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In vivo exposure to *Porphyromonas gingivalis* up-regulates nitric oxide but suppresses tumour necrosis factor-α production by cultured macrophages

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SUMMARY

The present study was designed to test whether the functional response of mouse macrophages elicited by chronic exposure to bacteria will be different from that of cells elicited by a non-bacterial irritant. Macrophage elicitation was conducted by *Porphyromonas gingivalis*, a major periodontal pathogen, in comparison to a standard elicitation by thioglycollate (TG). We measured lipopolysaccharide (LPS)-induced nitric oxide (NO) and tumour necrosis factor-α (TNF-α) secretion by the elicited macrophages, and the expression of inflammatory cytokines in the whole elicited cell population. In addition, we tested the response of TG-elicited macrophages to pretreatment with *P. gingivalis* LPS *in vitro*. Mouse peritoneal macrophages were harvested 4 days after intraperitoneal injection of TG or heat-killed *P. gingivalis*. TG-elicited macrophages produced undetectable levels of TNF-α and approximately 0·5 μM of NO. The stimulation of the macrophages with LPS resulted in the secretion of NO and TNF-α in a dose-dependent manner. The *P. gingivalis*-elicited macrophages produced basal levels of approximately 5 μM NO, but TNF-α was not detectable. LPS stimulation of these cells further increased the secretion of NO eightfold while TNF-α remained undetectable. The NO secretion by *P. gingivalis*-elicited cells was significantly higher than that by TG-elicited cells. Examination of cytokine expression in the whole elicited cell population revealed that both *P. gingivalis*-elicited cells and TG-elicited cells expressed messenger RNA for interleukin-2 (IL-2), TNF-α and interferon-γ (IFN-γ), but not for IL-4. IL-6 was expressed in *P. gingivalis*-elicited cells only. Pretreatment of TG-elicited macrophages with *P. gingivalis* LPS for 24 hr prior to a second LPS challenge resulted in down-regulation of TNF-α secretion and up-regulation of NO secretion, a response similar to that seen in *P. gingivalis*-elicited peritoneal macrophages. The results suggest that the *in vivo* exposure of resident macrophages to *P. gingivalis* induces functional changes in peritoneal macrophages. These changes might be due to the effect of *P. gingivalis* LPS.

INTRODUCTION

Tumour necrosis factor-α (TNF-α) and nitric oxide (NO) are known as important mediators of inflammation. Both mediators have been implicated in the pathogenesis of several inflammatory diseases, such as septic shock,[1] rheumatoid arthritis[2,3] and multiple sclerosis.[4,5] Inflamed periodontal tissues contain elevated levels of TNF-α[6] and NO,[7] suggesting that they are also important factors in the pathogenesis of periodontal disease.

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Abbreviations: IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; NO, nitric oxide; TG, thioglycollate; TNF, tumour necrosis factor.

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One of the major sources of TNF-α and NO in inflamed tissues is the tissue macrophage.[8,9] The precursor of the tissue macrophage is the circulating blood monocyte, which emigrates from the vasculature to the extravascular compartment under the influence of a variety of exogenous irritants. Once in the extravascular compartment, the monocytes differentiate into macrophages, which are characterized by their enhanced phagocytic and antibacterial properties, enabling them actively to participate in the immune response.

In vitro studies have examined the secretory activity of macrophages in response to bacterial antigens. Many of these studies are based on cultures of macrophages derived from varying sources. Classically, thioglycollate (TG)-elicited peritoneal murine macrophages, myeloid leukaemic cell lines and peripheral blood monocytes have been used,[10] each having their own special characteristics depending on their derivation. The results from these *in vitro* models have been extrapolated to provide information on macrophage function during inflammation. The relevance of this information is questionable because in a chronic inflammatory response there is a

continuous long-term exposure of the macrophages to the irritant. In addition the interaction of the macrophage with the inflammatory environment, which is lacking in macrophage cultures, probably has a major effect on the functional activity of the macrophage.

Chronic inflammatory periodontal diseases are chronic infections associated with Gram-negative bacteria, such as *Porphyromonas gingivalis*.[11] The accumulation and the continuous presence of bacteria and their products in the periodontal environment are thought to be an important factor in disease pathogenesis.[12–14]

The present study was carried out in order to test the hypothesis that the bacterial pathogens involved in periodontal disease can modify the secretion of inflammatory mediators by the associated inflammatory macrophages. The experiments were based on the widely accepted TG-elicited peritoneal inflammatory macrophage model. In this model, the inflammatory macrophages have a 4-day exposure to the non-bacterial irritant (TG) before they are harvested for *in vitro* testing. Replacement of the TG with heat-killed *P. gingivalis* cells allowed us to compare the behaviour of macrophages chronically exposed to a bacterial irritant with macrophages exposed to a non-bacterial irritant. Specifically, macrophages were elicited by intraperitoneal injection of heat-killed *P. gingivalis* cells or TG 4 days prior to cell harvesting. Lipopolysaccharide (LPS)-induced NO and TNF-α secretion and the expression of inflammatory cytokines by these cells were compared. In addition, we tested whether the response of TG-elicited macrophages to LPS *in vitro* could be shifted by long-term exposure to *P. gingivalis* LPS.

MATERIALS AND METHODS

Preparations of heat-killed P. gingivalis

Porphyromonas gingivalis A7436 (Boston University culture collection) was grown under anaerobic conditions[15] to the log phase. They were then washed three times with phosphate-buffered saline (PBS) and killed by incubation at 80° for 10 min (heat-killing).[15] The heat-killed bacteria were stored at 4° until further use.

Preparation of mouse macrophages

Female Sabra mice, 6–7 weeks old (Harlane, Jerusalem, Israel), were injected intraperitoneally with 0·5 ml of 3% sterile TG broth (Difco, Detroit, MI) or with 0·5 ml of PBS containing heat-killed *P. gingivalis* (10^9 bacteria/ml). Four days after injection, macrophages were harvested from the peritoneal cavity of the mice by washing with 7 ml of PBS followed by aspiration of the wash from the peritoneal cavity. Macrophages were pooled for each treatment group, washed twice and counted using a haemocytometer. Cell vitality was verified using the trypan blue exclusion technique. Macrophages were suspended in RPMI-1640 medium supplemented to a final concentration of 100 U/ml penicillin, 100 μg streptomycin, 2 mM L-glutamine and 5% fetal serum (C-RPMI, Biological Industries, Beit-i-i-Emek, Israel). Cells were plated in 24-well culture plates at a concentration of 10^6 cells per well, and incubated for 60 min at 37° in 5% CO_2. Non-adherent cells were removed by aspiration and the adherent cells were washed three times with PBS prior to their use in the following experiments. All tissue culture materials used were of endotoxin-free grade.

Stimulation of macrophages

LPS was extracted from *P. gingivalis* strain A7436 by a hot phenol–water method, and was further purified by caesium chloride isopyknic density gradient centrifugation as previously described by Morrison & Leive.[16] *Salmonella typhosa* LPS (phenol extract) was purchased from Sigma Chemicals (St Louis, MO). An LPS stock solution (1 mg/ml in PBS) was sonicated for 3 min before being diluted into working solutions. Stimulation of the cells by *P. gingivalis* or *S. typhosa* LPS was performed by adding 10 μl aliquots from the LPS working solution into 990 μl C-RPMI. Following stimulation, macrophages were incubated at 37° in humidified 5% CO_2 in four to six replicate wells for each tested condition. At the indicated time periods, cell culture supernatants were collected and either assayed immediately for NO and TNF-α or stored at −70° until the assay.

Pretreatment of macrophages with LPS in culture

In some experiments, we examined the effect of chronic exposure *in vitro* of macrophages to LPS on the macrophage response. TG-elicited macrophages were incubated with or without *P. gingivalis* or *S. typhosa* LPS (1 μg/ml) or in medium alone. After 24 hr (0–24-hr period), the medium was discarded, the cells were washed once and then cultured for an additional 24 hr (24–48-hr period) in medium containing LPS (1 μg/ml) or in medium alone. At the end of the 24–48-hr period medium was collected and analysed for NO and TNF-α.

NO_2^- and TNF-α determination

Accumulation of NO_2^- was used as an indicator of NO production in the medium and was assayed by Gries reagent (1% sulphanilamide/0·1% naphthylethylene diamine dihydrochloride/2·5% H_3PO_4; all from Sigma),[17] and read in a Vmax enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices, Palo Alto, CA) at 550 nm against a standard curve (1 μM–100 μM) of $NaNO_2$ (Sigma) in culture medium.

Mouse TNF-α was determined by ELISA using antibody pairs from Pharmingen (San Diego, CA) as previously described.[18] Briefly, 96-well ELISA plates (Maxisorp, NUNC, Naperville, IL) were coated with 1 μg/ml anti-mouse TNF-α monoclonal antibodies, and blocked by 3% bovine serum albumin (BSA). A rat anti-TNF-α biotinylated antibody was used as detecting antibody, followed by strepavidin–horseradish peroxidase conjugate (Jackson ImmunoResearch Laboratories, West Grove, PA). The substrate used was *o*-phenylenediamine. The reaction was terminated by the addition of 2 M, sulfuric acid, and optical density read in a Vmax microplate reader (Molecular Devices) at 490–650 nm against a standard curve of 15–2000 pg/ml.

RNA extraction and detection of cytokine mRNA

The reverse transcription–polymerase chain reaction (RT-PCR) method was used for the detection of cytokine mRNA. Total cellular RNA was extracted from 2×10^6 peritoneal cells by a one-step method.[19] RNA was reverse-transcribed using SuperScript RNase H^- reverse transcriptase (Gibco BRL, Gaithersburg, MD), followed by amplification

in a thermocycler using Taq polymerase (Promega, Madison, WI), and specific primers for the proinflammatory cytokines. Interferon-γ (IFN-γ), interleukin-2 (IL-2), IL-4, IL-6 and TNF-α.[20] The presence of $CD4^+$ T cells was verified by transcripts of CD4.[21] β-actin gene expression was used as internal control. The PCR products were separated on 3% NuSieve (FMC Bioproducts, Rockland, ME)/1% agarose gel and were stained with ethidium bromide.

Data analysis

Duplicate samples from each culture well were assayed for NO and TNF-α, and their mean values were used for calculations of the mean value and standard deviation of each tested condition. Statistical significance was analysed by Repeated Measurements analysis of variance (ANOVA) followed by *t*-test. The level of significance was determined at $P<0{\cdot}05$. RT-PCR products were classified as present (+) or absent (−) by examination of the stained gels using an ultraviolet transilluminator.

RESULTS

Preliminary experiments were performed to determine the dose-and time-dependent secretion of NO and TNF-α from TG-elicited mouse macrophages in response to *P. gingivalis* LPS. The results revealed that the induction of NO and TNF-α secretion occurred at *P. gingivalis* LPS concentrations above 10 ng/ml, with significant levels being achieved at concentrations of 100 ng/ml LPS and above (Fig. 1). From the dose–response curve obtained and on the basis of previous experience,[22,23] a concentration of 1 μg/ml *P. gingivalis* LPS was used to stimulate the production of NO and TNF-α from peritoneal mouse macrophages in this study. Kinetic experiments (0–72 hr, data not shown) have found that TNF-α peaked 6–8 hr after LPS stimulation, and declined slowly. NO was detectable 18 hr after LPS stimulation, and its levels increased with time. Based on these results and on data from other studies,[23,24] the 24-hr culture supernatants were used for the simultaneous detection of NO and TNF-α.

TG-elicited macrophages secreted approximately 0·5 μM of NO (Fig. 2) after 24 hr in culture. Stimulation with either *P. gingivalis* or *S. typhosa* LPS significantly increased the NO secretion 20–30-fold. The *P. gingivalis*-elicited macrophages produced basal levels of approximately 5 μM of NO (Fig. 2) and LPS stimulation significantly enhanced their NO secretion seven- to eightfold. Under all the tested conditions, *P. gingivalis*-elicited macrophages constantly secreted significantly more NO than did TG-elicited cells. The 10-fold differences in NO secretion at baseline between *P. gingivalis*-elicited macrophages and TG-elicited cells was reduced to a two- to threefold difference after LPS stimulation.

Unstimulated cultures of TG- or *P. gingivalis*-elicited macrophages did not produce detectable levels of TNF-α (<15 pg/ml). After stimulation with either *P. gingivalis* or *S. typhosa* LPS there was a significant increase in the levels of TNF-α secreted by TG-elicited cells, while the levels remained undetectable in the cultures of *P. gingivalis*-elicited macrophages (Fig. 3).

No significant differences were noted between the responses to *P. gingivalis* or *S. typhosa* LPS preparations in any of the above experiments (Figs 2 and 3).

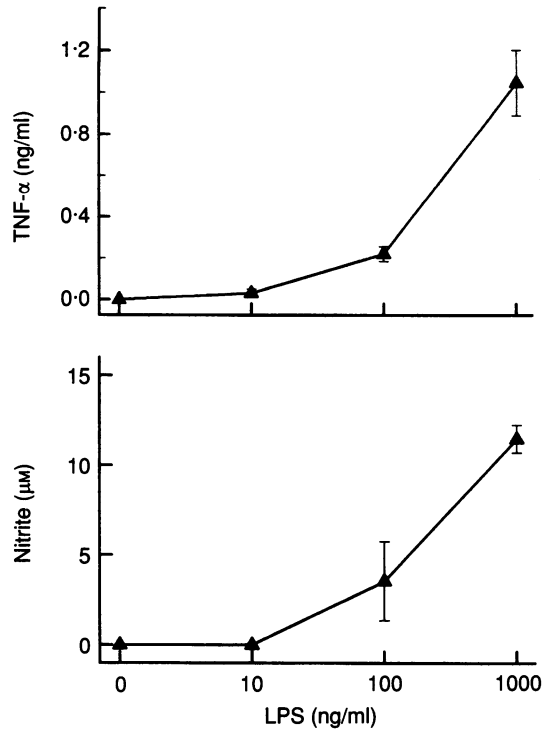

Figure 1. Dose-effect of *P. gingivalis* LPS stimulation of macrophages. Macrophages were stimulated with increasing doses of *P. gingivalis* LPS for 24 hr. Media were harvested and NO (bottom) or TNF-α (top) secretion was measured as described. Results are means ± standard deviation of the mean of four replicates, each assayed for NO or TNF-α in duplicates.

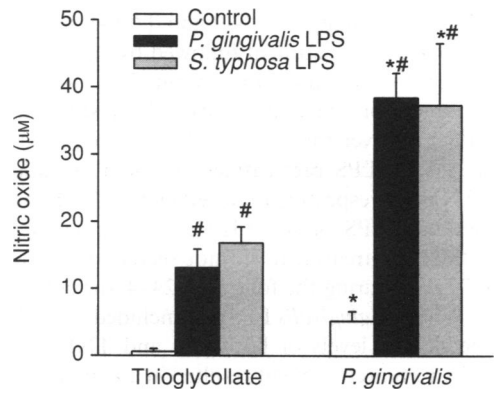

Figure 2. NO secretion by LPS-stimulated macrophages with and without pre-exposure to *P. gingivalis in vivo*. TG- or *P. gingivalis*-elicited macrophages were stimulated with 1 μg/ml *P. gingivalis* or *S. typhosa* LPS for 24 hr. Media were harvested and NO secretion was measured as described. Results are means ± standard deviation of the mean of six replicates, each assayed for NO in duplicates; *statistically significant difference from TG-elicited cells, #statistically significant difference from control (unstimulated) cells.

The presence of T-helper ($CD4^+$) cells and the expression of inflammatory cytokines in the TG-elicited and *P. gingivalis*-elicited peritoneal cells was evaluated by assessing the presence of the specific mRNA (Table 1). Both cell preparations were positive for transcripts of CD4. Messenger RNA of IL-2, TNF-α and IFN-γ was expressed in both TG- and *P. gingivalis*-

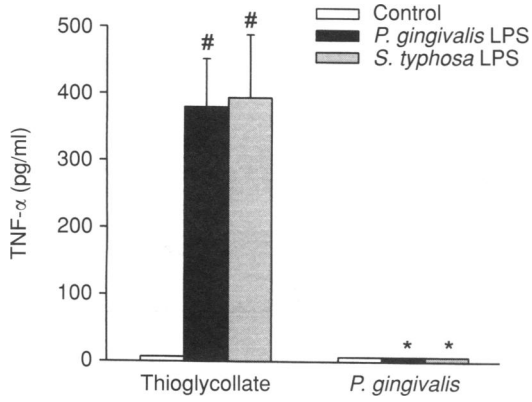

Figure 3. TNF-α secretion by LPS-stimulated macrophages with and without pre-exposure to *P. gingivalis in vivo*. TG- or *P. gingivalis*-elicited macrophages were stimulated with 1 μg/ml *P. gingivalis* or *S. typhosa* LPS for 24 hr. Media were harvested and TNF-α secretion was measured by ELISA. Results are means ± standard deviation of the mean of six replicates, each assayed using ELISA in duplicates; *statistically significant difference from TG-elicited cells, #statistically significant difference from control (unstimulated) cells.

elicited macrophages, while IL-4 was absent in both preparations. The only difference detected was the expression of IL-6 mRNA in *P. gingivalis*-elicited peritoneal cells while it was not detectable in TG-elicited cells (Table 1).

To examine whether the observed *in vivo* phenomena are due to chronic exposure of the macrophages to LPS, we used an *in vitro* model of 'LPS pretreatment'.[24] Pretreatment of TG-elicited macrophages by culturing them in medium containing 1 μg/ml *P. gingivalis* LPS over the 0–24-hr period resulted in marked changes in NO and TNF-α secretion over the next 24–48 hr. Pretreatment followed by culturing in plain medium over the second 24 hr resulted in low levels of NO secretion (approximately 1 μM). When stimulated with *P. gingivalis* LPS over this second 24-hr period, levels of NO increased 11-fold. LPS pretreatment resulted in undetectable levels of TNF-α irrespective of whether the 24–48-hr culture media contained LPS or not (Table 2). In contrast, macrophages without pretreatment did not secrete detectable levels of NO or TNF-α during the following 24–48-hr culture period (Table 2). When *P. gingivalis* LPS was included in the 24–48-hr culture media, the levels of both NO and TNF-α increased significantly. LPS pretreatment resulted in a significant increase

Table 1. Cytokine mRNA analysis by RT-PCR for peritoneal cells. Four days after *in vivo* injection of *P. gingivalis* or TG, cells were harvested from the peritoneum, total RNA was extracted and analysed by RT-PCR as described

	P. gingivalis	Thioglycollate
β-actin	+	+
IL-2	+	+
IL-4	−	−
IL-6	+	−
TNF-α	+	+
IFN-γ	+	+
CD4	+	+

Table 2. Effect of pretreatment of TG-elicited macrophages with *P. gingivalis* LPS on the secretion of NO and TNF-α

	Without pretreatment		Pretreatment	
Pre-treatment (0–24 hr)	Media	Media	LPS	LPS
Treatment (24–48 hr)	Media	LPS	Media	LPS
Nitric oxide (μM)	0	6·1 ± 1·0	1·0 ± 0·1	11·6 ± 2·1
TNF-α (pg/ml)	0	470 ± 50	0	0

TG-elicited macrophages were incubated with or without *P. gingivalis* LPS (1 μg/ml) or media alone for 24 hr (0–24-hr period). The media were then discarded, the cells washed once and then cultured for an additional 24 hr (24–48-hr period) in medium containing LPS (1 μg/ml) or medium alone. At the end of the 24–48-hr period the media were collected and analysed for NO and TNF-α. Results are mean ± standard deviation of four wells (each assayed in duplicate).

in NO secretion and a significant decrease in LPS-stimulated TNF-α secretion. Similar results were obtained when *S. typhosa* LPS was used to pretreat the cultured cells.

DISCUSSION

Numerous studies have shown that macrophages and monocytes obtained from different *in-vitro* sources, including peripheral blood monocytes, alveolar macrophages and peritoneal macrophages, have different functional responses *in vitro* to the same stimulus.[10,25,26] The findings of this study suggest that macrophages from the same source can also express different functional responses and that these differential responses are dependent on the eliciting agent. The differences between the TG-elicited and the *P. gingivalis*-elicited cells are both quantitative and qualitative. *P. gingivalis*-elicited cells produce small but detectable amounts of NO in culture whereas TG-elicited cells do not. In response to LPS stimulation, the differences become more obvious. *P. gingivalis*-elicited cells respond by producing quantitatively more NO than do TG-elicited cells. Although both cell types express TNF-α mRNA, only the TG-elicited cells respond by secreting significant amounts of TNF-α into the culture medium and TNF-α was not detectable in the culture medium from *P. gingivalis*-elicited cells.

The quantitative differences in the secretion of NO between the two cell types is probably due to an priming effect *in vivo* of *P. gingivalis* LPS released from the heat-killed *P. gingivalis* cells used to elicit the peritoneal macrophages. The ability of LPS to prime macrophages to secrete greater amounts of NO *in vitro* has been demonstrated previously[24,27] and is confirmed in our *in vitro* experiments reported here using *P. gingivalis* LPS. The inability of LPS to stimulate TNF-α secretion from *P. gingivalis* -elicited cells is in contrast to the ability of LPS to stimulate TNF-α secretion from TG-elicited cells. In most of the models used to study macrophage function, LPS stimulates the secretion of increased amounts of TNF-α.[10,17,24,28] It can therefore be deduced that elicitation of macrophages by heat-killed *P. gingivalis* cells blocks the LPS-induced TNF-α signal transduction pathway. The mechanism of this blockage may also be the result of the extended exposure of the cells to LPS.[24,29]

Another interesting, and qualitative, difference between the cells elicited by the two different irritants is the expression of IL-6 mRNA in the *P. gingivalis*-elicited cells and its absence in the TG-elicited cells. IL-6 is known to inhibit LPS-stimulated TNF-α production by human monocytes in culture and in mice.[30,31] It is therefore tempting to postulate that the lack of LPS-stimulated TNF-α secretion by *P. gingivalis*-elicited cells is the result of the action of IL-6 on these cells *in vivo*.

These qualitative and quantitative differences in the secretory function of the cells elicited by the two different irritants could be due to the elicitation of cells of different lineage suggesting that there are basic differences in the mechanisms of macrophage elicitation or due to the modification of cells of the same lineage by the different irritants. Evidence to support the latter possibility is provided by the *in vitro* studies on the TG-elicited cells. The results show that the pretreatment of TG-elicited macrophages with *P. gingivalis* LPS results in an increased secretion of NO in response to a second challenge by LPS whereas the TNF-α secretion is inhibited to non-detectable levels. This response is similar to that seen in *P. gingivalis*-elicited peritoneal macrophages.

In conclusion, the present study supports the hypothesis that the functions of macrophages associated with local inflammatory response are modified by the causative irritant. Clearly, *P. gingivalis*-elicited macrophages have a distinct functional response. These functional changes in macrophages may play an important role in *P. gingivalis*-associated periodontal disease.

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