Local Anesthetics Impair Human Granulocyte Phagocytosis Activity, Oxidative Burst, and CD11b Expression in Response to Staphylococcus aureus

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Background: With invasion of bacteria, the host defense system is activated by a complex cascade of various mechanisms. Local anesthetics previously were shown to interact with diverse components of the immune response, such as leukocyte adherence on endothelial monolayers, oxidative burst, or crosstalk within lymphocyte subset populations. However, effects of newer local anesthetics like bupivacaine and ropivacaine on antibacterial host defense—primarily phagocytosis activity, oxidative burst, or CD11b expression—still remain unclear.

Methods: Whole blood samples were preincubated with local anesthetics (lidocaine, 9.2, 92.2, and 1,846 μM; bupivacaine, 6.1, 61, and 770 μM; ropivacaine, 6.4, 64, and 801 μM). For the oxidative burst and CD11b assay, dihydroethidium was added to the probes. After viable Staphylococcus aureus was added in a 5 to 1 ratio following leukocyte count, phagocytosis was stopped at different times, and staining with monoclonal antibodies was performed for subsequent flow cytometric analysis of phagocytosis activity, oxidative burst, and CD11b expression.

Results: Granulocyte phagocytosis activity, CD11b expression, and generation of reactive oxygen species were significantly reduced by lidocaine (P < 0.0002) and bupivacaine (P < 0.005) in the highest concentration (1,846 μM and 770 μM, respectively). The capability of granulocytes to ingest bacteria was significantly depressed only by lidocaine (P < 0.0003). Ropivacaine had no significant effect on any parameter investigated.

Conclusions: Local anesthetic dose and structure dependently inhibit inflammatory and immunologic parameters of granulocyte functions. Ropivacaine shows low interference with granulocyte immunologic and inflammatory functions.

APART from their ability to block sodium channels, local anesthetics interact with various cell systems relevant to immune functions. These immunomodulatory actions of local anesthetics potentially may have both positive and negative effects.

Antiinflammatory actions of local anesthetics may be beneficial in settings of sterile inflammation. Depressed generation of reactive oxygen species and expression of CD11b in leukocytes in the presence of local anesthetics are associated with decreases in ischemic damage after myocardial infarction, tissue inflammation in acute respiratory distress syndrome, and ulcerative colitis in animal models and humans, although the latter finding has recently been contradicted.

In contrast, examples of potential negative effects include retardation of wound healing and increased infectious complications, such as epidural abscess; whether such effects are clinically relevant remains to be shown. Epidural abscesses represent a rare but severe complication of regional anesthesia. Staphylococcus aureus has been identified as the most common pathogen. Although local anesthetics show a concentration-dependent antimicrobial activity on several types of bacteria in vitro, S. aureus grows in the presence of local anesthetics. This insensitivity of S. aureus to local anesthetics, in combination with an impaired host defense, has been suggested to facilitate infectious complications.

In vitro, local anesthetic interactions with leukocyte functions in inflammatory models have been described repeatedly. Responses (chemotaxis, phagocytosis activity, cellular metabolic activity, and oxidative burst) to various experimental stimuli in the presence of lidocaine are inhibited. In addition, CD11b expression, which is important for granulocyte adhesion, transmigration, and leukocyte-endothelial interactions, was shown to be impaired by local anesthetics. However, local anesthetic interactions with the bacterial host defense, a possibly more relevant end point, have not been investigated in detail.

Based on these data, we hypothesized that, besides the well-known growth of S. aureus in the presence of local anesthetics, an interference of local anesthetics with leukocyte functions also might compromise host defense against this pathogen. To test this hypothesis, we investigated, in a model of whole blood infection with S. aureus, the impact of local anesthetics on three key leukocyte functions: expression of CD11b surface binding sites, the main recognition and binding sites for S. aureus; phagocytosis, which internalizes the bacterium; and oxidative burst, which kills the bacterium.

Materials and Methods

Local Anesthetics

Three different concentrations of lidocaine (Xylocaine®, Astra, Wedel, Germany), bupivacaine (Carbostesin®, Astra, Wedel, Germany), and ropivacaine (Naropin®; Astra, Wedel, Germany) were chosen for investigation of their effect on granulocyte phagocytosis activity, oxidative burst,
and CD11b expression. Preservative-free preparations of the local anesthetics were diluted in isotonic saline to a total volume of 100 µl each. Final working concentrations of local anesthetic in whole blood were as follows: lidocaine, 9.2, 92.2 and 1,846 µM; bupivacaine, 6.1, 61.6, and 770 µM; and ropivacaine, 6.4, 64, and 801 µM. The pH was 7.4 in all experiments. Eleven experiments were conducted for each local anesthetic in each concentration.

**Blood Samples**

After obtaining institutional and ethical committee approval (Medical Faculty, Eberhard-Karls University, Tuebingen, Germany) and informed consent, blood samples (10 ml) were acquired from 14 healthy volunteers (aged 32.5 ± 7 yr) via an 18-gauge needle in a large antecubital vein. Blood was heparinized with 5 U/ml heparin sodium (Vetren 200®; Byk Gulden, Konstanz, Germany). One hundred microliters of the samples was used for leukocyte count (using trypan blue solution with 3% acetic acid for lysis of erythrocytes). The remaining blood was divided into volumes of 2 ml and mixed with different concentrations of local anesthetic or with isotonic saline (not bacteriostatic) as control. Blood samples were incubated with the different local anesthetic concentrations for 30 min at 37°C.

**Bacteria**

*S. aureus* (ATCC 25923; American Type Culture Collection, Rockville, MD) was cultivated in Tryptone Soya Broth (Oxoid, Wesel, Germany) for 3 h and centrifuged (375 g) for 5 min. The pellets were resuspended in phosphate-buffered saline without calcium or magnesium (Sigma, Deisenhofen, Germany) and then washed twice to remove the growth medium. For the detection of intracellular bacteria by flow cytometry, the pellets were incubated with Calcein-AM (Calcein-AM special packing; Molecular Probes, Eugene, OR) for 50 min at 37°C. Calcein-AM, an acetoxyethyl ester with a high and stable green fluorescence signal, penetrates the cell wall, is cleaved by cytoplasmic esterases to a hydrophilic product, and is thereby trapped in the cell. Stained bacteria were washed twice in phosphate-buffered saline, adjusted to a final concentration of 2 × 10⁶ colony-forming units (CFU) per milliliter, and stored in aliquots at −20°C until use. Bacterial count was performed by plating out appropriate dilutions of bacterial preparations on mannitol salt agar (Oxoid, Wesel, Germany) and counting CFU after 24 h of incubation at 37°C. Repeated assays were performed to ascertain stable fluorescence intensity and bacterial viability during all experiments. For the oxidative burst and CD11b assay, unstained bacteria were diluted to 2 × 10⁹ CFU/ml and stored at −20°C.

**Phagocytosis**

To provide a constant 5 to 1 ratio of bacteria to leukocytes for each assay, bacteria were diluted based on whole blood leukocyte count. The culture medium was RPMI (RPMI 1640; Sigma, Deisenhofen, Germany) containing 10% autologous serum (to preopsonize bacteria and to provide for faster phagocytosis). Diluted bacteria and local anesthetic-treated whole blood samples were separately incubated for 30 min at 37°C. Bacteria were then added to the blood samples and incubated at 37°C for 10, 30, and 60 min before phagocytosis was stopped by adding N-ethylmaleimide (10 µM; Sigma, Deisenhofen, Germany). Between steps, the samples were stored on ice.

For flow cytometric detection, the granulocytes were stained with the monoclonal antibody against CD13 (Myeloïd Cell, CD13 RPE; DAKO, Glostrup, Denmark), and the erythrocytes were lysed (Lysing Solution; Becton Dickinson, Heidelberg, Germany). The samples then were centrifuged (375 g for 5 min), resuspended in phosphate-buffered saline, and kept on ice until flow cytometric analysis.

**Oxidative Burst and CD11b Expression**

For determination of oxidative burst activity and CD11b expression of leukocytes, unstained bacteria were mixed with leukocytes to a final ratio of 5 to 1 and preopsonized, as in the phagocytosis assay. Blood samples were mixed with local anesthetic and treated as described previously. To determine the generation of reactive oxygen species, dihydroethidium (2.5 µg/ml; Sigma, Deisenhofen, Germany) was added. Dihydroethidium is reduced to hydroethidium by reactive oxygen metabolites, emitting a stable fluorescence signal of 550–720 nm wavelength. Because of the broad emission spectrum of dihydroethidium during flow cytometric analysis (detection in channels 2 and 3), a staining with three fluorescence dyes is not feasible. As the uptake of bacteria was already quantified in the phagocytosis assay, unstained bacteria were used (in the same ratio as in the phagocytosis assay) to determine CD11b expression during phagocytosis.

After phagocytosis was stopped (10 and 30 min), as described previously, the samples were stained with a monoclonal antibody against CD11b (Mouse anti-Human CD11b; Caltag Laboratories, San Francisco, CA) for 20 min at room temperature, the erythrocytes were lysed (as in the phagocytosis assay), and the samples were prepared for flow cytometric analysis.

**Flow Cytometry**

Flow cytometric analysis was performed using FACS SORT (Cell Quest software package, version 3.1f; Becton Dickinson, Heidelberg, Germany). Unstained samples served as a control for the background fluorescence of dihydroethidium, collected through the 620-nm band.
pass filter. Isotype controls for each monoclonal antibody were used to define the cutoff for positive fluorescence.

Phagocytosis activity was determined in two different ways: as the ratio between the number of neutrophils ingesting bacteria and the total amount of neutrophils and as the fluorescence intensity of intracellular bacteria; the latter served as a semiquantitative measurement of bacterial uptake. Ten thousand granulocytes were analyzed in each sample.

**Statistical Analysis**

Statistical analysis was performed using the JMP® statistical software package (SAS Institute Inc., Cary, NC). Analysis of variance (ANOVA) was performed, including blood donors as random effect and time, local anesthetic concentration, and their respective interaction as fixed factors. For each time point, differences between the local anesthetic concentration and the respective control were detected by Student t-test with Bonferroni correction for multiple comparisons. Alpha was set at 0.05.

Comparisons among the local anesthetics were performed by ANOVA as well, including blood donors as random effect and local anesthetic concentrations and the different local anesthetics as fixed facts. To avoid multiple comparison problems, time effects were not

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**Fig. 1.** Ratio of granulocytes (in percent) actively ingesting bacteria to total amount of granulocytes after 10, 30, and 60 min of exposure to local anesthetic. Data are presented as mean and SD. Significant inhibition of lidocaine occurred after 30 and 60 min ($P < 0.0002$), and significant inhibition of bupivacaine occurred after 0 and 30 min ($P < 0.005$).

**Fig. 2.** Flow cytometric evaluation of fluorescence intensity of intracellular bacteria. Bacteria were stained with Calcein-AM (green channel fluorescence signal; Molecular Probes, Eugene, OR). Data after 10, 30, and 60 min are presented as mean and SD. Significant inhibition ($P < 0.003$) of bacterial uptake occurred at 60 min of lidocaine exposure.
evaluated. Normal distribution of the ANOVA residuals was assured (Shapiro–Wilk W test).

Results

Phagocytosis Activity

Ropivacaine, at all concentrations and at all time points investigated, had no effect on the percentage of granulocytes ingesting bacteria. Lidocaine and bupivacaine inhibited phagocytosis but with different time courses. After 10 min of incubation, the percentage of granulocytes ingesting bacteria was decreased to 80% by bupivacaine (770 μM; \( P < 0.002 \) compared with control) but not by lidocaine. After 30 min of incubation, the percentage of granulocytes that ingested \( S. \) aureus was decreased to 71% by lidocaine (1,846 μM; \( P < 0.0002 \)) and to 85% by bupivacaine (770 μM; \( P < 0.001 \); fig. 1). After 60 min of incubation, only lidocaine (1,846 μM) significantly reduced the number of granulocytes that ingested bacteria (to 71%; \( P < 0.0001 \)). Hence, the effects of the three local anesthetics at these concentrations were significantly different from one another (\( P < 0.02 \)). At lower concentrations, none of the local anesthetics affected the number of granulocytes that ingested bacteria.

In addition, the uptake of labeled \( S. \) aureus was semi-quantitatively determined by measuring the intracellular

Fig. 3. Granulocyte oxidative burst during phagocytosis of \( S. \) aureus and local anesthetic exposure. Fluorescence intensity of reactive oxygen metabolites is shown. Significant inhibition of lidocaine and bupivacaine occurred after 30 min (\( P < 0.0001 \)).

Fig. 4. Granulocyte CD11b expression during phagocytosis of \( S. \) aureus and local anesthetic exposure. Significant inhibition of CD11b upregulation occurred during exposure to lidocaine (\( +P < 0.0001 \)) and bupivacaine (\( P < 0.006 \)) in the highest concentration.
fluorescence signal. The fluorescence intensity of S. aureus was significantly decreased to 68% by lidocaine at 60 min (1,846 μM; P < 0.005). For bupivacaine (770 μM), a trend toward fewer intracellular bacteria could be observed but did not reach statistical significance (fig. 2). As a result, the effects of lidocaine and bupivacaine at these concentrations were significantly different from those of ropivacaine (P < 0.0001).

**Oxidative Burst**

A significant reduction in oxidative burst—to 61% and to 69%—was observed at 30 min for lidocaine (1,846 μM; P < 0.0001) and bupivacaine (770 μM; P < 0.0001), respectively. Ropivacaine did not have any effect on oxidative burst (fig. 3). However, due to substantial variability, no significant differences among the local anesthetics were observed.

**CD11b Expression**

CD11b expression was reduced at 10 and 30 min by lidocaine (1,846 μM; to 50 and 52%) and by bupivacaine (770 μM; to 65 and 70%), respectively (P < 0.006). For ropivacaine (801 μM), a trend toward reduced expression of CD11b to 79% (10 min) and to 78% (30 min) was observed but did not reach statistical significance (fig. 4). The effect of lidocaine was significantly different from that of the other local anesthetics (P < 0.0001), whereas no differences in effect were observed between bupivacaine and ropivacaine.

**Discussion**

The present study investigated the effects of lidocaine, bupivacaine, and ropivacaine on granulocyte phagocytosis activity, oxidative burst, and CD11b expression. All three parameters were significantly attenuated by lidocaine or bupivacaine in their highest concentrations (1,846 and 770 μM, respectively). The uptake of intracellular bacteria was significantly affected following lidocaine exposure only. Treatment with ropivacaine showed only a nonsignificant trend toward reduced CD11b expression in the highest concentration (801 μM).

Lidocaine previously has been shown to inhibit phagocytosis of radiolabeled S. aureus,14 heat-killed Escherichia coli,16 or latex particles15 in isolated leukocytes. This study confirms these findings for a whole blood assay and adds new evidence suggesting comparable inhibitory effects of bupivacaine but lack of ropivacaine interference with bacterial ingestion. In addition, we observed a significant decrease in the number of leukocytes actively participating in phagocytosis after lidocaine exposure. Ropivacaine, in contrast, affected neither bacterial ingestion nor the overall number of active phagocytes.

The effects of lidocaine on oxidative burst stimulated by bacterial surface antigens,16 yeast particles,13,18 or intracellular stimuli, like phorbol myristate acetate, have been well documented.13,15,17,22 Our data show that lidocaine similarly affects respiratory burst activated by S. aureus phagocytosis. In contrast, the effects of bupivacaine and ropivacaine on oxidative burst are variably reported. Mikawa et al.19 did not observe any effect of bupivacaine on zymosan-stimulated oxidative burst, whereas Sinclair et al.18 reported suppression by bupivacaine in concentrations comparable to those used in this study. Cederholm et al.25 could not show significant effects of bupivacaine or ropivacaine on respiratory burst induced by phorbol myristate acetate, whereas Hollmann et al.22 and Fischer et al.24 demonstrated inhibitory activity of both local anesthetics on chemotaxis and oxygen production in granulocytes primed with platelet-activating factor and activated with N-formyl-methionine-leucine-phenylalanine. The differences between these studies may be a result of the various stimuli and incubation times used. Hollmann et al.25,26 recently demonstrated that local anesthetics influence intracellular signaling by interfering with Gα proteins, resulting in the blockage of neutrophil priming. In our study, differentiation between priming and activation effects was not possible because viable S. aureus was applied as a single stimulus.

CD11b, an adhesion molecule in the β2-integrin family, is rapidly upregulated after neutrophil activation and plays a crucial role by allowing firm adhesion of rolling leukocytes to the endothelium,27,28 thereby initiating the first step in extravasation of leukocytes to sites of inflammation. Moreover, CD11b is the predominant leukocyte surface-binding site for opsonized S. aureus and is responsible for both recognition and binding. Bound S. aureus is rapidly internalized into phagocytes (via phagosomes) and degraded (via oxidative burst and enzymatically) after interaction with the CD11b receptor. The most pronounced local anesthetic effect observed in our study was on CD11b expression. Inhibition of CD11b expression by lidocaine and ropivacaine has already been reported for N-formyl-methionine-leucine-phenylalanine-stimulated or tumor necrosis factor α-stimulated leukocytes.20,29 In our study, CD11b expression was decreased by lidocaine and bupivacaine during phagocytosis of viable S. aureus. This would be expected to result in compromised ingestion of viable S. aureus by leukocytes.

We observed significant differences in effect among the various anesthetics. This could imply that either nonequipotent concentrations were used or that various local anesthetics have different pharmacologic effects on leukocyte function. Statements regarding equipotency are difficult to make since we investigated a novel phenomenon for which anesthetic sensitivity has not been described. Clearly, “equipotent” concentrations, as described for analgesia, are irrelevant in the present setting since local anesthesia is largely mediated by the blockage...
of sodium channels, which are not expressed in leukocytes. Therefore, we decided to study a wide range of concentrations, anchored in clinical relevance. For each of the local anesthetics, we chose the lowest concentration to correspond to plasma concentrations observed following regional anesthetic procedures.\textsuperscript{31–35} In addition, we studied a concentration 10-fold greater than and a concentration corresponding to 100 times the peak plasma levels observed after regional anesthesia. As a result, we studied a greater than 100-fold range of concentrations. Unfortunately, almost no data exist on a more useful measure in the current context: tissue concentrations of local anesthetic at the site of injection (e.g., after regional blocks, which are performed with high volumes and concentrated local anesthetics). After subarachnoid block, millimolar concentrations are observed near the spinal cord.\textsuperscript{36} After brachial plexus subarachnoid block, millimolar concentrations are observed after regional anesthesia. As a result, we studied a greater than 100-fold range of concentrations. Unfortunately, almost no data exist on a more useful measure in the current context: tissue concentrations of local anesthetic at the site of injection (e.g., after regional blocks, which are performed with high volumes and concentrated local anesthetics). After subarachnoid block, millimolar concentrations are obtained near the spinal cord.\textsuperscript{36} After brachial plexus block, tissue ropivacaine concentrations were approximately 50 times greater than those subsequently observed in plasma.\textsuperscript{34,35} By extrapolating, it can be assumed that tissue concentrations are at least 10-fold greater than systemic concentrations. In addition, local anesthetics are frequently applied over the period of days or weeks. \textit{In vitro} studies describe an increasing susceptibility of leukocytes to local anesthetics with increasing exposure time.\textsuperscript{37} Thus, even lower local anesthetic concentrations may result in compromised leukocyte function in cases involving prolonged exposure times. Together, these data indicate that the concentrations used in this study may, indeed, be relevant in the clinical setting.

Although the results of this \textit{in vitro} study cannot be extrapolated to local anesthetic actions \textit{in vivo} and in clinical practice, our data at least suggest the possibility of clinically relevant effects on leukocyte functions at sites with high local anesthetic concentrations. However, the limitations of the \textit{in vitro} model must be emphasized since under experimental conditions, only effects on an isolated system are observed, whereas \textit{in vitro}, leukocytes and \textit{S. aureus} interact with a variety of cellular and noncellular targets, including the tissue immune system and circulating blood cells other than neutrophils. Nonetheless, our findings may be of clinical relevance in subpopulations of patients with preexisting compromised immune status, diabetes mellitus, malignancies, use of antiinflammatory medication, or long-term analgesia for chronic pain syndromes, in whom a 10-fold increase in the incidence of epidural abscess, as compared with healthy subjects, has been reported.\textsuperscript{38} Our data suggest that ropivacaine, which showed limited effects on leukocyte function, may be the preferred local anesthetic for prolonged regional anesthesia in such patients.

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