann (1986) was followed using a pool of fresh human sera as source of complement. Veronal-buffered saline (pH 7.2), as described by Harrison & Lachmann (1986), was employed for the haemolytic assay; EDTA-containing PBS (pH 7.4) was made with 0.15 M-NaCl, 5 mM-sodium phosphate, and 10 mM EDTA and was employed to block the lytic reaction at the end of the incubation time. Mixtures of 200 µl of Veronal-buffered saline containing 1/20 normal serum and various amounts of PG were added to 50 µl of sensitized sheep erythrocytes (3×10^8 cells ml⁻¹) according to Harrison & Lachmann (1986) and incubated for 30 min at 37 °C. The extent of erythrocyte lysis was measured at 415 nm after the addition of 1 ml ice-cold PBS-EDTA. The results of two repeated assays were expressed as CH50 units (ml serum)⁻¹, where 1 CH50 Unit was defined as that serum activity able to produce 50% haemolysis according to the Mayer technique described in the same reference.

Peripheral blood mononuclear cells (PBMC) preparation. Mononuclear cells were isolated from heparinized blood of healthy donors by Ficoll-Hypaque separation (Seromed), then washed and resuspended at a final concentration of 5×10^5 cell ml⁻¹ in RPMI-1640 (Seromed), supplemented with 2% of the synthetic serum Ultrosor G (Gibco-BRL), antibiotics and glutamine. Cultures were incubated at 37° for 48 h in the presence of *E. coli* LPS (Sigma) or PG at 0.1, 1, or 10 µg ml⁻¹. After the incubation, supernatants were removed by centrifugation at 750 *g*.

Mitogenic assay: antigen Ki-67 measurement. Ki-67 antigen is expressed by all human proliferating cells, preferentially during the G₁-G₂ phases and not in G₀ (Gerdes *et al.*, 1984). A fluorescein-isothiocyanate (FITC)-conjugated monoclonal antibody to Ki-67 (DAKO-Ki-67; Dakopatts) detects the amount of this antigen expressed by proliferating cells (Gerdes *et al.*, 1984) in a cell suspension. Flow cytometric analysis of fluoresceinated Ki-67 was performed to measure the growth fraction of cell populations with the same sensitivity as assay of [³H]thymidine uptake (Schwartz *et al.*, 1986). After exposure to various amounts of LPS and PG, PBMC were fixed with a paraformaldehyde/lysine/periodate-solution for 30 min at 40 °C. Paraformaldehyde/lysine/periodate solution was prepared according to the method described by McLean & Nakane (1974). The preparation was then washed, resuspended in PBS and stained with FITC-conjugated DAKO-Ki-67 monoclonal antibody, as described by the manufacturer (Dakopatts). Fluorescence intensity was read by flow cytometry and expressed as the percentage of proliferating cells (Schwartz *et al.*, 1986). Experiments were repeated in triplicate.

Assay for the simultaneous quantification of phagocytosis and oxidative products. We used the method described elsewhere (Perticarari *et al.*, 1991), with minor modifications. Briefly, leucocytes were isolated by dextran sedimentation of heparinized blood samples (5 IU heparin ml⁻¹) obtained from healthy volunteers. After centrifugation at 100 *g* for 10 min of the leucocyte-rich plasma, the remaining erythrocytes were lysed with 0.8% (w/v) NH₄Cl. The pellet was washed once and resuspended in Hanks' balanced salt solution (HBSS; Seromed), and resuspended in the same medium. FITC-labelled *Staphylococcus aureus* cells, prepared as described previously (Perticarari *et al.*, 1991), were opsonized with a pool of fresh human sera and used at a concentration of 10^8 cells ml⁻¹ in HBSS. For the assay, 100 µl HBSS containing 5×10^5 leucocytes were incubated for 15 min with 100 ng hydroethidine ml⁻¹ (HE; Soc. Italiana Chimici), which was converted to the red fluorescent ethidium bromide in the presence of oxidative products. Bioassays were performed in the presence or absence of 100 µl fluoresceinated bacteria and in the presence or absence of 15 µg LPS or 20 µg PG. After incubation, 500 µl cold PBS and 100 µl 0.25 g crystal violet l⁻¹ were added to each tube to quench the fluorescence of non-ingested bacteria. Two independent experiments were performed.

Flow cytometry. Analyses of fluorescence were done by using a FACScan flow cytometer (Becton-Dickinson). Ten-thousand events per sample were acquired in the list mode by linear amplification for forward (FSC) and side scatter (SSC) signals. Fluorescence signals were then logarithmically amplified. Data were analysed by a Consort 32 with LYSIS II software. Consort 32 is a laboratory data analysis system used with the FACScan to perform data acquisition and analysis on cell samples. It uses Hewlett-Packard hardware with the Pascal operating system. Leucocyte populations were selected as R1 and R2 regions for polymorphonuclear leucocytes (PMN) and monocytes, respectively, on the basis of FSC and SSC properties. Measures of phagocytosis and of the oxidative burst were performed by two colour analysis: green fluorescence (FL1) indicating the rate of phagocytosis, and red fluorescence (FL2) the rate of production of oxidative metabolites (Perticarari *et al.*, 1991). Compensations for FL1 to FL2 and FL2 to FL1 were 6% and 30%, respectively.

Fig. 1

Fig. 1. Complement activation by PG. Values of CH50 are means $\pm$ SD of three independent assays ($15 \text{ CH50} \times 10^2 \text{ ml}^{-1}$ corresponded to 50% haemolysis by distilled water).

Fig. 2

Fig. 2. Cell fraction of PMBC preparations stimulated by PG or *E. coli* LPS. Values represent the percentage of Ki-67-antigen-positive cells detected by flow cytometry, and are means $\pm$ SD of three independent assays. $\square$, Medium; ▨ , PG; ▩ , LPS.

Results

Activation of complement by leptospira PG

Fig. 1 shows the degree of complement consumption triggered by PG in normal human serum. It can be seen that PG, though already effective at $4 \mu\text{g ml}^{-1}$ induces an almost 50% depletion of complement activity at $8 \mu\text{g ml}^{-1}$.

Mitogenic activity on PBMC by *Leptospira* PG

The mitogenic activity of PG was determined by measuring the amount of the Ki-67 antigen expressed on PBMC after exposure to PG or LPS. This determination identifies proliferating cells in a PBMC population. After incubation for 2 d, PG was able to induce a dose-dependent cellular response that was at least as high as the corresponding effect triggered by *E. coli* LPS (Fig. 2). In fact, at $1 \mu\text{g}$ about 69% of the cells underwent multiplication, compared to 62% of the PBMC population stimulated by LPS and to the 35% growth fraction observed in unstimulated cells.

Stimulation of phagocytosis and of the respiratory burst of PMN and monocytes

Fig. 3 shows one of the two experiments performed on the effect of PG on phagocytosis and on the metabolic activation of PMN and monocytes. The rate of phagocytosis of fluoresceinated *Staph. aureus* is represented by a shift of green fluorescence along the abscissa, from quadrant 3 to 4; the respiratory burst is indicated by an

increase of red fluorescence along the ordinate, from quadrant 4 to 2. The fraction of stimulated cells is expressed in percentage values in Table 1. When PMN were preincubated with PG, two effects were observed: (1) induction of the metabolic activation on resting cells (plot c) followed by amplification of this effect during phagocytosis, and (2) an increase in the number of phagocytosing cells, as indicated by the shift of contour plots towards square 4 (plot f). Percentage values of metabolic activation observed after incubation with PG in two experiments were higher than those observed after exposure to *E. coli* LPS, since PG directly stimulated 36% of the resting and 83% of the phagocytosing PMN, whereas 8% and 73% of the corresponding PMN populations were stimulated by LPS (Table 1).

A metabolic stimulation by PG was also observed on the monocyte population (plots h–n). Moreover, during phagocytosis (plot n) it was greater (70%) than that induced by LPS (57%).

Discussion

Clinical features of leptospirosis-like haemorrhages, fever and inflammatory symptoms were postulated in the past to be caused by an endotoxin released from leptospires during the infection (Alston & Broom, 1958). However, though several attempts have been made to demonstrate leptospiral endotoxicity, only limited biological effects were observed in various *Leptospira* LPS preparations, not sufficient to justify the complex and severe development of the inflammatory response of the host (Arean *et al.*, 1964; Shimizu *et al.*, 1987; Cinco *et*

Green fluorescence

Fig. 3. Stimulation of the oxidative metabolism of PMN (A) and monocytes (B) by 15 µg PG or 20 µg *E. coli* LPS. Contour plots represent green fluorescence (abscissa, FL1) versus ethidium bromide red fluorescence (ordinate, FL2) of cells incubated with *Staph. aureus*, representing phagocytosis and the oxidative burst, respectively. Contour lines of the plots provide the third dimension by indicating the numbers of events with similar parameter values. Cells were resting (*r*) or phagocytosing (*ph*). Each plot is divided into four quadrants by markers: these lines were set up to separate the autofluorescence of *r* cells (quadrant 3), stimulated red fluorescent cells (quadrant 1), green fluorescent *ph* cells (quadrant 4) and red plus green emitting fluorescence cells, which are simultaneously phagocytosing and stimulated (quadrant 2). Contour plots: (a) PMNr; (b) PMNr plus LPS; (c) PMNr plus PG; (d) PMNph; (e) PMNph plus LPS; (f) PMNph plus PG; (g) monocytesr; (h) monocytesr plus LPS; (i) monocytesr plus PG; (j) monocytesph; (k) monocytesph plus LPS; (l) monocytesph plus PG; (m) monocytesph plus LPS; (n) monocytesph plus PG.

Table 1. *Metabolic stimulation of PMN and monocytes after incubation with PG or E. coli LPS*

Values indicate the percentage fraction of the cell population emitting fluorescence: 100% of PMN was 4.7×10^6 cells and of monocytes 2.9×10^5 cells ml^{-1} . Values are means of duplicate assays.

Treatment	PMN		Monocytes	
	Resting	Phago-cytosing	Resting	Phago-cytosing
None	3	59	7	44
LPS (20 μg)	8	73	26	57
PG (15 μg)	36	83	20	67

al., 1988; De Souza & Koury, 1991). In the present investigation, we describe some relevant biological effects exerted *in vitro* by PG extracted from a strain of *Leptospira interrogans*, which are typical of the biologically active peptidoglycans. We demonstrate here that leptospiral PG is capable of activating human complement at very low concentrations when compared to peptidoglycans of other micro-organisms; in fact only $8 \mu\text{g PG ml}^{-1}$ were sufficient to reduce by 50% the haemolytic activity of complement, whereas 200 $\mu\text{g T. pallidum PG}$ (Umemoto *et al.*, 1981) and about 0.5 mg of *Staph. aureus PG* (Peterson *et al.*, 1978) were required to produce a comparable effect. Such a finding identifies PG as a cellular fraction responsible for the activation of complement, which was known already to be triggered by whole leptospires (Cinco & Banfi, 1983). Since leptospires are known to be complement resistant (Cinco & Banfi, 1983), we may postulate that complement activation by PG acts as an indirect factor in the stimulation of phagocytic cells.

A direct stimulation of PMN and monocytes was also observed using similar amounts of PG, since 15 $\mu\text{g PG}$ increased the release of oxidative products from the cells during both phagocytosis and the resting state, and at a rate which is slightly higher than that obtained with *E. coli LPS*. Moreover PG enhanced PMN uptake of *Staph. aureus*. Taken together, these direct and indirect stimulatory effects on the leucocytes may result in an amplification of the phagocytic process. Unlike peptidoglycans of other spirochaetes (Umemoto *et al.*, 1981; Beck *et al.*, 1990) but in agreement with results obtained for the majority of peptidoglycan preparations (Stewart-Tull, 1980), leptospiral PG was mitogenic for peripheral mononuclear cells. This *in vitro* observation, previously reported also for leptospiral LPS (Shimizu *et al.*, 1987), suggests an *in vivo* role for leptospiral PG in activation of the immunocompetent system.

In summary, the biological activities of leptospiral PG reported in this study suggest that it plays a role in

modulating the function of immunocompetent cells. We recently demonstrated that PG, but not LPS, both extracted from *Leptospira* strain Teramo, was able to trigger a proadhesive effect on endothelial cells towards PMN (A. Dobrina and others, unpublished data): this could suggest that PG is involved also in generalized vasculitis and the late symptoms which can develop in leptospirosis. These biological characteristics of PG and the few documented effects induced by leptospiral LPS may account for the multi-systemic course of leptospirosis. That both agents activate complement and stimulate leucocytes could contribute to the amplification of the biological response induced by spirochaetes.

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