Lipopolysaccharide from the Periodontal Pathogen *Porphyromonas gingivalis* Prevents Apoptosis of HL60-Derived Neutrophils In Vitro

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Lipopolysaccharide (LPS) from *Porphyromonas gingivalis* prevented apoptosis of HL60-derived neutrophils, which could not be restored upon the addition of interleukin-10. Signaling of *P. gingivalis* LPS through Toll-like receptor 2 (TLR2), not TLR4, may account for the inhibiting effect of *P. gingivalis* LPS on apoptosis and provide a mechanism for the development of destructive periodontal inflammation.

Porphyromonas gingivalis is a strongly implicated periodontal pathogen and is a predominant species in the gingival pockets of some individuals with advanced and severe periodontal disease. It is mostly found in deep periodontal pockets and active sites (13, 21), and elevated levels of opsonic activity to *P. gingivalis* have been found in the serum of patients with a history of destructive periodontal disease (42).

P. gingivalis has developed various mechanisms to evade the immune response. These include production of a trypsin-like protease that degrades many serum proteins including immunoglobulins, complement components, acute-phase proteins, and proteinase inhibitors (5, 13). Neutrophils are the predominant line of defense in the gingival crevice. *P. gingivalis* culture products have been reported to interfere with neutrophil elimination of the organism by causing inappropriate stimulation of the neutrophil, enhancing complement receptor 3 expression, and inhibiting phagocytosis (40, 41).

High levels of lipopolysaccharide (LPS) and lipid A from *P. gingivalis* have been reported to delay neutrophil apoptosis (10, 27) but induce lymphocyte apoptosis (7). *P. gingivalis* LPS has also been reported to increase the production of interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), and IL-8 by neutrophils (33, 43).

In this study, the effects of *P. gingivalis* LPS on the apoptosis of neutrophils derived from the human promyelocytic cell line, HL60, were determined. The use of HL60 cells allowed us to look at a homogenous neutrophil population over an extended time period. The results showed that *P. gingivalis* LPS prevented apoptosis of HL60-derived neutrophils and that apoptosis could not be restored upon the addition of IL-10.

HL60 cells (European Collection of Cell Cultures, Wiltshire, United Kingdom) are nonadherent and were grown in suspension culture containing RPMI 1640 medium plus L-glycerol (300 mg/liter) with 10% fetal bovine serum and 1% antibiotic-antimycotic solution (all from Life Technologies, Paisley, United Kingdom). Cells were maintained at 1×10^5 to 5×10^5

cells per ml at 37°C in a humidified environment with 5% CO_2 in a vented tissue culture flask (Life Technologies) and replated when the density reached 1×10^6/ml. To obtain neutrophils, cells were stimulated with 10^{-7} M all-*trans* retinoic acid (Sigma-Aldrich Company Ltd., Dorset, United Kingdom). Cells were plated at a concentration of no more than 2.5×10^5/ml so that after differentiation the cell density obtained was no higher than 10^6/ml. The ability of differentiated HL60 cells to undergo apoptosis was determined by fluorescence microscopy with an Annexin V-FITC (fluorescein isothiocyanate) apoptosis detection kit (Oncogene Research Products, Calbiochem-Novabiochem Ltd., Nottingham, United Kingdom) following the manufacturer's RAPID annexin binding protocol. At 3-h intervals a 0.5-ml sample was taken from each flask and the percentage of apoptotic cells was determined. In each experiment three flasks were differentiated and three slides were prepared from each flask. The experiments were repeated twice, and the results were expressed as the means ± standard deviations. Total apoptosis was reached when 85% of the cells were apoptotic, as after this point cells began to undergo secondary necrosis and disintegrate.

To ensure that HL60-derived neutrophils responded in a manner similar to peripheral blood neutrophils, LPS from *Escherichia coli* was used as a positive control. At differentiation *E. coli* 055:B5 LPS (catalogue number L2637; Sigma-Aldrich Company Ltd.) was added at concentrations of 1, 10, 100, or 1,000 ng/ml. *E. coli* LPS delayed apoptosis by 12 h at a concentration of 1 ng/ml, by 15 h at 10 ng/ml, by 18 h at 100 ng/ml, and by 21 h at 1,000 ng/ml, indicating a dose-dependent effect (Fig. 1). This result supports the findings of other in vitro (3, 8, 14, 16, 19) and in vivo (4) studies of humans. However, in about 30% of the cells, there was an induction of apoptosis at time points earlier than these.

Purified *P. gingivalis* W50 LPS (prepared by Haroun Shah, Public Health Laboratory Service, Colindale, London [30, 31], and donated by Veronica Booth, London Hospital Dental School, London, United Kingdom) was added to the cell cultures at concentrations of 0.1, 1, 10, or 100 ng/ml. LPS from *P. gingivalis* prevented apoptosis of the majority of neutrophils, with a maximum of 35% of cells apoptotic after 45 h in culture (Fig. 2). Apoptosis in these cultures occurred at earlier time

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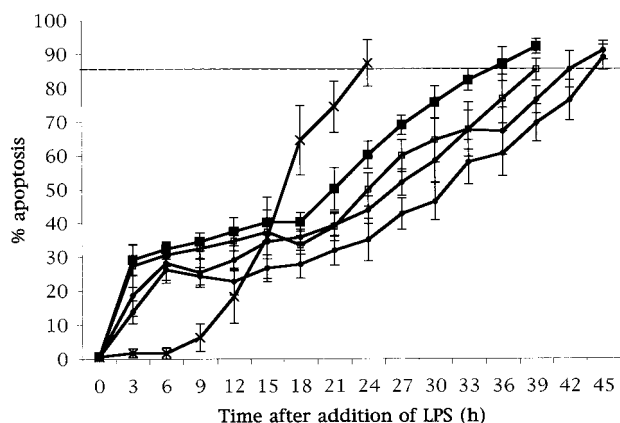
FIG. 1. *E. coli* LPS delays HL60-derived neutrophil apoptosis. *E. coli* LPS was added to differentiated HL60 cells at concentrations of 1 ng/ml (■), 10 ng/ml (□), 100 ng/ml (●), and 1,000 ng/ml (○). Differentiated cells that received no LPS were used as an internal control (X). The addition of *E. coli* LPS induced apoptosis of 35% of the population at earlier time points compared to time for the control but delayed apoptosis of the remaining population in a dose-dependent manner.

FIG. 3. IL-10 counteracts delay in apoptosis by *E. coli* LPS. *E. coli* LPS (1 ng/ml) was added to differentiated HL60 cells along with IL-10 at concentrations of 0.001 ng/ml (■), 0.01 ng/ml (□), 0.1 ng/ml (●), or 1 ng/ml (○). Cells which received neither LPS nor IL-10 were used as a control (X). The addition of IL-10 with *E. coli* LPS induced apoptosis of the total cell population by 6 h in culture, compared to 24 h for unstimulated cells and 36 h for *E. coli* LPS without IL-10.

points and then remained at a stable level. Such a profound effect on neutrophil apoptosis by LPS has not previously been reported. Clearly, such a delay in apoptosis would prolong an acute inflammatory response with a consequently increased potential for tissue damage. These results confirm those of a previous study which also showed *P. gingivalis* LPS, albeit at a concentration 1 log higher, to delay neutrophil apoptosis (27). The experiments reported here extend those results as they were carried out over an extended time frame and were tested at concentrations more likely to be found in the subgingival plaque and gingival crevice/pocket (6 and clinical observation).

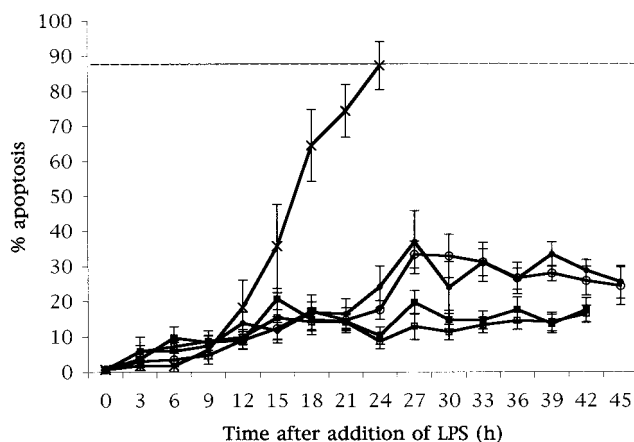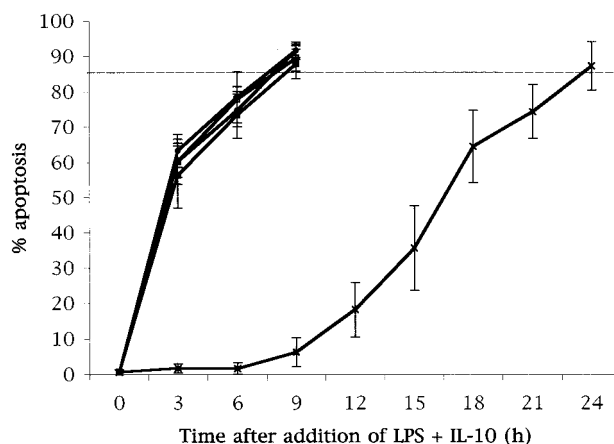IL-10 was originally discovered as a Th2-secreted product

FIG. 2. *P. gingivalis* LPS prevents HL60-derived neutrophil apoptosis. *P. gingivalis* LPS was added to differentiated HL60 cells at concentrations of 0.1 ng/ml (■), 1 ng/ml (□), 10 ng/ml (●), and 100 ng/ml (○). Differentiated cells which received no LPS were plotted as a control (X). *P. gingivalis* LPS prevented apoptosis of the majority of the cell population, with a maximum of 35% of cells undergoing apoptosis after 45 h. Apoptosis occurred at earlier time points and remained at a stable level for the remaining culture period.

that inhibited cytokine production from Th1 cells (20, 22). It has since been shown that IL-10 has effects on various cell types including T cells, B cells, macrophages, NK cells, mast cells, and neutrophils (2, 12, 20, 22). The effects of IL-10 on neutrophils include negative modulation of the production of IL-1, IL-8, IL-12, and TNF-α, up-regulation of IL-1 receptor antagonist production, inhibition of prostanoid and platelet-activating factor synthesis, and inhibition of phagocytosis. IL-10 is reported to counteract the delay in neutrophil apoptosis caused by LPS and proinflammatory cytokines such as granulocyte colony-stimulating factor, TNF-α, and interferon gamma but itself has no effect on basal survival rates (4, 14). It has been suggested that, as IL-10 can down-regulate proinflammatory cytokine production by macrophages and induce apoptosis in LPS- and cytokine-stimulated neutrophils, it may have a protective role in disorders such as endotoxic shock (17), pulmonary inflammation (4), and severe sepsis (14).

At differentiation, *E. coli* LPS was added to the cell cultures at a concentration of 1 ng/ml. IL-10 was then added at concentrations of 0.001, 0.01, 0.1, or 1 ng/ml. The combination of IL-10 and *E. coli* LPS induced apoptosis after 3 h in culture (Fig. 3). This effect was shown at all doses of IL-10 tested. In addition, the IL-10–LPS combination induced apoptosis in 85% of the total cell population after 9 h compared with the 24 h needed for the spontaneous, unstimulated apoptosis to reach the same level. When compared to the delayed apoptosis with LPS alone, total apoptosis with the IL-10–LPS combination was induced 27 h earlier. In our system, as in previous reports, IL-10 alone had no effect on basal survival rates (data not shown).

As IL-10 induced apoptosis when it was added to cultures containing *E. coli* LPS, we tested the effect of IL-10 on the delay in apoptosis caused by *P. gingivalis* LPS by adding 0.1 and 10 ng of LPS/ml to cell cultures together with 0.1 ng of IL-10/ml. IL-10 did not affect the kinetics of the profoundly delayed apoptosis induced by *P. gingivalis* LPS (Fig. 4). This result
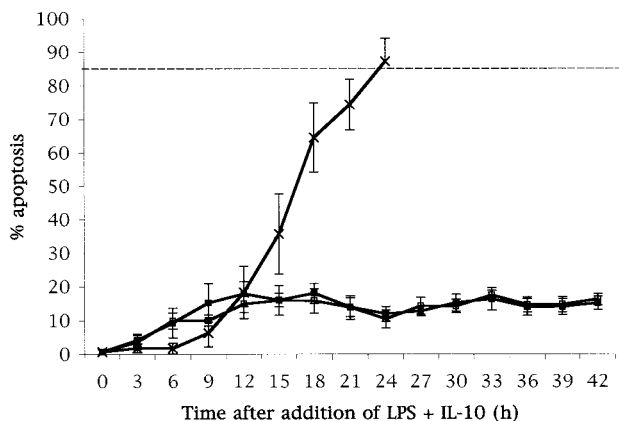
FIG. 4. No effect of IL-10 on prevention of apoptosis by *P. gingivalis* LPS. IL-10 (0.1 ng/ml) was added to differentiated HL60 cells with *P. gingivalis* LPS at a concentration of either 0.1 ng/ml (■) or 10 ng/ml (□). Differentiated cells which received no additional stimulus were used as a control (X). The addition of IL-10 did not alter the effect of *P. gingivalis* LPS in preventing neutrophil apoptosis.

would indicate that the down-regulatory effects of IL-10 previously reported do not extend to *P. gingivalis* LPS. This result also demonstrates that LPS from different bacterial species, while showing an array of biological effects, also exhibits substantial differences for a given effect.

Differential effects of *P. gingivalis* and *E. coli* LPS have been reported in various cell types, including monocytes and epithelial cells, both in vitro (1, 18, 26, 29) and in vivo (28). These differences are most likely due to distinct signaling pathways through the Toll-like receptors (TLRs) as LPS from *P. gingivalis* was recently shown to bind to TLR2, not the common LPS receptor TLR4 (9, 11, 15, 25, 32, 35). LPS from *P. gingivalis* has also been reported as antagonistic for TLR4 (44). It has been suggested that the binding of different LPS moieties to TLRs may be, at least in part, due to the secondary structure of lipid A (23). However, studies with fibroblasts have shown *P. gingivalis* LPS to signal through TLR4 (24, 36, 37). This finding may indicate a difference in signaling in this particular cell type or reflect a difference between parenchymal and lymphoid-derived cells. IL-10 has been found to inhibit the effect of *P. gingivalis* LPS-induced IL-6 production in fibroblasts (39). As this study showed no effect of IL-10, it further suggests use of a different signaling pathway in these two cell types.

The ability of *P. gingivalis* LPS to stimulate cells through TLR2 may have skewed the immune response, allowing the bacteria to persist and invade the stratified epithelium. The ability of *P. gingivalis* to prolong the life span of neutrophils while causing inappropriate activation could possibly result in increased and prolonged inflammation and damage to surrounding tissue. This ability is further highlighted by the finding that *P. gingivalis* can induce expression of cell adhesion molecules as well as chemokine and cytokine release from gingival epithelium, thereby recruiting neutrophils (34, 38). These findings support the role of *P. gingivalis* in the initiation and exacerbation of destructive periodontal disease.

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