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Coexistence of CD14-Dependent and Independent Pathways for Stimulation of Human Monocytes by Gram-Positive Bacteria

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The cell wall is a key inflammatory agent of gram-positive bacteria. Possible receptors mediating cell wall-induced inflammation include CD14 and platelet-activating factor (PAF) receptor. To delineate the conditions under which these various receptors might be used, human monocytic THP-1 cells and heparinized whole human blood were stimulated with lipopolysaccharide (LPS), intact *Streptococcus pneumoniae* bacteria, or purified pneumococcal cell wall. THP-1 culture supernatant or cell-free plasma was analyzed for the presence of tumor necrosis factor, interleukin-1β (IL-1β), and IL-6. For the cultured monocytes, anti-CD14 inhibited induction of the inflammatory cytokines by the cell wall and LPS but not by intact pneumococcal bacteria. Despite the difference in CD-14 usage, the intracellular pathways induced by the three agents demonstrated similarities, as revealed in the presence of specific signal transduction inhibitors such as cholera toxin, pertussis toxin, and genistein. Cytokine production in whole human blood indicated that anti-CD14 failed to block responses to cell wall and intact pneumococci, whereas while LPS-induced responses were inhibited. PAF receptor antagonist had no effect under any conditions in both assays. These results indicate that although cell walls bind to both CD14 and PAF receptor, only CD14 appears to engender a cytokine response under restricted conditions. Furthermore, host cell responses to intact pneumococci are consistently independent of CD14 and PAF receptor.

Gram-positive bacteria are being recognized increasingly as the cause of shock-like syndromes which, in general, are not clinically distinguishable from those seen in association with gram-negative endotoxic shock. Gram-positive bacteria account for as many as half of the cases of bacterial sepsis, a figure which is rising substantially (2). Invasive *Streptococcus pneumoniae* infections are characterized by particularly intense inflammatory host reactions, reflected in the high mortality and morbidity rate associated with pneumococcal meningitis (18). The majority of both experimental and clinical data strongly link tumor necrosis factor (TNF) with the pathogenesis of gram-negative shock, whereas evidence exists that interleukin-1 (IL-1) is a pivotal player in gram-positive inflammation (15, 16).

In gram-negative sepsis, the outer membrane lipopolysaccharide (LPS; endotoxin) molecule is responsible for triggering both cellular and physiological responses. Thus far, three different mammalian membrane proteins that interact with LPS have been described. The principal endotoxin receptor, CD14, represents a high-affinity receptor with inflammatory effects downstream (21). Macrophage scavenger receptor and CD11b/CD18, a member of the leukocyte integrin family, have been demonstrated to actively bind LPS, although these interactions seem nonessential for the cellular responses to LPS (8, 12). Recent evidence suggests the existence of other pathways, since CD14-deficient mice show some responses to LPS (9).

CD14 is a glycosylphosphatidylinositol (GPI)-anchored membrane protein (mCD14) that is surface exposed on cells of the myeloid lineage only (21). It lacks a transmembrane or cytoplasmic domain. Two isoforms of soluble CD14 (sCD14) lacking the GPI anchor are released from both stimulated and unstimulated monocytes and can be detected in human plasma or serum (6). They mediate LPS-induced effects via an unidentified receptor in cells devoid of mCD14, such as endothelial and epithelial cells (13). Under physiological conditions, a liver-derived soluble serum protein termed LPS-binding protein binds with high affinity to LPS and subsequently promotes and facilitates its interaction with either mCD14 or sCD14 in a catalytic fashion (17, 21). Binding of LPS to CD14 eventually results in the development of inflammation. The molecule responsible for transduction of the LPS signal across the plasma membrane in both myeloid (GPI-anchored mCD14 lacks an intracellular region) and nonmyeloid (devoid of mCD14) cells has yet to be identified.

In contrast to the well-researched mechanism for LPS-induced cellular stimulation, the few studies that have addressed the molecular mechanism of gram-positive eukaryotic cellular activation have produced strikingly contradictory results. Products and components of gram-positive bacteria capable of activating eukaryotic host cells include exotoxins and cell wall components such as peptidoglycan and (lipo)teichoic acid. The vast majority of the work on gram-positive inflammatory responses has concentrated on *Staphylococcus aureus*, including conflicting results about whether CD14 can mediate staphylococcal cell wall-dependent cytokine stimulation (4, 10, 14, 20). Very few studies have addressed the receptor usage and activation in human blood cells by *S. pneumoniae*. Moreover, whereas one study described the absolute requirement for CD14 to stimulate IL-8 secretion induced by the pneumococcal cell wall in THP-1 monocytes, others demonstrated the inability of anti-CD14 antibody to reduce the level of TNF released by human peripheral blood monocytes stimulated with *S. pneumoniae* or *S. pyogenes* cell wall preparations (10, 14).

In the present study, we sought to determine the similarity or difference in receptors and intracellular transduction pathways used by the gram-positive pneumococcal bacteria and gram-negative LPS to evoke an inflammatory cytokine response. We included an investigation of the platelet-activating factor

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(PAF) receptor since pneumococci have been described to adhere to human cells bearing this receptor via the cell wall (5). We systematically tested possible sources of differences between studies, including the nature of the stimulus (intact bacteria versus cell wall), type of cell stimulated (cell line versus whole blood), and the cytokine used as a readout (TNF, IL-1, or IL-6). We present evidence that supports the existence of several pathways, both known and unknown, for activation of cellular cytokine production by gram-positive bacteria.

MATERIALS AND METHODS

Bacterial strains and growth conditions. An unencapsulated strain of *S. pneumoniae*, R6x, was grown in semisynthetic medium supplemented with 0.5% yeast extract in a stationary water bath at 37°C (18). At the mid-logarythmic growth phase, bacteria were heat killed (10-min incubation at 60°C), washed, and resuspended in phosphate-buffered saline to a concentration of 10^9 CFU/ml.

Cell culture and THP-1 assay. Human acute monocytic leukemia THP-1 cells were maintained in RPMI 1640 tissue culture medium containing 10% fetal bovine serum, penicillin-streptomycin, L-glutamine, and 5×10^{-5} M β-mercaptoethanol. The cells were seeded into 24-well cell culture plates in complete RPMI 1640 culture medium supplemented with an extra 10% human serum. As inflammatory stimuli, various concentrations of *Escherichia coli* LPS (Sigma, St. Louis, Mo.), intact but heat-killed *S. pneumoniae* bacteria, or purified pneumococcal cell wall were added. Cell wall preparations were obtained as previously described for the preparation of peptidoglycan-teichoic acid complex, including digestion with proteases to remove adducted proteins (18). At specific time points after the addition of stimuli, aliquots of supernatant were collected and stored at −20°C. The cytokine protein concentration was determined in the supernatant by enzyme-linked immunosorbent assay techniques specific for human TNF, IL-1β, or IL-6 (Quantikine; R&D Systems, Minneapolis, Minn.) as recommended by the manufacturer. The experiments were repeated at least 10 times, and the mean and standard deviation were calculated. For comparison between experiments, values were transformed to a percentage of the values for LPS. Representative absolute values for LPS are 2,000 pg/ml (TNF), 300 pg/ml (IL-1), 90 pg/ml (IL-6), and 2500 pg/ml (IL-8).

Whole-blood cytokine assay. Heparinized blood from a healthy human donor was distributed into V-shaped microtiter plates and incubated at 37°C with various concentrations of *E. coli* LPS, *S. pneumoniae* bacteria, or purified pneumococcal cell wall. Cell-free plasma was obtained by centrifugation. The plasma was stored at −20°C before the enzyme-linked immunosorbent assay was performed as above. Four independent experiments were performed.

Inhibition of receptor binding and signal transduction pathways. To demonstrate the participation of CD14 or the PAF receptor in the induction of a cytokine response, cultured THP-1 monocytes or whole blood was preincubated for 2 h at 37°C with 10 μg of the anti-CD14 monoclonal antibody MY4 or isotype-matched control per ml (Coulter Immunology, Hialeah, Fla.) or 12 μg of the PAF receptor antagonist L659,989 per ml (kindly provided by Merck, Sharp & Dohme Research Laboratories, Rahway, N.J., and tested for bioactivity in the platelet aggregation assay [3]).

To study the downstream signaling pathways, experiments were performed with cells pretreated for 2 h with 10 μg of cholera toxin per ml (Sigma), 10 μg of pertussis toxin per ml (Sigma), or 1 mM genistein (ICN Biomedicals Inc., Aurora, Ohio). Doses of 0.5 and 0.25 times these amounts showed no difference in the results (data not shown).

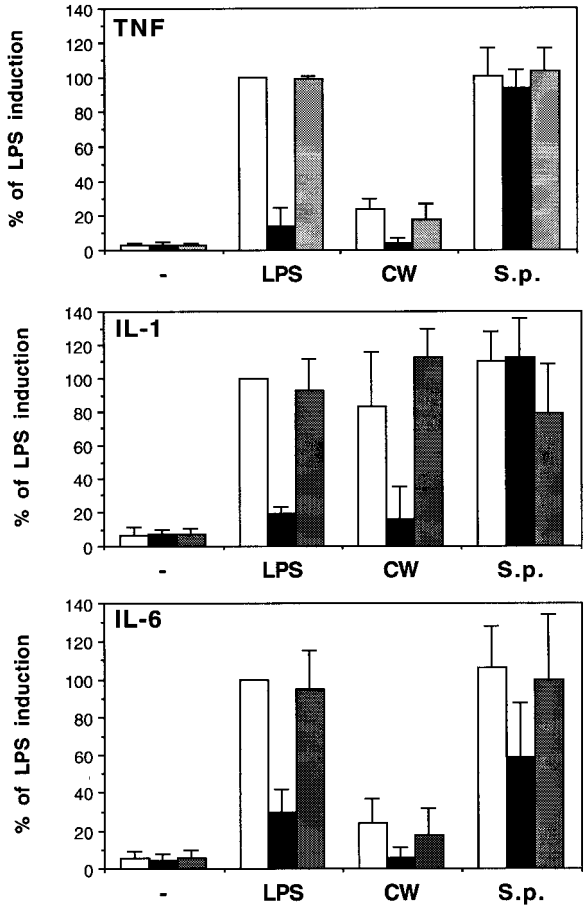

FIG. 1. Involvement of CD14 and PAF receptor in cytokine production by THP-1 monocytes stimulated by gram-positive components. TNF concentrations were determined 4 h after stimulus, whereas IL-1β and IL-6 levels were examined at 24 h from supernatant of 10^6 THP-1 monocytes/ml. The stimulants were 1 μg of LPS per ml, 10 μg of pneumococcal cell wall (CW) per ml, or 10^7 CFU of intact *S. pneumoniae* bacteria (S.p.) per ml. The cytokine concentration observed after stimulation with 1 μg of LPS per ml was arbitrarily defined as 100%, and the data are presented as a percentage of this value (mean ± SD of four to six individual experiments). When indicated, the cell cultures were pretreated for 2 h with 10 μg of the anti-CD14 MAb MY4 per ml (solid bars) or 12 μg of PAF receptor antagonist per ml (shaded bars). Controls (−) received RPMI complete culture medium only.

RESULTS AND DISCUSSION

Role of CD14 and PAF receptor in cytokine production by THP-1 cells. Monocytes-macrophages are generally considered the main cellular source of inflammatory cytokines, such as TNF and IL-1. The kinetics of cytokine release from THP-1 cells was determined for each of the three stimuli LPS, pneumococci, and cell wall. TNF released in the culture supernatant after stimulation with either LPS, intact pneumococcal bacteria, or purified cell wall clearly peaked at 4 h, gradually declining thereafter, whereas IL-1β and IL-6 secretion showed a progressive accumulation for all three stimuli (data not shown). Hence, for subsequent analytical experiments, 4-h supernatant was used to compare TNF induction whereas supernatants were harvested 24 h after stimulus to measure IL-1β and IL-6 production.

Relatively high concentrations of LPS (≥1 μg/ml), pneumococcal cell wall (≥10 μg/ml), and intact *S. pneumoniae* (≥10^7 CFU/ml) were required to induce substantial amounts of cytokines from THP-1 cells (Fig. 1). This may be attributed to the relative immaturity of THP-1 monocytes, including low levels of mCD14 expression (1). Comparing the specific activities of the stimuli, cell wall from 10^8 bacteria (i.e., 10 μg) was five times less efficient than LPS from 10^8 bacteria (i.e., 1 μg) at inducing TNF and IL-6, respectively. Cell wall was roughly equivalent to LPS for induction of IL-1β, consistent with previous studies indicating that IL-1β is the predominant cytokine generated by cell walls (in contrast to TNF for LPS) (15). Induction of an amount of each cytokine approximating that seen with LPS at 10^8 bacterial equivalents required only 10^7 intact pneumococci, indicating a 10-fold-greater potency for the intact gram-positive bacteria.

To address the role of CD14 in the cytokine response to pneumococci, monoclonal anti-CD14 antibody was used to inhibit cytokine production. In parallel, since *S. pneumoniae* cells anchor themselves to the PAF receptor via the cell wall, an antagonist of PAF receptor G-protein signalling was tested for inhibition of cytokine production. As shown in Fig. 1, PAF

receptor antagonist failed to influence any cytokine release. This is consistent with the lack of G-protein signal generated upon anchorage of pneumococci to the PAF receptor (5). In contrast and as expected, the MY4 anti-CD14 antibody clearly abolished the LPS-induced TNF, IL-1β, and IL-6 release (Fig. 1). The antibody also inhibited the release of all cytokines induced by purified pneumococcal cell wall but failed to inhibit the response induced by intact pneumococcal bacteria. Hence, we hypothesize that pneumococcal cell wall components can use CD14 as an inflammatory receptor on THP-1 monocytes, a finding consistent with the results of Heumann et al. (10) and Pugin et al. (13). However, whole bacteria appear to be capable of inducing inflammatory responses which either are independent of CD14 or use an epitope for binding CD14 that is not recognized by MY4. This latter observation indicates either that the presentation of the cell wall on the intact bacterium influences bioactivity or that components other than the cell wall can induce cytokines. A case for the involvement of pneumococcal surface proteins can be suggested based on the results of Geelen et al. (7), where pneumococci were shown to bind to human macrophages in a manner sensitive to trypsin and pronase (binding decreased by 89% ± 4% and 88% ± 4%, respectively) but not to inhibition by exogenous cell wall (binding decreased by 25% ± 5%).

Intracellular signalling generated by cell walls in THP-1 cells. Eukaryotic cell surface-localized receptors responsible for transducing signals across the plasma membrane can be classified into three functionally distinct classes: (i) receptors with intrinsic catalytic activity, including the large family of receptor tyrosine kinases; (ii) receptors coupled to a G protein; and (iii) receptors that may associate with dedicated signal-transducing molecules in the cytoplasm. An indication of the involvement of G proteins and tyrosine phosphorylation in the generation of an inflammatory output signal can be gleaned from the use of generalized inhibitors. Cholera toxin pretreatment causes ADP-ribosylation and hence irreversible activation of the α-subunit of G_s proteins, leaving them unresponsive to subsequent stimuli. Pertussis toxin treatment, on the other hand, leads to ADP-ribosylation of the α-subunit of G_i proteins and thus blocks their intrinsic enzymatic activity. Both toxins eventually cause an increase of intracellular cyclic AMP (cAMP) concentrations and can therefore function as controls for each other to exclude the involvement of mere cAMP-mediated events. To evaluate the possibility of tyrosine phosphorylation, genistein was used as an inhibitor of receptor tyrosine kinase-associated tyrosine phosphorylation. As demonstrated in Fig. 2, the patterns of inhibition by pertussis toxin, cholera toxin, or genistein were very similar for all three different inflammatory stimuli (LPS, intact pneumococcus, and purified pneumococcal cell wall). The induction of TNF (Fig. 2A) by all three different stimuli was compatible with the involvement of both a G_s protein and tyrosine phosphorylation. Since cholera toxin but not pertussis toxin pretreatment resulted in a substantial decrease in the level of induced TNF, inhibition was not related to increases in cAMP. None of the applied drugs reduced the secretion of IL-1β by any stimulus (Fig. 2B). Finally, phosphorylation of tyrosine seemed to be crucial for the production of IL-6, as demonstrated by the almost complete inhibition of IL-6 secretion by genistein for all three stimulatory cases (Fig. 2C). Thus, each cytokine had a distinct pattern of inhibition, but for a given cytokine, signal transduction pathways were qualitatively similar for both gram-positive pneumococci and gram-negative stimuli. This implies that although the surface receptors used by *S. pneumoniae* and gram-negative pathogens may be completely or partially different, the signal transduction pathways may share elements intracellularly.

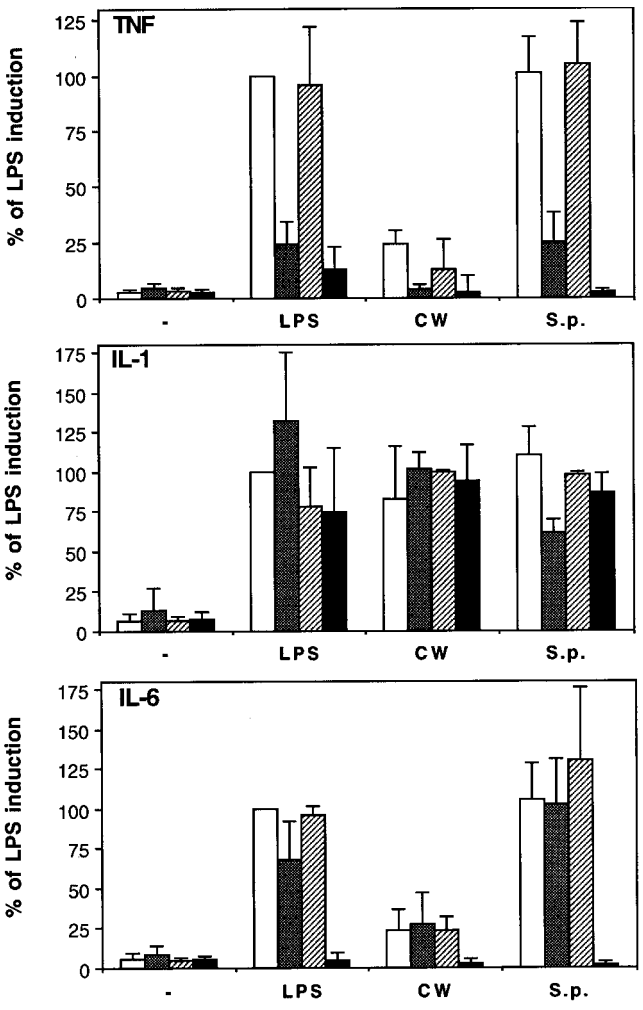

FIG. 2. Effect of inhibition of G proteins and tyrosine phosphorylation on cytokine induction in THP-1 cells. The assay and plotting conditions were identical to those in Fig. 1. When indicated, monocytes were pretreated for 2 h with no inhibitor (open bars), 10 μg of cholera toxin per ml (shaded bars), 10 μg of pertussis toxin per ml (striped bars), or 1 mM genistein (solid bars).

Cytokine responses to gram-positive components in whole human blood. When isolated from whole blood and treated with cell walls in vitro, human mononuclear cells generate multiple cytokines (11, 15). Since pneumococci attach avidly to these cells in a manner that is both dose dependent and saturable (7), it seemed reasonable that in the physiologically relevant environment of whole blood, bacteria and leukocytes would interact and generate a cytokine response. Since an additional variable in the studies with THP-1 cells is the variation of sensitivity to endotoxin with the maturity of the cell, the experiments were extended by using heparinized whole human blood exposed ex vivo to LPS, intact pneumococci, or purified pneumococcal cell wall.

First, the kinetics of cytokine release were reestablished for TNF, IL-1β, and IL-6, indicating a peak at 4 h followed by stabilization without decline until at least 8 h after the stimulation with either LPS, bacteria, or cell wall (data not shown). It has been suggested that monokines induced by LPS can mediate indirectly the production of other cytokines. To min-

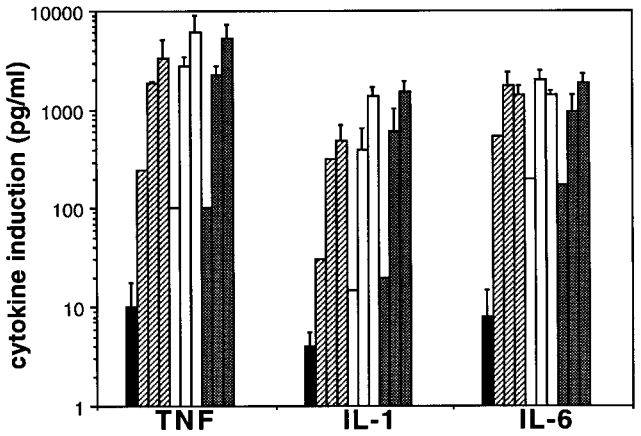

FIG. 3. Dose-response analysis for cytokine induction in whole blood. Heparinized whole blood was stimulated ex vivo with LPS (striped bars; 1, 5, or 10 ng/ml [left to right]), pneumococcal cell wall (open bars; 100, 1,000, or 5,000 ng/ml), or intact pneumococci (shaded bars; 1×10^4, 1×10^5, or 5×10^5 CFU/ml). Solid bars represent blood stimulated with phosphate-buffered saline as a control. Cytokine concentrations were determined in cell-free plasma harvested after centrifugation of the blood.

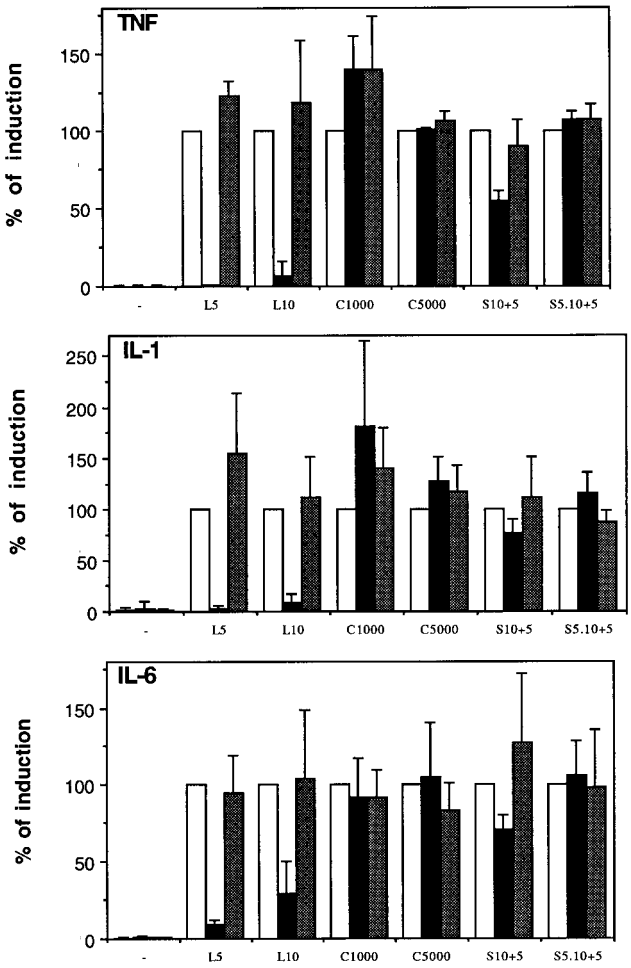

FIG. 4. Involvement of CD14 and PAF receptor in cytokine production in whole blood exposed to gram-positive components. In parallel experiments, heparinized whole human blood was treated for 2 h with either saline alone (control; open bars), 10 μg of anti-CD14 MY4 MAb (solid bars), or 12 μg/ml PAFra (shaded bars). The samples were then exposed to 5 or 10 ng of LPS per ml (L5 and L10, respectively), 1,000 or 5,000 ng of cell wall per ml (C1000 and C5000, respectively), and 1×10^5 or 5×10^5 CFU of pneumococci per ml (S10+5 and S5.10+5, respectively) for 4 h. The cytokine concentration was determined in the cell-free plasma supernatant.

imize the risk that any cytokine (especially TNF) could act as a proximal mediator to induce the production of other cytokines, like IL-1β or IL-6, all cytokine concentrations were measured 4 h after stimulus.

Second, the dose response was redetermined in whole blood. The whole-blood assay system was about 1,000-fold more sensitive than the system involving THP-1 cells and responded to as little as 1 ng of LPS. As shown in Fig. 3, increasing concentrations of LPS, purified cell wall, or whole pneumococci correlated directly with increasing amounts of cytokines produced. The cytokine response to 5 ng of LPS (5×10^5 bacteria) was similar to that to 1 μg of cell wall (10^7 bacteria) and 10^5 intact bacteria, indicating that LPS was ~200 times more potent than the cell wall for induction of comparable levels of TNF, IL-1β, or IL-6, but roughly equipotent to whole pneumococci.

Role of CD14 in cytokine responses in whole blood. Inhibitors were again used to assess the role of CD14 and PAF receptor as inflammatory receptors in whole blood. As plotted in Fig. 4, LPS-induced TNF, IL-1β, or IL-6 was readily blocked by the anti-CD14 Ab. In contrast to the results obtained with cultured THP-1 monocytes, however, cytokine production was not inhibited by anti-CD14 for both intact pneumococci and pneumococcal cell wall. This suggests that although purified cell wall clearly signals via CD14 in cultured monocytes (Fig. 1), other receptors may play a major role in whole blood. This non-CD14-dependent effect, arising from either nonmyeloid blood cells or cells of the monocyte-macrophage lineage which are more mature than the cultured THP-1 cells, differs from the non-CD14-mediated stimulation by whole *S. pneumoniae* on THP-1 cultured cells.

These experiments raise the question of the possibility of more than one non-CD14-mediated pathway for induction of cytokines by gram-positive but not gram-negative bacterial components. One pathway is detectable on relatively immature monocytes stimulated with pneumococci but not cell walls. The second pathway is stimulated by both pneumococci and cell wall components and can be detected in whole blood. Considering the heterogeneity of cells in blood, the details of these differences remain to be determined. Neither of these pathways appears to involve PAF receptor G-protein-mediated effects, as evidenced by the lack of effect of the PAF receptor antagonist (Fig. 4). Participation of the PAF receptor independent of G-protein signalling remains to be assessed.

As presented in Fig. 5, inhibitors of intracellular signalling events showed the same activities as in THP-1 cells for TNF and IL-1β, i.e., involvement of G_s protein and tyrosine phosphorylation in the induction of TNF by any of the applied inflammatory stimuli. The main intracellular difference between the THP-1 assay and the ex vivo system, however, was the inability of genistein to thoroughly inhibit IL-6 production in whole blood. Only at the lower doses of stimuli used (5 ng of LPS, 1 μg of cell wall) was a modest 50% inhibition of IL-6 secretion observed in the ex vivo assay (Fig. 5). This is in contrast to the almost 100% inhibition of IL-6 production by genistein in the THP-1 monocytes (Fig. 2). This difference may be attributed to participation of other blood cell types in the whole-blood assay, a possibility consistent with LPS-induced production of IL-6, but not of TNF or IL-1, by B cells (11, 19, 22). Induction of IL-6 in B cells might be less dependent on

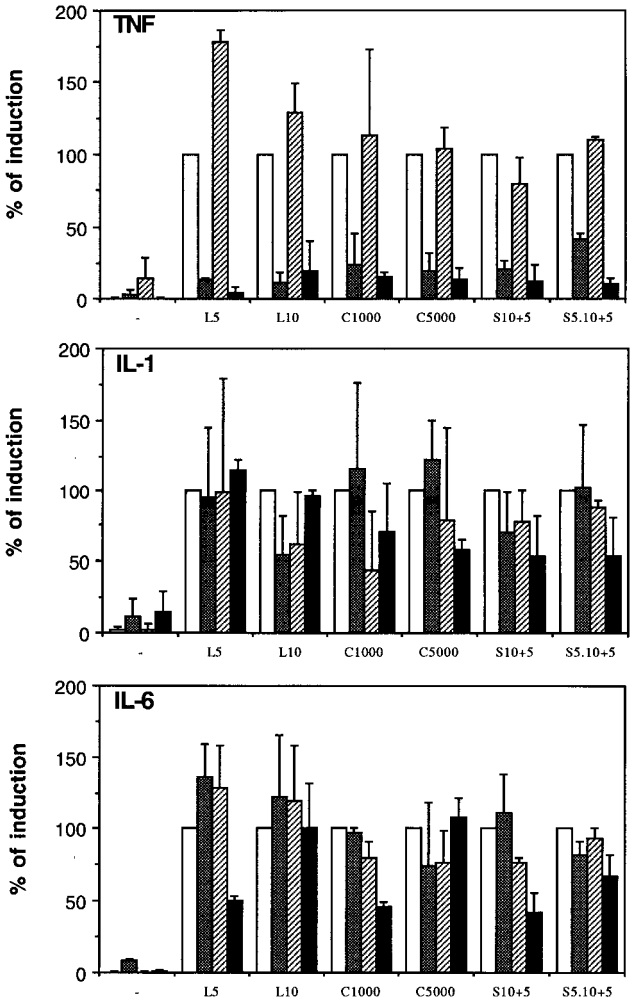

FIG. 5. Effect of inhibition of G proteins and tyrosine phosphorylation on cytokine induction in whole human blood. Blood was pretreated for 2 h with either 10 μg of cholera toxin per ml (shaded bars), 10 μg of pertussis toxin per ml (striped bars), 1 mM genistein (solid bars), or saline alone (open bars). Stimulation and cytokine measurements were performed as described in the legend to Fig. 4.

tyrosine phosphorylation than in monocytes and could therefore account for the persistent IL-6 release in LPS-induced whole blood pretreated with genistein.

Model for induction of cytokines by gram-positive bacteria. We sought to identify overlap in receptor usage and intracellular signaling cascades between *S. pneumoniae* and LPS-induced inflammatory cytokine induction. Inconsistencies in the literature can now be attributed to variations in several parameters important for cytokine production: cell type stimulated, type of stimulus, and cytokine measured. CD14 was critical for all responses to LPS under all conditions used herein. Conversely, CD14 could not be shown to participate in cytokine induction upon stimulation with intact pneumococci under all conditions used herein. Finally, CD14-dependent effects could be demonstrated for cell walls in the relatively immature THP-1 human monocyte cell line but not ex vivo when whole human blood was used. Thus, as many as two receptors in addition to CD14 appear to lead to cytokine production by gram-positive components: one on THP-1 cells for pneumococci (probably pneumococcal proteins) and not cell walls, and another for both pneumococci and cell walls in whole-blood cellular elements. Despite the clear-cut differences in extracellular receptor utilization, however, the signaling pathways used by LPS and pneumococci might converge intracellularly, as demonstrated by parallel results obtained with cholera toxin, pertussis toxin, and genistein. This observation might be important for future research on therapeutic inhibitory drugs aimed at possibly blocking both gram-negative and gram-positive sepsis.

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