The Effect of Minocycline and Lysostaphin on the Intracellular Killing of *Staphylococcus aureus* by Polymorphonuclear Leukocytes

M. J. Raff, P. A. Barnwell, J. A. Van Arsdall, II, and J. C. Melo

From the Section of Infectious Diseases, Department of Medicine and The Department of Microbiology and Immunology, University of Louisville School of Medicine, Louisville, KY 40292, USA

Several investigators have examined the effects of tetracyclines on the phagocytosis and intracellular killing of bacteria by polymorphonuclear leukocytes [1–4]. Munoz and Geister [1] reported that chlortetracycline produced dose-related inhibition of phagocytosis of *Staphylococcus epidermidis* by neutrophils. Subsequent studies have indicated that tetracyclines do not affect neutrophil phagocytosis or intracellular killing of *S. aureus* [2–4]. More recently, Forsgren et al. [5] demonstrated a significant reduction in the phagocytosis of preopsonized yeasts and *Escherichia coli* by neutrophils exposed in vitro to tetracycline or doxycycline. None of these studies has explored the influence of minocycline on the interactions between staphylococci and human neutrophils. The present investigation utilized a method reported to distinguish phagocytosis from intracellular killing [6]. We attempted to determine if the phagocytosis and intracellular killing of staphylococci would be affected by minocycline.

**Methods**

**Antibiotic**

Minocycline was provided as a powder of known potency by Lederle Laboratories. This material was dissolved in distilled water to provide a concentration of 1000 µg/ml, filter sterilized, and diluted to working concentrations in bicarbonate-buffered Hanks’ balanced salt solution containing 0.1% gelatin (HBSS gel).

**Bacteria**

The Wood-46 strain of *S. aureus* was used in these experiments. This organism was shown to be sensitive to minocycline; the minimal inhibitory concentration (MIC) is 0.25 µg/ml. Cultures were maintained on refrigerated agar slants and
Minocycline, Lysostaphin, and Intraleukocytic Killing of *S. aureus*

Prepared for use by overnight (15–18 h) incubation of an inoculum in tryptic soy broth. Bacteria were centrifuged at 1200 g for 5 min, washed once with HBSS gel, and resuspended to a concentration of approximately \(10^8\)/ml in a medium containing 50% HBSS gel and 50% fetal calf serum (FCS). The FCS contained 1% chick embryo extract. Minocycline was added in the desired concentrations, and these suspensions were incubated at 37° C for 2 h. Bacteria were then washed twice with HBSS gel, resuspended in HBSS gel, and enumerated by conventional dilution and plating techniques, with counts expressed as colony-forming units (CFU)/ml.

Leukocyte Isolation

Blood was drawn from healthy adult volunteers after obtaining informed consent. Polymorphonuclear leukocytes were isolated by a modification of the Hypaque-Ficoll gradient technique described by Kimball et al. [7]. Blood was diluted with three volumes of HBSS gel containing sufficient heparin to yield a final concentration of 10 units/ml. This suspension was dispensed in 25 ml aliquots into sterile plastic centrifuge tubes. Using a 10-cm, 12-gauge unbeveled needle, the suspensions were carefully underlaid with 10-ml volumes of Hypaque-Ficoll (Ficoll-Paque, Pharmacia, Piscataway, N.J.) and centrifuged at 400 g for 40 min at 25° C. Under these conditions granulocytes and erythrocytes formed a pellet, with the mononuclear cells producing a band at the interface between the plasma-HBSS and Hypaque-Ficoll layers. All material above the pellet was discarded and the remaining cells washed with 200 ml of cold HBSS gel. After centrifugation at 1400 g for 10 min, erythrocyte lysis was accomplished by shaking the cell button gently for 20 s with 100 ml of cold 0.2% NaCl. Isotonicity was immediately restored with 100 ml of cold 1.6% NaCl, and the cells recentrifuged for 10 min at 1400 g. This hypotonic lysis was repeated once to remove remaining erythrocytes, and the resulting granulocyte pellet washed with cold HBSS gel. These cells were resuspended to a concentration of approximately \(10^7\)/ml in an incubation medium consisting of 45% HBSS gel, 45% FCS containing 1% chick embryo extract, and 10% autologous serum. Leukocyte viability was assessed by trypan blue dye exclusion.

Phagocytosis and Intracellular Killing

The neutrophil suspension was dispensed in 0.9 ml aliquots into plastic tubes, and 0.1 ml of the staphylococcal suspension was added to each. A ratio of 3–5 bacteria per neutrophil was used in these experiments. Control tubes without neutrophils were prepared to assess the effect of the medium on staphylococci. Staphylococci had been pretreated with minocycline or HBSS as a control. Tubes were rotated at 8 rpm in a 37° C incubator. At intervals, tubes were removed and centrifuged at 110 g for 4 min. Unphagocytosed staphylococci in the supernatant were enumerated by dilution and plating. Extraleukocytic staphylococci remaining in the pellet were destroyed by adding lysostaphin, 20 units/ml. After 20 min incubation at 37° C, 5 mg of trypsin was added and the suspension reincubated for 10 min to inactivate the lysostaphin. This quantity of