**Streptococcus pneumoniae** Autolysis Prevents Phagocytosis and Production of Phagocyte-Activating Cytokines

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**Streptococcus pneumoniae** is a major pathogen in humans. The pathogenicity of this organism is related to its many virulence factors, the most important of which is the thick pneumococcal capsule that minimizes phagocytosis. Another virulence-associated trait is the tendency of this bacterium to undergo autolysis in stationary phase through activation of the cell wall-bound amidase LytA, which breaks down peptidoglycan. The exact function of autolysis in pneumococcal pathogenesis is, however, unclear. Here, we show the selective and specific inefficiency of wild-type **S. pneumoniae** for inducing production of phagocyte-activating cytokines in human peripheral blood mononuclear cells (PBMC). Indeed, clinical pneumococcal strains induced production of 30-fold less tumor necrosis factor (TNF), 15-fold less gamma interferon (IFN-γ), and only negligible amounts of interleukin-12 (IL-12) compared with 3 other closely related **Streptococcus** species, whereas the levels of induction of IL-6, IL-8, and IL-10 production were similar. If pneumococcal LytA was inactivated by mutation or by culture in a medium containing excess choline, the pneumococci induced production of significantly more TNF, IFN-γ, and IL-12 in PBMC, whereas the production of IL-6, IL-8, and IL-10 was unaffected. Further, adding autolyzed pneumococci to intact bacteria inhibited production of TNF, IFN-γ, and IL-12 in a dose-dependent manner but did not inhibit production of IL-6, IL-8, and IL-10 in response to the intact bacteria. Fragments from autolyzed bacteria inhibited phagocytosis of intact bacteria and reduced the in vitro elimination of pneumococci from human blood. Our results suggest that fragments generated by autolysis of bacteria with reduced viability interfere with phagocyte-mediated elimination of live pneumococci.

The pneumococcus **Streptococcus pneumoniae** is a leading cause of community-acquired pneumonia, meningitis, otitis media, and sinusitis and is a common cause of infection-related mortality in children and elderly people (28, 37). There is a large number of streptococcal species whose taxonomic classification is debated (14, 31). A number of streptococci, including alpha-hemolytic and nonhemolytic variants, constitute the viridans group, which can be further subdivided into the mitis, sanguinis, anginosus, salivarius, and mutans species, whereas the levels of induction of IL-6, IL-8, and IL-10 production were similar. If pneumococcal LytA was inactivated by mutation or by culture in a medium containing excess choline, the pneumococci induced production of significantly more TNF, IFN-γ, and IL-12 in PBMC, whereas the production of IL-6, IL-8, and IL-10 was unaffected. Further, adding autolyzed pneumococci to intact bacteria inhibited production of TNF, IFN-γ, and IL-12 in a dose-dependent manner but did not inhibit production of IL-6, IL-8, and IL-10 in response to the intact bacteria. Fragments from autolyzed bacteria inhibited phagocytosis of intact bacteria and reduced the in vitro elimination of pneumococci from human blood. Our results suggest that fragments generated by autolysis of bacteria with reduced viability interfere with phagocyte-mediated elimination of live pneumococci.

The ability of phagocytes to kill microbes is augmented by the phagocyte-activating cytokines gamma interferon (IFN-γ) and tumor necrosis factor (TNF), which boost the bactericidal machinery and enhance killing and digestion of bacteria present within the phagosome (4, 39, 47). TNF is produced by monocytes/macrophages and activated T cells, while IFN-γ is produced by NK cells and T cells in response to interleukin-12 (IL-12) from macrophages. Thus, production of TNF, IFN-γ, and IL-12 is necessary for host defense against intracellular bacteria (8, 11, 21, 34, 48). More recently, these phagocyte-activating cytokines have also been shown to be essential for controlling extracellular gram-positive bacteria, including **S. pneumoniae** (36, 42, 50, 52, 54). Thus, a patient with an IL-12 deficiency was shown to suffer from recurrent episodes of pneumococcal infection (20). Phagocyte activation by TNF and/or IFN-γ might be required for decomposition of the thick, sturdy peptidoglycan (PG) layer of gram-positive bacteria after phagocytosis, while gram-negative bacteria may be more easily digested. Thus, human leukocytes produce more TNF, IFN-γ, and IL-12 when they are stimulated with gram-positive bacteria than when they are stimulated with gram-negative bacteria (23, 24). A peculiar property of **S. pneumoniae** is its tendency to undergo autolysis when it reaches the stationary phase of growth. This process is mediated by enzymes called autolysins (ALs), which, when activated, degrade cell wall PG. The major AL is an N-acetyl-muramyl-L-alanine amidase called LytA (27). Other pneumococcal ALs include LytB and LytC, which are believed to be involved mainly in modification of the cell wall during growth and division (16, 17). ALs are anchored to the cell wall via interactions with choline moieties on teichoic

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TABLE 1. Serotype and MLST of seven clinical S. pneumoniae isolates, wild-type strain D39, and autolysin- and pneumolysin-deficient mutants of this strain

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotype</th>
<th>MLST</th>
<th>Source or reference</th>
</tr>
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<tr>
<td>A6</td>
<td>19F</td>
<td>ND*</td>
<td>Otis media patient</td>
</tr>
<tr>
<td>A7</td>
<td>9V</td>
<td>ND</td>
<td>Otis media patient</td>
</tr>
<tr>
<td>A17</td>
<td>14</td>
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<td>481</td>
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<td>ND</td>
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<td>CCUG 23261</td>
<td>11A</td>
<td>62</td>
<td>CCUG</td>
</tr>
<tr>
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<td>5</td>
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<td>CCUG</td>
</tr>
<tr>
<td>D39 WT</td>
<td>2</td>
<td>128</td>
<td>51</td>
</tr>
<tr>
<td>D39 AL</td>
<td>2</td>
<td>128</td>
<td>2</td>
</tr>
<tr>
<td>D39 PLY</td>
<td>2</td>
<td>128</td>
<td>3</td>
</tr>
</tbody>
</table>

* ND, not determined.

acid and lipoteichoic acid (LTA). Choline is necessary for pneumococcal growth, but culture in the presence of high concentrations of choline renders the bacteria incapable of undergoing autolysis (6, 19).

Studies with mice have shown that S. pneumoniae with mutated LytA is less virulent than wild-type pneumococci (2, 7, 25). The reason for this is not clear, but two main hypotheses have been put forward. First, autolysis promotes the release of the intracellular toxin pneumolysin (Ply) (5, 33). Ply is an important determinant of virulence (3, 41) and interferes with several defense systems, including inhibition of ciliary beating (15), complement activation (38), and induction of intracellular oxygen radical production (33). Second, cell wall degradation products, such as soluble PG fragments and LTA released upon autolysis, have been suggested to augment the inflammatory response (9, 10, 44, 49).

Here we examine a third possibility, that autolysis interferes with the generation of phagocyte-activating cytokines. We have previously shown that intact gram-positive bacteria provide a very efficient stimulus for IL-12 production by human monocytes, regardless of whether they are dead or alive (1, 23, 24), but that decomposed bacteria are inactive in this process and soluble components of the gram-positive cell wall, such as PG and LTA, even downregulate the production of IL-12 in response to intact bacteria in a dose-dependent manner (1). These observations led us to speculate that autolysis may promote virulence by generating bacterial cell wall fragments that block IL-12 production and thereby reduce IFN-γ production and phagocyte activation. Indeed, our data demonstrate that AL-mediated disintegration of pneumococci inhibits production of IFN-γ and also TNF in response to intact bacteria. Further, the presence of autolyzed bacteria reduced elimination of live pneumococci by blood cells in vitro.

MATERIALS AND METHODS

Bacterial strains. The pneumococcal strains used in the present study are listed in Table 1. The virulent strain S. pneumoniae D39 (D39 WT) and an AL-deficient mutant (D39 AL−) and pneumolysin-deficient mutant (D39 PLY−) of this strain have been described previously (2, 3). Seven clinical virulent wild-type strains have been described previously (2, 3). Seven clinical virulent wild-type strains were also analyzed by multilocus sequence typing (MLST) as previously described (12). In brief, internal fragments of the aroE, gdh, gpi, recP, spi, spt, and ddl genes were amplified by PCR by use of the primer pairs indicated at http://spneumoniae.mlst.net/misc/info.asp#experimental. Sequences were obtained for both DNA strands by use of an ABI 3730xl DNA analyzer. Alleles from the MLST website (http://spneumoniae.mlst.net) were downloaded for alignment analysis and sequence type determination.

The following seven strains representing species in the Streptococcus viridans group were obtained from Virginia Polytechnic Institute and State University (VPI), the National Collection of Type Cultures (NCTC), the American Type Culture Collection (ATCC), or the Culture Collection of the University of Gothenburg (CCUG): S. mutans VPI 8290, S. salivarius VPI 13484, S. anginosus NCTC 10713, S. intermedius ATCC 27353, S. sanguis ATCC 10556, S. oralis ATCC 35057, and S. mitis CCUG 31611.

Culture and preparation of bacteria. For comparison of cytokine release in response to Streptococcus viridans group strains and pneumococci, viridans group streptococci and S. pneumoniae CCUG 33774 were cultured on horse blood agar overnight. The bacterial colonies were then harvested, washed twice in Dulbecco's endotoxin-free phosphate-buffered saline (PBS) (PAA Laboratories, Linz, Austria), and suspended to an optical density at 580 nm (OD580) of 0.87, which corresponded to 7 × 10^8 bacteria/ml as determined by counting with a microscope. The suspended bacteria were killed by exposure to UV light for 18 min (which was confirmed by a negative viable count) and stored at −70°C until they were used in the experiments.

For studies of the role of autolysis, the pneumococcal strains and the control strain S. mitis CCUG 31661 were cultured to late log phase for 12 to 14 h in Todd-Hewitt broth supplemented with 0.5% yeast extract in the presence or absence of 2% choline chloride (Substrate Department, Clinical Bacteriology Laboratory, Sahlgrenska University Hospital). High concentrations of choline in this broth inhibit the action of LytA and thus prevent bacterial autolysis (6, 19). After two washes, bacteria grown in the presence of choline were suspended in Dulbecco's endotoxin-free PBS with 1% sterile filtered choline chloride, while bacteria grown in the absence of choline were suspended in PBS without choline. Bacteria were counted, the OD650 was adjusted to 0.87 (equivalent to 7 × 10^8 bacterial cells/ml as determined by counting separate cells with a microscope), and then the bacteria were inactivated by UV light and frozen.

UV inactivation of wild-type pneumococci leads to bacterial death followed by autolysis, the latter mediated by ALs. To measure the proportion of autolysis occurring during UV inactivation, freezing, and thawing, the bacterial concentrations before and after this treatment were compared by using OD650 measurements and microscopic enumeration. UV inactivation of the Streptococcus viridans group strains, the LytA-negative mutant D39 AL−, and the strains grown in the presence of choline had little or no effect on the number of bacterial cells (7 × 10^8 cells/ml before treatment and 6 × 10^8 to 7 × 10^8 cells/ml after treatment). In contrast, when wild-type strains grown in plain broth had been UV inactivated, frozen, and thawed, only 0.5 × 10^9 to 2 × 10^9 intact bacterial cells/ml remained in the preparations. Similarly, the OD650 values were markedly reduced (from 0.87 to 0.3 to 0.5) in the autolysis-positive preparations but not in the autolysis-negative preparations.

For stimulation of peripheral blood mononuclear cells (PBMC), different dilutions of the thawed bacterial preparations were added directly to PBMC cultures. The concentrations indicated below refer to the numbers of bacteria present before UV inactivation (i.e., before autolysis took place). Thus, in preparations of strains with active ALs, approximately one-fifth of the bacteria were intact, while 80% of the bacteria had lysed and generated cell wall fragments and cytoplasmic constituents that were present in the preparations.

In some experiments, the autolysin-positive preparations were centrifuged at 13,000 × g for 10 min at 4°C to crudely separate the remaining, more or less intact bacteria and soluble fragments. Each supernatant was collected, and the pellet was resuspended in the original volume of PBS with choline to prevent further autolysis.

Purified PG and LTA were also used for stimulation of PBMC. These compounds originated from S. aureus and were purchased from Sigma (St. Louis, MO). N-Acetylmuramyl-l-alanine-␥-D-glutamic acid (MDP) was also purchased from Sigma.

To generate bacterial fragments of S. mitis, UV-inactivated bacteria were sonicated for 10 min with strong cooling at amplitude 70 (Vibra cell; Sonics & Natick, MA).
Materials Inc., Danbury, CT). Less than 10% of the bacterial cells remained intact, as determined by enumeration with a microscope.

Preparation and stimulation of blood mononuclear cells. PBMC were prepared from blood donor buffy coats (Blood Bank, Sahlgrenska University Hospital) by density gradient centrifugation (Lymphoprep; Axis-Shield, Norway) for 20 min at 820 × g at room temperature. The cells were washed three times in ice-cold endotoxin-free RPMI 1640 medium with 2 mM t-glutamine (PAA Laboratories GmbH, Linz, Austria) and suspended in RPMI 1640 medium with t-glutamine, 5% inactivated fetal calf serum (Invitrogen, San Diego, CA), and 0.1% gentamicin (50 mg/ml; Sigma) to a final concentration of 2 × 10⁶ cells/ml. The cells were stimulated with 3 × 10⁶ or 3 × 10⁷ UV-inactivated bacteria/ml (based on the original numbers of bacteria determined before autolysis took place [see above]) in 96-well flat-bottom plates (Nunc, Roskilde, Denmark) at 37°C in a humidified atmosphere with 5% CO₂. Supernatants were harvested after 24 h and 5 days, the latter time point was used to measure IFN-γ.

The viability of PBMC was assessed after 24 h of bacterial stimulation. The culture plates were placed on ice. Cells were removed using a rubber policeman, washed with PBS, and incubated with the nucleic acid dye 7-amino-actinomycin D (BD Pharmingen), which permeates nonviable cells. The proportion of stained cells was analyzed by flow cytometry (Becton Dickinson FACScan equipped with FlowJo software).

Cytokine determination. The concentrations of IL-12, TNF, IFN-γ, IL-6, IL-8, and IL-10 were measured by enzyme-linked immunosorbent assays as described in detail elsewhere (29). In brief, Costar plates (Invitrogen) were coated with the following anti-human monoclonal antibodies: anti-IL-12p70 (20C2), anti-TNF-α (MAb1), anti-IFN-γ (NIB42), anti-IL-6 (MO2-13A5), anti-IL-8 (G265-5), and anti-IL-10 (JES3-9D7), all obtained from BD Pharmingen (San Diego, CA). The plates were blocked with 5% bovine serum albumin (BSA) in PBS. Standard curves were generated by diluting recombinant human IL-12, TNF-IFN-γ, IL-6, IL-8, and IL-10 (BD Pharmingen) in high-performance enzyme-linked immunosorbent assay dilution buffer (CLB, Amsterdam, The Netherlands).

The following biotinylated anti-human monoclonal antibodies were diluted in this buffer and used for detection: anti-IL-12p40/p70 (20C2), anti-TNF-α (Mab1), anti-IFN-γ (NIB42), anti-IL-6 (MO2-13A5), anti-IL-8 (G265-5), and anti-IL-10 (JES3-9D7), all obtained from BD Pharmingen (San Diego, CA). The plates were blocked with 5% bovine serum albumin (BSA) in PBS. Standard curves were generated by diluting recombinant human IL-12, TNF, IFN-γ, IL-6, IL-8, and IL-10 (BD Pharmingen) in high-performance enzyme-linked immunosorbent assay dilution buffer (CLB, Amsterdam, The Netherlands).

To examine whether bacteria were internalized by monocytes, PBMC were prepared from blood donor buffy coats as described above. Red blood cells were lysed by incubation in distilled H₂O for 30 s. The cells were suspended in RPMI 1640 medium with t-glutamine and 5% inactivated fetal calf serum (Invitrogen, San Diego, CA) and suspended in RPMI 1640 medium with t-glutamine and 5% inactivated fetal calf serum (Invitrogen) at a concentration of 1 × 10⁶ cells/ml and incubated with 3 × 10⁷ bacteria/ml (enumerated before UV inactivation) in 24-well plates (Nunc) for 30 min at 37°C. The cells were washed, subjected to cytocentrifugation onto glass slides (Cytospin; Shandon Southern, Runcorn, United Kingdom), and then stained with Diff-quick (Dade Behring AG, Duedingen, Switzerland). At least 80 monocytes were examined, and the proportion of monocytes containing surface-associated or internalized bacteria was determined.

Assays for phagocytosis and intracellular killing by whole blood. The capacity of whole blood to eliminate live pneumococci was assessed as described previously, with minor modifications (13). S. pneumoniae CCUG 10708 was grown to early exponential phase (A₅₅₀ 0.15 to 0.2) in Todd-Hewitt broth with yeast extract supplement with 2% choline chloride, and approximately 10⁷ CFU was added to 1 ml of heparinized human blood. After incubation with end-over-end rotation at 37°C for 2 to 3 h, serial dilutions were plated in duplicate on horse blood agar. To include intracellular bacteria in the counts, Triton X-60 was added at a final concentration of 0.1%, and the blood samples were vortexed vigorously before the dilution and plating process was repeated.

To elucidate the effect of intact and autolyzed bacteria on elimination of bacteria by blood, 6 × 10⁷ cells/ml UV-inactivated D39 WT or D39 AL- (as determined before autolysis) were added to the human blood cells 10 min before addition of 10⁹ CFU/ml live S. pneumoniae CCUG 10708. Killing of S. pneumoniae CCUG 10708 was assessed as described above.

Statistical analysis. The Wilcoxon matched-pair test was used to compare cytokine production induced by AL-positive bacteria and cytokine production induced by AL-negative bacteria. The same test was used to compare cytokine induction by intact bacteria alone and cytokine induction by intact bacteria mixed with autolyzed, sonicated, or soluble or pellet fractions of autolyzed bacteria. The Mann-Whitney test was used to compare the cytokines induced by S. pneumoniae with the cytokines induced by the related viridans group streptococci and D39 WT with D39 PLY-. Further, the Mann-Whitney test was used to compare the mean numbers of phagocytosed pneumococci or pneumococci interacting with monocytes with and without addition of autolyzed pneumococci. The Mann-Whitney test was also used to compare the numbers of viable bacteria remaining after incubation with blood cells for 2 to 3 h with and without addition of UV-inactivated intact or autolyzed pneumococci.

RESULTS

PBMC produce less phagocyte-activating cytokines when they are stimulated with S. pneumoniae than when they are stimulated with related Streptococcus species. TNF and IFN-γ are the major activators of phagocyte-mediated bacterial killing, while IL-12 is a trigger of IFN-γ production. In general, gram-positive bacteria induce production of large amounts of TNF, IFN-γ, and IL-12 in freshly isolated human PBMC (23, 24).

Pneumococci are closely related to streptococci in the viridans group, but they have a range of unique virulence factors, including the peculiar ability to undergo spontaneous autolysis in stationary culture. One strain each of S. pneumoniae (CCUG 33774) and seven related Streptococcus species (S. mutans, S. salivarius, S. anginosus, S. intermedius, S. sanguinis, S. oralis, and S. mitis) were added to human blood donor PBMC. Whereas all viridans group streptococci induced production of massive quantities of TNF and IFN-γ (8 to 38 ng/ml and 50 to 160 ng/ml, respectively), the amounts induced by the S. pneumoniae strain were 10 to 50 times lower (Fig. 1). TNF is produced by monocytes after direct contact with bacteria, while IFN-γ is produced by T cells and NK cells in response to IL-12 from monocytes and related cells. In accord with this, we observed no IL-12 in S. pneumoniae-stimulated cultures, while all cultures stimulated with viridans streptococci contained very large amounts of IL-12 after 24 h (1 to 5 ng/ml) (Fig. 1). In sharp contrast, pneumococci induced the production of levels of IL-6, IL-8, and IL-10 similar to the levels obtained with the other Streptococcus species tested (Fig. 1).

To investigate whether these findings could be generalized, the cytokine production induced by six additional S. pneumoniae strains belonging to various sero- and genotypes (A6, A7, A17, A22, A42, and CCUG 23261 [Table 1]) was examined. All of the pneumococcal strains tested induced production of little TNF and IFN-γ (0.3 to 1.2 ng/ml and 1 to 20 ng/ml, respectively), and none of them induced production of detectable IL-12 (≥ 20 pg/ml). Thus, on average, the pneumococcal strains induced production of 30 times less TNF, 15 times less IFN-γ, and at least 50 times less IL-12 than related streptococcal species. On the other hand, the levels of IL-6, IL-8, and IL-10 were comparable to the levels induced by the other Streptococcus species (data not shown). Thus, the pattern of cytokines generated by human PBMC in response to pneumococci was characterized by conspicuous reductions in the levels of cytokines which promote bacterial killing by macrophages (39, 40) and neutrophils (4).

Chemical inhibition of autolysis restores the ability of pneumococci to induce production of phagocyte-activating cytokines. A fraction of S. pneumoniae cells spontaneously lyse when a culture reaches stationary phase due to activation of PG-degrading enzymes called ALs, and the dominant AL is LytA (27). We have previously shown that soluble components of the gram-positive cell wall, such as PG and LTA, readily

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induce IL-6 and IL-8 production in monocytes but induce production of much less TNF and IL-12 than the corresponding amount of intact gram-positive bacteria (1, 23). We therefore speculated that the specific failure of pneumococci to induce production of TNF, IFN-γ, and IL-12 may be related to the capacity of pneumococci to autolyze.

To determine if spontaneous autolysis was responsible for the unique cytokine pattern induced by pneumococci, the seven *S. pneumoniae* isolates were grown to stationary phase in normal broth or broth supplemented with 2% choline chloride. Growth in medium with excess choline is known to inhibit the function of ALs (6, 19). The bacteria were washed, and the concentration was adjusted to $7 \times 10^8$ cells/ml (in choline-containing PBS for bacteria cultured with an excess of choline), after which the preparations were UV inactivated and frozen at $-70^\circ$C. After thawing, preparations of pneumococci cultured in the presence of high levels of choline (CC+) contained almost the original number of intact bacteria, as determined by counting with a microscope, while for bacteria cultured and prepared without added choline (CC−), ~30% of the original number of bacteria were in the intact form and the rest of the bacteria had been degraded (Fig. 2A). Preparations containing variable proportions of intact and fragmented bacteria were added to human blood donor cells at 1/20 and 1/200 dilutions (corresponding to $3 \times 10^7$ and $3 \times 10^8$ bacteria/ml, respectively, in the original preparations before autolysis occurred).

Pneumococcal strains prevented from autolysis by culture and preparation in the presence of high concentrations of choline (CC+) induced the production of significantly more TNF, IFN-γ, and IL-12 than the same strains cultured and prepared under standard conditions (CC−) (Fig. 2). In contrast, the induction of production of IL-6, IL-8, and IL-10 was the same regardless of the culture conditions. The same pattern was seen for all seven pneumococcal strains, but the strongest induction of IL-12, TNF, and IFN-γ production among the CC+ bacteria and the largest difference related to culture conditions (CC+/CC−) were seen for strain A17, which is a serotype 14 and MLST 124 strain (Fig. 2B). To exclude the possibility that choline had an effect on PBMC function, *S. mitis*, which is incapable of autolysis, was grown and prepared in media with and without choline and used to stimulate PBMC. The presence of choline did not affect the production of any of the cytokines in response to *S. mitis* (data not shown).

We compared the cytokine responses to intact pneumococci and to the soluble cell wall components PG and LTA, as well as MDP, which is the smallest basic unit of PG interacting with NOD2 (18). We assumed that PG accounts for 50% of the gram-positive bacterial cell mass and that the bacteria are spherical with a diameter of 1 μm. Using these assumptions, $3 \times 10^7$ bacteria would contain 10 μg of PG and probably less LTA. Stimulation of PBMC with 10 μg/ml of PG or LTA induced production of levels of IL-6, IL-8, and IL-10 that were similar to or higher than the levels produced when $3 \times 10^7$ cells/ml of *S. pneumoniae* or *S. mitis* were used for induction, while intact bacteria were much more potent inducers of TNF, IFN-γ, and IL-12 production than corresponding amounts of
FIG. 2. Pneumococci with inhibited ALs induce production of higher concentrations of TNF, IFN-γ, and IL-12 from PBMC than their AL-positive counterparts. Seven clinical isolates of *S. pneumoniae* (A6, A7, A17, A22, A24, CCUG 23261, and CCUG 33774 [Table 1]) were cultured, washed, and prepared in normal broth (CC- or in broth containing high levels of the autolysis inhibitor choline chloride (CC+). The AL-negative mutant D39 AL- and the wild-type control D39 WT (WT) were cultured and prepared in normal broth. (A) The amounts of autolysis that occurred in the bacterial preparations during the process that included UV inactivation, freezing, and thawing were determined by counting the remaining intact cells with a microscope and comparing the results to the original concentrations. Each cell in bacterial chains was counted separately, and the percentages of intact cells (based on the $7 \times 10^8$ cells/ml which were present in the preparations before UV inactivation; mean ± standard deviation for three individual counts) are shown. (B) PBMC from six blood donors were stimulated with the seven clinical isolates.
isolated bacterial components (Table 2). MDP was a poor inducer of cytokine production at doses up to 10 μg/ml (Table 2).

Although the AL-inhibited pneumococci induced the production of levels of the phagocyte-activating cytokines that were higher than the levels induced by strains allowed to autolyze, they were still inferior inducers of production of these cytokines compared to related viridans group streptococci. This finding was most pronounced for IL-12 and may be related to the poor phagocytosis of encapsulated *S. pneumoniae*, as we have previously shown that phagocytosis is an absolute requirement for IL-12 production by human monocytes in response to gram-positive bacteria (1). Indeed, when the nonencapsulated strain *S. pneumoniae* CCUG 10708 was grown in a manner that inhibited autolysis, it induced the production of levels of TNF and IFN-γ similar to the levels induced by *S. mitis* and the production of only two to three times less IL-12 (Table 2).

The ability to stimulate production of phagocyte-activating cytokines is increased in a LytA-deficient strain of *S. pneumoniae*. Autolysis is achieved mainly by activation of the LytA enzyme (27). We compared the cytokine patterns induced by the LytA-negative mutant D39 AL− and the wild-type control strain D39 WT. Stationary-phase wild-type and AL− cultures were adjusted so their concentrations were the same and then UV inactivated and frozen. After the preparations were adjusted so their concentrations were the same and then

### Table 2. Cytokine production in human PBMC in response to bacteria and doses of bacterial products

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Cytokine concn (ng/ml)</th>
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<tbody>
<tr>
<td></td>
<td>TNF</td>
</tr>
<tr>
<td><em>S. pneumoniae</em> A17 CC+ (3 × 10⁷ bacteria/ml)</td>
<td>11 ± 9.7</td>
</tr>
<tr>
<td><em>S. pneumoniae</em> CCUG 10708 CC+ (3 × 10⁷ bacteria/ml)</td>
<td>30 ± 12</td>
</tr>
<tr>
<td><em>S. mitis</em> CCUG 31661 (3 × 10⁷ bacteria/ml)</td>
<td>30 ± 14</td>
</tr>
<tr>
<td>PG (S. aureus) (10 μg/ml)</td>
<td>1.9 ± 1</td>
</tr>
<tr>
<td>MDP (10 μg/ml)</td>
<td>0.18 ± 0.12</td>
</tr>
<tr>
<td>LTA (S. aureus) (10 μg/ml)</td>
<td>1.2 ± 1.2</td>
</tr>
</tbody>
</table>

| a | PBMC were incubated with various stimuli, and the production of cytokines was measured using the supernatant after 24 h (TNF, IL-12, IL-6, IL-8, and IL-10) or after 5 days (IFN-γ) by an enzyme-linked immunosorbent assay. CC+ indicates that the organism was grown and prepared in the presence of high levels of choline. The data are the means ± standard deviations for three to six blood donors.

Release of pneumolysin is not responsible for the modulated cytokine pattern induced by autolyzed pneumococci. Pneumolysin (Ply) is an intracellular toxin known to be released during autolysis of pneumococci (5). To investigate if released Ply was responsible for the “pneumococcus-specific” cytokine pattern, we examined the cytokines produced in response to a pneumolysin mutant strain, *S. pneumoniae* D39 Ply−, and the wild-type control strain D39 WT. D39 Ply− and D39 WT both have the capacity to autolyze and induced practically identical cytokine patterns (see Fig. S1 in the supplemental material). Release of Ply into the supernatant of a wild-type strain but not of a Ply− mutant strain was previously confirmed by Western blotting (33). We concluded that release of Ply during autolysis was not responsible for the reduction in the production of phagocyte-activating cytokines induced by pneumococci capable of autolysis.

Reduced PBMC viability cannot explain the altered cytokine production. The viability of PBMC from six blood donors was assessed after culture for 24 h with D39 WT, D39 AL−, D39 Ply−, and clinical isolates A17 and CCUG 23261 cultured with or without added choline. The viability of PBMC was assessed by determining the resistance to uptake of the DNA-binding dye 7-amino-actinomycin D and was analyzed by flow cytometry. Cultures stimulated with bacteria contained slightly more dead cells than nonstimulated cultures contained (6.4% ± 2.1% versus 2.9% ± 0.6%; P < 0.01). However, cultures stimulated with pneumococci capable of autolysis contained no more dead cells than cultures stimulated with bacteria prevented from autolyzing (data not shown). Thus, cell death could not account for the selectively reduced production of phagocyte-activating cytokines in response to autolyzed pneumococci.

Autolyzed pneumococci induce the production of less phagocyte-activating cytokines than intact pneumococci at all time points tested. To exclude the possibility that our findings were due to differences in the kinetics of cytokine production in of *S. pneumoniae* grown under normal conditions or in the presence of excess choline using 3 × 10⁷ bacteria/ml (measured before autolysis took place; 3 × 10⁷ bacteria/ml for IL-12). The cytokine production was measured using the supernatant after 24 h (TNF, IL-12, IL-6, IL-8, and IL-10) or 5 days (IFN-γ). The cytokine responses to each *S. pneumoniae* strain when cells were grown in normal broth or in broth with added choline are expressed as the cytokine response ratio (CC+/CC−) (mean and standard deviation) and were compared using the Wilcoxon matched-pair test, **, P < 0.05; ***, P < 0.01. (C) PBMC were stimulated with 3 × 10⁷ bacteria/ml (before autolysis) of *S. pneumoniae* D39 WT or the LytA-deficient mutant D39 AL−, and the cytokine production was measured using the supernatant after 24 h (TNF, IL-12, IL-6, IL-8, and IL-10) or 5 days (IFN-γ). Each symbol indicates the response of one of nine blood donors. The cytokine responses to wild-type and mutant bacteria in the same donor were compared using the Wilcoxon matched-pair test, ***, P < 0.01.
response to intact and autolyzed pneumococci, we measured cytokines in supernatants at 4, 8, 12, 18, and 24 h and 3, 5, and 7 days after stimulation with pneumococci capable (D39 WT and A17 CC) or not capable (D39 AL and A17 CC) of autolysis. Confirming our findings for the 24-h and 5-day time points, larger amounts of TNF and IFN-γ were produced in response to intact bacteria at all time points tested. IL-12 was not always detected, but when it was present, the level was maximal after 24 h and the cytokine was produced only in response to bacteria prevented from autolysis (D39 AL or A17 CC). Production of equal amounts of IL-6, IL-8, and IL-10 was induced in response to autolyzed and intact bacteria at all time points examined. Similar results were obtained using high and low doses of bacteria (3 × 10^7 and 3 × 10^6 bacteria/ml before autolysis) (see Fig. S2 in the supplemental material; data not shown). We concluded that a shift in kinetics in response to intact and autolyzed bacteria could not account for the low level of TNF at 24 h or the low level of IFN-γ at 5 days after stimulation with autolyzed pneumococci. Furthermore, the absence of IL-12 or production of a very low level of IL-12 in response to pneumococci, particularly pneumococci capable of autolysis, was confirmed at all time points.

The presence of bacteria that have undergone autolysis reduces the production of phagocyte-activating cytokines in response to intact bacteria. Although the preparations of AL-positive strains contained up to 20% intact bacterial cells when they were examined with a microscope, a high dose of AL-positive pneumococci (3 × 10^7 bacteria/ml before autolysis) still induced production of significantly less TNF and IFN-γ than a 10-fold-lower dose of intact pneumococci (data not shown). Thus, the absence of intact bacteria might not be the only explanation for the low levels of phagocyte-activating cytokines produced. We hypothesized that bacterial degradation products released during autolysis inhibited the cytokine production which otherwise would be induced by intact bacteria.

To test this hypothesis, PBMC from six blood donors were stimulated with two intact clinical pneumococcal isolates (CCUG 23261 and the otitis media strain A17 cultured and prepared with choline) at a concentration of 3 × 10^7 or 3 × 10^6 bacteria/ml in the presence of various concentrations of the corresponding autolyzed bacteria (3 × 10^7, 3 × 10^6, and 3 × 10^5 cells/ml before autolysis). Figure 3 shows that addition of preparations containing autolyzed bacteria reduced the amounts of TNF, IFN-γ, and IL-12 induced by intact bacteria in a dose-dependent manner. Compared to intact bacteria, significant inhibition of the TNF (P < 0.05) and IFN-γ (P < 0.01) responses was obtained by adding a 10-fold-lower dose of autolyzed bacteria (Fig. 3). Thus, if only 1 bacterium in 10 autolyses, the TNF and IFN-γ responses to the remaining intact bacteria are significantly reduced. The IL-12 responses were optimal when 3 × 10^6 intact bacteria/ml were used, and addition of an equal amount of autolyzed bacteria caused significant inhibition (P < 0.05) (Fig. 3). Production of IL-6, IL-8, and IL-10 was not affected or was slightly increased when autolyzed bacteria were added to intact pneumococci (Fig. 3), which was expected, as both fragmented and intact bacteria could induce production of these cytokines.

The same pattern of strong and dose-dependent inhibition of the phagocyte-activating cytokines was seen when D39 AL- was mixed with graded concentrations of D39 WT. Addition of a 10-times-lower dose of autolyzed wild-type bacteria significantly reduced the production of TNF in response to D39 AL-, and adding a 100-fold-lower dose of wild-type bacteria significantly inhibited the IFN-γ response to D39 AL- (see Fig. S3 in the supplemental material). Most donors did not produce IL-12 in response to this strain, but when IL-12 was detected in response to intact bacteria, the amount was reduced by adding bacteria capable of autolysis (data not shown). The levels of IL-6, IL-8, and IL-10 were the same or increased when autolyzed bacteria were added to the intact bacteria (see Fig. S3 in the supplemental material).

The soluble fraction of autolyzed bacteria inhibits induction of phagocyte-activating cytokines by intact bacteria. The preparations of wild-type bacteria grown in normal broth contained both intact bacteria and presumably both larger and smaller fragments of bacterial cell walls. Autolyzed S. pneumoniae D39 WT and A17 were separated into soluble and pellet fractions by centrifugation (15,000 × g, 10 min). Various amounts of the soluble or pellet fractions were mixed with intact D39 AL- or A17 CC- and added to PBMC, after which cytokine production was measured. The inhibition of TNF and IFN-γ production was most pronounced when the supernatant fraction was added. Thus, supernatant originating from only 3 × 10^6 autolyzed bacteria/ml significantly inhibited TNF production induced by 3 × 10^7 intact bacteria/ml, while the pellet from 3 × 10^7 autolyzed bacteria was required for a similar inhibitory effect (Fig. 4A). The level of IL-12 produced was low or undetectable, but, when IL-12 was present, its production was inhibited by both the pellet and the supernatant fraction (data not shown).

Sonicated bacteria and soluble bacterial components inhibit production of phagocyte-activating cytokines in response to intact bacteria. While phagocytes may have to be activated in order to efficiently disrupt microbial cell walls, such activation is not necessary once the bacteria have been degraded. Thus, it would be logical for immune cells to turn off production of TNF and IFN-γ in response to bacterial fragments. Sonicated gram-positive bacteria induce less TNF, IFN-γ, and IL-12 production than the same amount of intact bacteria (1; unpublished data). We tested whether fragments of streptococci generated by sonication also downregulated these cytokines in response to intact bacteria. S. mitis, which is not capable of autolysis, was subjected to sonication, and PBMC from six blood donors were stimulated with 3 × 10^7 bacteria/ml of intact UV-inactivated S. mitis in the presence or absence of various doses of sonicated S. mitis. Dose-dependent inhibition of production of TNF, IFN-γ, and IL-12 (i.e., cytokines yielding phagocyte activation) but not of IL-6, IL-8 or IL-10 was seen when intact and sonicated S. mitis cells were mixed (see Fig. S4 in the supplemental material).

We also determined whether purified PG, MDP, and LTA (i.e., components of the gram-positive cell wall) could inhibit TNF, IFN-γ, and IL-12 production induced by intact pneumococci. PBMC from six blood donors were stimulated with S. pneumoniae A17 CC in the presence or absence of various amounts of PG, MDP, and LTA. Indeed, both PG and MDP significantly inhibited production of TNF and IFN-γ but not production of IL-6, IL-8, and IL-10 in response to intact S. pneumoniae A17. LTA significantly inhibited IFN-γ produc-
tion but not production of the other cytokines in response to A17 CC
(H11001). Since the level of IL-12 was low or production of IL-12 was undetectable in response to A17 CC
(H11001), PBMC were also stimulated with S. mitis together with PG, MDP, and LTA. The S. mitis-induced IL-12 production was inhibited by PG, MDP, and LTA strongly and in a dose-dependent manner (data not shown). These data confirm and extend our previous findings that IL-12 production by monocytes in response to bifidobacteria is downregulated by bacterial sonicates, as well as by soluble cell wall components, such as PG and LTA (1).

Autolyzed bacteria block access of intact bacteria to the monocyte surface and phagocytosis. We speculated that fragments of the pneumococcal cell wall, released during autolysis, block interactions between intact bacteria and monocytes. PBMC were incubated with intact pneumococci (3 × 10^7 bacteria/ml) in the presence or absence of autolyzed bacteria (originally 3 × 10^7 bacteria/ml) for 30 min, after which they were spun onto glass slides, stained, and examined microscopically. However, the bacteria were not internalized after 30 min of incubation, reflecting the resistance of encapsulated pneumococci to uptake by phagocytes in the absence of capsule-specific antibodies. S. pneumoniae D39 AL-H11002 and clinical isolates of pneumococci prevented from autolysis bound in chains to the monocyte surface (Fig. 5A), but when the same bacteria were cultured under conditions which allowed them to autolyze, they exhibited negligible interactions with monocytes. After 30 min of incubation of PBMC with D39 AL-H11002, 22% ± 4% (mean ± standard deviation) of the monocytes had D39 AL-H11002 chains bound to their surfaces. Addition of both intact D39 AL-H11002 and autolyzed D39 wild-type bacteria simultaneously reduced the percentage of monocytes interacting with bacteria to 9% ± 3% (P < 0.05 compared with the AL mutant alone). Similarly, interactions between monocytes and bacteria were significantly reduced when equal amounts of intact and autolyzed S. pneumoniae A17 or CCUG 23261 bacteria were added to monocytes compared to the results for incubation with only intact A17 or CCUG 23261 (data not shown). Thus, fragments released during autolysis appeared to block access of intact pneumococci to the monocyte surface.

As encapsulated virulent pneumococci were not phagocytosed under the conditions employed, we used a capsule-neg-

FIG. 3. Autolyzed bacteria selectively inhibit the production of TNF, IFN-γ, and IL-12 in response to intact pneumococci. PBMC from six blood donors were stimulated with 3 × 10^7 bacteria/ml (or 3 × 10^6 bacteria/ml for measurements of IL-12) of clinical isolates A17 and CCUG 23226 cultured in the presence of excess choline chloride (CC+). Graded concentrations of the corresponding bacteria cultured in normal broth (CC−) were added, and the cytokines produced were measured using the supernatant after 24 h or after 5 days (for IFN-γ). The medians and quartiles are indicated by boxes, and the error bars indicate the 10th and 90th percentiles. The responses to intact bacteria (CC+) with and without addition of autolyzed bacteria (CC−) in the same donor were compared using the Wilcoxon matched-pair test. *, P < 0.05; **, P < 0.01.
ative pneumococcal strain (CCUG 10708) to study the effect of autolyzed bacteria on phagocytosis. PBMC from four blood donors were incubated with UV-inactivated *S. pneumoniae* CCUG 10708 cultured and prepared in the presence of an excess choline. In contrast to the encapsulated strains, this pneumococcal strain was frequently seen inside the monocytes within vacuoles (Fig. 5B), and 18% ± 3% (mean ± standard deviation) of the monocytes had phagocytosed bacteria after 30 min of incubation. When an equal amount of autolyzed *S. pneumoniae* CCUG 10708 grown without excess choline was added, only 11% ± 2% of the monocytes had internalized bacteria after 30 min of incubation (*P* < 0.05 compared to CCUG 10708 CC⁺ alone). Also, the presence of autolyzed *S. pneumoniae* D39 and A17 inhibited internalization of *S. pneumoniae* CCUG 10708 to at least a similar degree (data not shown).

**FIG. 4.** Production of phagocyte-activating cytokines in response to intact bacteria is selectively inhibited by soluble cell wall fragments (A) The soluble and pellet fractions of autolyzed *S. pneumoniae* A17 were separated by centrifugation (see Materials and Methods). PBMC from six blood donors were stimulated with 3 × 10⁷ bacteria/ml of intact *S. pneumoniae* A17 (A17 CC⁺) in the presence or absence of the soluble or pellet fraction originating from 3 × 10⁵, 3 × 10⁶, or 3 × 10⁷ autolyzed bacteria/ml (A17 CC⁺). Production of TNF and IFN-γ was measured using the 1-day and 5-day supernatants, respectively. The responses to intact bacteria with and without addition of the soluble or pellet fraction of autolyzed bacteria in the same donor were compared using the Wilcoxon matched-pair test. *, *P* < 0.05. (B) PBMC from six blood donors were stimulated with 3 × 10⁷ bacteria/ml of intact *S. pneumoniae* A17 (A17 CC⁺) in the absence or presence of various doses of PG, MDP, or LTA. Cytokines were measured using the supernatant after 24 h (TNF, IL-6, IL-8, and IL-10) or 5 days (IFN-γ). The responses to *S. pneumoniae* A17 with and without addition of PG, MDP, or LTA in the same donor were compared using the Wilcoxon matched-pair test. *, *P* < 0.05.

**DISCUSSION**

Gram-positive bacteria are, in general, very strong inducers of TNF and IL-12 production in human monocytes and IFN-γ production in PBMC (24). Here we show that *S. pneumoniae* is a striking exception to this rule. Eight *S. pneumoniae* wild-type strains, representing various sero- and genotypes, induced, on average, production of 30-fold less TNF and 15-fold less IFN-γ in PBMC than *S. mitis* and other closely related species in the viridans *Streptococcus* group. Furthermore, most pneumococcal strains completely failed to induce IL-12 production. *S. pneumoniae* has previously been reported to induce production of much lower levels of TNF than group B streptococci in murine macrophages (22). As TNF and IFN-γ are the key activators of phagocyte microbicidal mechanisms and IL-12 induces IFN-γ production in T cells and NK cells, we regard the capacity of pneumococci to avoid triggering production of these cytokines as a putative virulence mechanism.

The poor ability of pneumococci to induce production of TNF, IFN-γ, and IL-12 was related, at least in part, to the capacity of these organisms to undergo autolysis in stationary culture. When PBMC were stimulated with pneumococci that were prevented from autolyzing, either by culture in the presence of excess choline or by mutation of the major LytA AL gene, significantly more TNF and IFN-γ were produced than when PBMC were stimulated with the AL-positive counterparts. Although most clinical pneumococcal strains induced production of low levels of IL-12 even when autolysis was
inhibited, slightly higher levels were produced in PBMC in response to intact bacteria than in response to autolyzed bacteria. In contrast, similar amounts of IL-6, IL-8, and IL-10 were produced in response to intact and autolyzed strains, and pneumococci induced production of as much IL-6, IL-8, and IL-10 as related *Streptococcus* species. Further, production of similar amounts of the latter cytokines was induced by both intact bacteria and purified PG or LTA, while production of much larger amounts of TNF, IFN-γ, and IL-12 was induced by intact bacteria than by a corresponding amount of isolated bacterial components (Table 2). Although the PG and LTA utilized in this study were isolated from *S. aureus* and not *S. pneumoniae*, previous studies have shown that LTA from both of these species stimulate Toll-like receptor 2 and that *S. aureus* LTA is an even more potent inducer of TNF production in macrophages than *S. pneumoniae* LTA (44).

Collectively, our data point to a dichotomy between the stimuli inducing production of the two groups of cytokines; production of TNF, IFN-γ, and IL-12 is induced chiefly by intact bacterial particles, and production of IL-6, IL-8, and IL-10 is induced by both intact bacteria and their fragments. This confirms and extends our previous findings that IL-12 production is induced only by intact gram-positive bacteria and not by PG, LTA, or bacterial sonicates, while IL-6 is produced in response to both intact gram-positive bacteria and their fragments and cell wall components (1). TNF and IFN-γ enhance the lytic capacity of phagocytes, and it is logical that their production is induced by intact bacteria but not by their fragments.

Little IL-12 was produced in response to most virulent pneumococcal strains, even when autolysis was prevented. We suggest that the low level of phagocytosis of encapsulated pneumococci could be the reason for their limited capacity to trigger IL-12 production. We have previously shown that cytochalasin inhibits IL-12 production in response to intact gram-positive bacteria (1). The capsule-negative strain *S. pneumoniae* CCUG 10708 was phagocytosed to a greater degree and induced production of higher levels of IL-12, TNF, and IFN-γ than any of the encapsulated pneumococcal strains tested, while production of similar levels of the other cytokines was induced. Accordingly, phagocytosis of lactobacilli has been shown to be necessary for induction of IL-12 production, and strains relatively resistant to lysis within the macrophages were the strongest inducers of IL-12 production (46). Furthermore, phagocytosis of chitin particles induces production of TNF, IFN-γ, and IL-12 but not production of other cytokines, like IL-10, in mouse spleen cells (35, 45).

One possibility is that production of IL-12, as well as production of high levels of TNF, is induced by the presence of bacteria within the phagosome inside the phagocyte.
mented bacteria or components of the bacterial cell wall were not only inefficient in inducing production of IL-12, TNF, and IFN-γ but also inhibited production of these cytokines in response to intact bacteria in a dose-dependent manner. It is logical that fragments of bacteria turn off production of TNF and IFN-γ, since the purpose of the phagocyte-activating cytokines is to promote killing and decomposition of phagocytosed particles (26, 32, 43, 47). This kind of activation is no longer necessary once the microbe has been decomposed. We cannot distinguish whether this inhibition results from blocking of the necessary interactions between intact bacteria and cyto-kine-inducing receptors or whether the fragments themselves trigger inhibitory pathways reducing cytokine production. Autoyolized bacteria reduced the tendency of blood cells to eliminate viable pneumococci in vitro. Further, interactions between monocytess and intact pneumococci were partially prevented by the presence of bacterial fragments. We propose that bacterial fragments released during autoysis do have synergistic functions, both blocking phagocytosis of bacteria and preventing the normal triggering of phagocyte-activating cytokines aiming to increase the bactericidal mechanisms of phagocytes. We suggest that in pneumococci the autolytic mechanisms that are present in all bacteria, as they are necessary for restructuring the cell wall during growth and fission, are adapted to generate fragments that paralyze phagocyte effector functions in a fraction of bacteria that have reduced viability. As little as 10% of the pneumococcal cells needed to decay to significantly downregulate production of phagocyte-activating cytokines in response to the majority of viable, intact cells. Thus, bacterial cells which die also serve a posthumous pur-pose in preventing elimination of their living relatives. We propose that this mechanism contributes to the highly patho-genic nature of pneumococci.

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