ORIGINAL ARTICLE

The effects of live *Streptococcus pneumoniae and* tumor necrosis factor-α on neutrophil oxidative burst and β2-integrin expression

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Objective To study the effects of TNF-α and live *Streptococcus pneumoniae* on human neutrophil oxidative burst and β2-integrin expression using flow cytometry.

Methods Six clinical isolates of *S. pneumoniae* (serotypes 3, 19A, 22F, 6A, 33F and 9N) from patients with bacteremic pneumonia or upper respiratory tract infections were studied. Whole blood was incubated either alone, with TNF-α or with *S. pneumoniae* or with both TNF-α and pneumococci at a ratio of one neutrophil per 1–5 bacteria. After 30 min of incubation, the tubes were put into ice, fixed and analysed.

Results *S. pneumoniae* caused an increase in oxidative burst but not greater than that caused by TNF-α alone. When whole blood was preincubated with TNF-α for 30 min before the addition of pneumococci, a further increase in the oxidative burst response was seen. The variation in CD11b expression was not significant. Both *S. pneumoniae* and TNF-α caused increases in CD18 expression. The addition of TNF-α directly with the bacteria caused no further increase, but preincubation of blood with TNF-α 30 min before the addition of the bacteria caused a significant increase in CD18 expression.

Conclusions Live *S. pneumoniae* stimulates polymorphonuclear leukocytes to produce an oxidative burst and increases expression of CD18, and these effects are enhanced by TNF-α.

Keywords Neutrophils, *Streptococcus pneumoniae*, oxidative burst, β2-integrin

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INTRODUCTION

Streptococcus pneumoniae is a common bacterial pathogen causing upper and lower respiratory tract infections, sepsis and meningitis [1]. The interplay between neutrophil granulocytes, monocytes and pneumococci is decisive for the outcome of infection, but the mechanisms determining a successful immune response are still incompletely understood [2]. Previous studies have shown that tumor necrosis factor-α (TNF-α) enhances neutrophil oxidative burst and phagocytosis of *Staphylococcus aureus*, non-typable *Haemophilus influenzae*, fungi and *Neisseria meningitidis* [3–6]. *Streptococcus pneumoniae* was previously shown not to stimulate superoxide production alone but also to inhibit neutrophil oxidative burst responses when neutrophils were stimulated with phorbol myristate acetate [7], and in another study pneumococcal cell walls did not degranulate neutrophils [8].

The β2-integrin CD11b/CD18 is found on the surface of leukocytes and has importance for phagocytosis and adhesion [9]. Previous studies have shown that expression of CD11b/CD18 increases when neutrophils are stimulated with lipopolysaccharide (LPS) or TNF-α [3,10]. In experimental pneumococcal meningitis, blockade of CD18 was shown to reduce inflammation and mortality, suggesting a central role for CD18 in the pathogenesis of pneumococcal meningitis [11].

In the present study we investigated the effects of recombinant human TNF-α on neutrophil oxidative burst responses to live *Streptococcus pneumoniae* and the effects of TNF-α and *Streptococcus pneumoniae* on neutrophil expression of CD11b/CD18.

MATERIALS AND METHODS

Bacteria

Six isolates of *Streptococcus pneumoniae* were used. Three isolates were from blood from patients with bacteremic pneumococcal

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pneumonia (serotypes 3, 19A, 22F) and three were from the upper respiratory tract of children in day care (serotypes 6A, 33F, 9N). The bacteria were serotyped at Statens Seruminstitut, Copenhagen, Denmark. The strains were kept frozen at −70 °C, thawed and grown on blood agar for 24 h, checked for purity and then cultured in fastidious anaerobic broth (FAB, Kemila, Sollentuna, Sweden) on a shaker overnight at 37 °C. After centrifugation at 3000 *g* for 20 min, the bacteria were washed and suspended in phosphate-buffered saline solution (PBS) with Ca^{2+} (0.9 mmol/L) and Mg^{2+} (0.5 mmol/L) [12].

Whole blood and isolated polymorphonuclear leukocytes (PMNLs)

Whole blood (WB) was obtained from healthy donors and collected in sterile tubes with sodium heparin as an additive (Venoject, Terumo Europe NV, Leuven, Belgium). The concentration of PMNLs was determined by counting in a hemacytometer (CellDyn Abbott, St. Clara, CA, USA). In each experiment, blood from three donors with the same ABO and Rhesus blood group was used. When isolated PMNLs were used, preparation was performed as previously described [6,12].

Pool serum

The pool serum defined previously was used in the experiments using isolated PMNLs [6,12]. The pool serum was tested for the presence of antibodies against the six serotypes included in the present study. The levels of total immunoglobulin (in percentages of international standard 89-SF) were as follows: serotype 3, 105%; serotype 19A, 38%; serotype 22F, 35%; serotype 6A, 111%; serotype 33F, 14%; serotype 9N, 35%. Analysis of antipneumococcal antibodies was performed at Statens Serum Institut, Copenhagen, Denmark.

TNF-α

Human recombinant TNF-α was obtained from Boehringer Mannheim, Mannheim, Germany. According to the manufacturer, the specific activity of the preparation was $>1.0 \times 10^8$ U/mg using a cell lytic assay with WEHI 164 actinomycin D-treated cells, and the purity was >95%. The TNF-α was produced in *Escherichia coli* and the endotoxin contamination was <10 EU/mL (LAL test).

FLOW CYTOMETRY

Flow cytometric analyses were performed with a FACScan flow cytometer (Becton Dickinson Immunocytometric Systems, San Jose, CA, USA) with computer-assisted evaluation of data (Lysis II software, Becton Dickinson). The instrument settings were those set with AutoComp test with minor modifications: The FSC threshold was raised to 180, and when CD11b was analysed the detector level for FL2 was lowered to 450. The fluorescence parameters from single cells were collected after gating on the combination of forward and sideward scatter. In total, 5000 cells were analyzed per tube. The fluorescence distribution was displayed as a single histogram, and the mean fluorescence channel was used for further calculations.

Preparation of bacteria for FACS

Bacterial culture (3 mL) grown in FAB overnight was centrifuged, washed with 10 mL of NaCl, centrifuged and resuspended in 1 mL of NaCl. Bacteria to be used in the oxidative burst assay were stained in order to verify the ratio of PMNLs/bacteria with FACS. A staining technique described previously was used with minor modifications [13]. Briefly, bacteria were incubated for 30 min at 37 °C with 30 μL of a 200 μM stock solution of bis-carboxyfluorescein pentaacetoxy-methylester (BCECF-AM, cat. no. 1214 683, Boehringer Mannheim). After three washes in NaCl, and centrifugation and resuspension in NaCl, the bacteria were suspended in 1 mL of PBS. The bacterial density was optically determined at 540 nm using a spectrophotometer (Titertek Multiscan, Labsystems Flow Laboratories, VA, USA), and the absorbance value was fed into a computer program containing a standard curve calibrated against CFU (MultiCalc, Wallac OY, Turku, Finland). The bacterial concentration was adjusted to a ratio of 1–5 bacteria to 1 PMNL in each experiment.

Monoclonal antibodies

FITC-conjugated mouse antihuman CD18 (cat. no. F0839) and RPE-conjugated mouse antihuman CD11b (cat. no. R0841) were obtained from DAKO, Stockholm, Sweden.

Analysis of oxidative burst, CD11b/CD18

PMNL oxidative burst response was determined using hydroethidine (HE, Molecular Probes, Europe BV, Leiden, The Netherlands), which is oxidized to ethidium bromide when reacting with oxidative metabolites within the PMNLs. Staining of PMNLs with HE was performed as described previously [14] with minor modifications. HE was dissolved in dimethylsulfoxide (DMSO) at a concentration of 1 mg/mL and stored at −70 °C. Heparinized whole blood (100 μL) was mixed with 20 μL of HE (200 ng) in plastic tubes (Falcon 2058, Becton Dickinson) and incubated at 37 °C for 15 min with mild agitation. Three sets of tubes were prepared for each bacterial strain. Bacterial suspension (20 μL) and 10 μL of TNF-α (15 000 U/mL) were added as indicated. In one set of tubes,

whole blood was preincubated with TNF-α for 30 min before the bacteria were added; in the second set, the bacteria and TNF-α were added simultaneously; and in the third set, only bacteria were added. One tube containing HE-pretreated whole blood without TNF-α, one tube with TNF-α and one tube preincubated with TNF-α for 30 min were used as controls. The tubes were then incubated for 0 and 30 min at 37 °C with mild agitation. At the end of each incubation period, the tubes were placed in icy water. When CD11b and CD18 expression were studied, 10 μL of each monoclonal antibody was added to the appropriate tubes. After 60 min in icy water, the samples were lysed and fixed with 2 mL of lysing solution (Becton Dickinson) at room temperature for 10 min. After centrifugation (400 *g*, 5 min) and removal of the supernatant, the pellet was resuspended in 2 mL of PBS with 2% fetal calf serum, followed by centrifugation and resuspension in 0.4 mL of PBS with 1% formaldehyde. The tubes were then stored at +4 °C for a maximum of 18 h until analyzed.

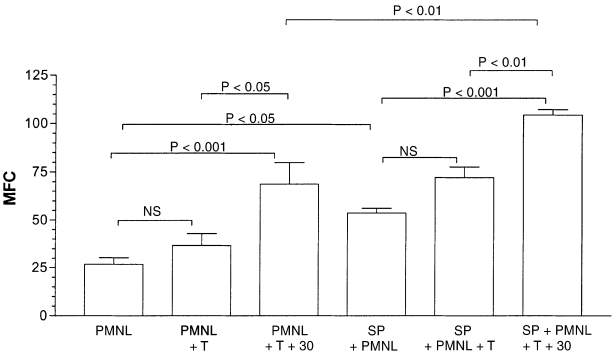

Figure 1 Histogram showing the neutrophil oxidative burst response to stimulation with 1000 U/mL TNF-α and live *Streptococcus pneumoniae*. Results are indicated as mean fluorescence channel (MFC). Five thousand PMNLs were counted. Values obtained after 30 min of incubation are shown. Means ± SE of six experiments are shown. PMNL, neutrophils; PMNL + T, neutrophils + TNF-α; PMNL + T + 30, neutrophils + TNF-α preincubated for 30 min; SP, pneumococci.

Analysis of the effect of complement and antibody on oxidative burst

To study the effects of complement and antibody, isolated PMNLs were added to PBS, pool serum or heat-inactivated pool serum [12] and experiments with pneumococci and FACS were performed as described previously.

Statistical methods

One-way ANOVA with Bonferroni post test was performed using GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego, California, USA.

Ethical considerations

This study was approved by the Örebro County Council Ethics Committee.

RESULTS

The results are presented as mean values ±1 standard error of the mean (SEM) in Figures 1 and 2. Only measurements after 30 min of incubation with or without 30 min of preincubation were used for the calculations. There was no significant difference between blood isolates and upper respiratory tract isolates with regard to oxidative burst or expression of CD11b and CD18.

Oxidative burst

The addition of TNF-α to WB caused significant increases in PMNL oxidative burst only when preincubated 30 min before measurement. *Streptococcus pneumoniae* added to WB caused a significant increase in oxidative burst, at a similar level as WB + TNF-α, compared to that caused by incubation of WB alone. A further increase was seen when TNF-α was preincubated 30 min before measurements both with and without *Streptococcus pneumoniae*. The addition of TNF-α + *Streptococcus pneumoniae* to WB caused a significant increase only when TNF-α was preincubated with WB for 30 min before the addition of the bacteria. The results are shown in Figure 1. When isolated PMNLs were studied, inactivation of complement caused a minor reduction in the oxidative burst response, but the oxidative burst was not abolished.

CD11b

The variation between the columns was not significant.

CD18

The addition of TNF-α to WB caused a significant increase in CD18 expression on PMNLs. Incubation for 30 min further increased the expression of CD18. Similarly, incubation with *Streptococcus pneumoniae* caused significant increases in CD18 expression. When WB was preincubated for 30 min with TNF-α before the addition of *Streptococcus pneumoniae*, a further significant increase in CD18 expression was seen. Incubation of TNF-α together with *Streptococcus pneumoniae* caused no further increase compared to incubation with bacteria only. The results are shown in Figure 2.

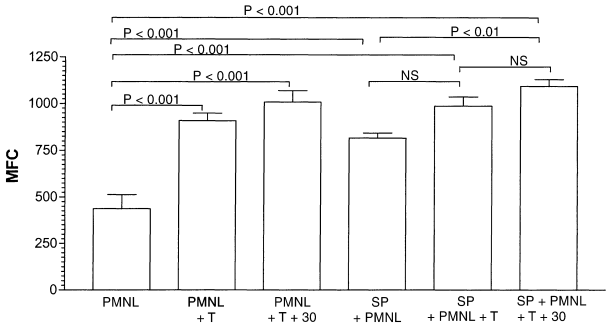

Figure 2 Histogram showing neutrophil expression of CD18 in response to stimulation with 1000 U/mL TNF-α and live *Streptococcus pneumoniae*. Results are indicated as mean fluorescence channel (MFC). Five thousand PMNLs were counted. Values obtained after 30 min of incubation are shown. Means ± SE of six experiments are shown. PMNL, neutrophils; PMNL + T, neutrophils + TNF-α; PMNL + T + 30, neutrophils + TNF-α preincubated for 30 min; SP, pneumococci.

DISCUSSION

In the present paper it is shown that live *Streptococcus pneumoniae* stimulates neutrophils to produce an increased oxidative burst response. It is also shown that the addition of TNF-α enhances this oxidative burst, especially when the PMNLs are primed for 30 min with TNF-α. In previous studies, *Streptococcus pneumoniae* was found not to stimulate superoxide production [7]. The difference in results may be explained by the differing methods used when measuring the neutrophil respiratory burst response. The method used in the present paper measures all of the respiratory burst, whereas the method used by Perry et al [7] measures only a specific part of the respiratory burst response. When isolated PMNLs were studied, we found the oxidative burst response to be reduced but not abolished in the absence of complement.

Live *Streptococcus pneumoniae* also stimulated increased expression of PMNL CD18, and this increase was statistically significant. For CD11b a similar pattern was seen, but the differences in mean channel fluorescence were not significant.

In a previous study it was shown that pneumococci stimulate the production of TNF-α in whole blood in vitro after 4 h of incubation but to a lesser degree than Gram-negative bacteria [15]. Others have studied the production of cytokines from peripheral blood mononuclear cells stimulated with *Streptococcus pneumoniae* and found that the maximum production of TNF-α occurred after 48 h of stimulation [16]. Thus, in the present experiments, measuring the effects within 30 min, we consider the effects of endogenously produced cytokine to be minimal.

In animal experimental models of meningitis, different pneumococcal serotypes were shown to induce different levels of inflammatory responses. Comparing pneumococcal serotypes 1, 3 and 9, three different degrees of severity of meningitis were demonstrated in one study [17]. In a later study investigating six different serotypes, three levels of inflammatory responses in experimental pneumococcal meningitis were found [18]. In the present study, the oxidative burst response was mainly dependent on the presence of serotype-specific antibodies, and the pneumococcal isolate that elicited the most vigorous oxidative burst response was a serotype 3 blood isolate. Serotype 3 was shown in the rabbit meningitis model to induce a mild inflammatory response, but in the previous studies the presence of serotype-specific antibodies was not investigated [17,18].

In the present study, we found similar responses to invasive isolates from blood and non-invasive isolates from the upper respiratory tract, which suggests that there is no difference in the handling of invasive and non-invasive pneumococci by PMNLs.

In conclusion, live *Streptococcus pneumoniae* stimulates PMNLs to produce an oxidative burst and increases expression of CD18, and these effects are enhanced by TNF-α. These findings may in part explain the vigorous local inflammation seen in pneumococcal meningitis. The oxidative burst response was found to be mainly dependent on the presence of serotype-specific antibodies.

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