Impairment of Antimicrobial Activity and Nitric Oxide Production in Alveolar Macrophages from Smokers of Marijuana and Cocaine

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Human alveolar macrophages (AMs) were recovered from the lungs of healthy nonsmokers (NS) or smokers of tobacco (TS), marijuana (MS), or crack cocaine (CS) and challenged in vitro with Staphylococcus aureus. AMs from NS and TS exhibited potent antibacterial activity that correlated with the production of nitric oxide (NO) and induction of NO synthase without the requirement for priming with exogenous cytokines. In contrast, AMs from MS and CS exhibited minimal antibacterial activity and failed to produce NO unless primed with additional cytokines. These results confirm that NO plays a significant role as an effector molecule used by normal human AMs, but this capacity is suppressed in AMs from MS and CS because of a lack of intrinsic cytokine priming.

Nitric oxide (NO) acts as an important effector molecule in activated rodent macrophages, and its inhibition reduces macrophage responses against fungi, bacteria, parasites, and tumor cells [1]. In addition to this role in innate immunity, NO functions as an important signaling and regulatory molecule in adaptive immunity [1, 2]. High-level synthesis of NO requires the expression of an inducible form of NO synthase (iNOS). Stimuli that induce expression of the iNOS gene in macrophages include infection by intracellular pathogens and dual stimulation by lipopolysaccharide (LPS) in combination with cytokines such as interferon (IFN)–γ, tumor necrosis factor–α, or granulocyte-macrophage colony-stimulating factor (GM-CSF) [1].

Although the generation of reactive nitrogen intermediates plays a critical role in antimicrobial defense mediated by rodent macrophages, the role of NO in the human response to inflammatory stimuli has remained controversial [1, 3, 4]. In vitro stimulation of human monocyte-derived macrophages by LPS and cytokines activates antimicrobial activity, but there is only limited evidence that a NO plays a mediating role. However, the response of human alveolar macrophages (AMs) to pathogens may be different. AMs are differentiated tissue macrophages that provide a first line of host defense against inhalled pathogens [5]. Biochemical evidence of NO, mRNA encoding for iNOS, and iNOS protein have been detected in AMs from patients with pulmonary fibrosis who are infected with bacille Calmette-Guérin in vitro and from patients with tuberculosis [1].

While investigating the impact of inhaled drug abuse on host defense, we observed that AMs from healthy nonsmokers (NS) exhibited potent in vitro antibacterial activity against Staphylococcus aureus that was abrogated by an iNOS inhibitor, Nω-monomethyl-1-arginine monooacetate (NGMMA) [6]. In contrast, AMs from long-term smokers of “crack” cocaine alone (CS) or marijuana alone (MS), but not tobacco alone (TS), exhibited much lower levels of antistaphylococcal activity that were not affected by NGMMA. We hypothesized that the capacity to produce NO is impaired by chronic exposure to marijuana and/or crack cocaine. To confirm this hypothesis, we cocultured AMs from different types of smokers with S. aureus and analyzed them simultaneously for antistaphylococcal activity, production of nitrite, and expression of mRNA encoding for iNOS.

Subjects and methods. All subjects were participants in ongoing studies evaluating the effects of habitual use of cocaine and/or marijuana on the lung and host defense; inclusion and exclusion criteria are described elsewhere [6]. In brief, subjects with normal pulmonary function and no known medical illnesses were selected for inclusion on the basis of their lifelong smoking history for tobacco, marijuana, or cocaine and their willingness to undergo bronchoscopy.

Fiberoptic bronchoscopies with bronchoalveolar lavage were...
Figure 1. Alveolar macrophages (AMs) recovered from the lungs of nonsmokers (NS), tobacco smokers (TS), marijuana smokers (MS), and "crack" cocaine smokers (CS) were cocultured with Staphylococcus aureus for 0, 30, 90, or 120 min in control medium or medium supplemented with 200 pM granulocyte-macrophage colony-stimulating factor (GM-CSF). Bar graphs show mean values for nitrite concentration in the supernatants at the indicated times, and line graphs reflect killing of intracellular S. aureus at the same time points. Bacterial killing was calculated using the formula 100 - (N/N₀ × 100), where N is the no. of colonies counted at each time point and N₀ is the no. of colonies counted at time zero. Cocultures were performed in the presence (black bars and black circles) and absence (white bars and white circles) of NG-monomethyl-L-arginine monoacetate, as described in "Subjects and methods." Assays were performed in duplicate, and data points represent mean ± SEM for 14 NS, 12 TS, 12 MS, and mean/SEM 10 CS.

performed in the Outpatient Medical Procedure Suite of the University of California, Los Angeles, Medical Center, as described elsewhere [6]. The lateral and medial subsegments of the right middle lobe were each lavaged with 150 mL of sterile saline. The recovered fluid was passed through a 100-μm sterile nylon filter (Becton Dickinson), pooled, and centrifuged at 200 g for 8 min at 4°C. The resulting cell pellets were washed in RPMI 1640 medium (Bio-Whittaker) and kept on ice in polypropylene tubes to avoid cell loss from adherence.

AMs (2 x 10⁶ cells) were suspended at a 1:1 ratio with S. aureus in a final volume of 2.0 mL of Hanks’ balanced salt solution (HBSS; Gibco). This suspension was then incubated, with gentle rocking, at 37°C in the presence and absence of (1) 250 ng/mL NGMMA (Calbiochem), an inhibitor of iNOS, and (2) 200 pM GM-CSF (Biosource International) or 100 U/mL IFN-γ (Boehringer Mannheim). Aliquots (0.1 and 0.25 mL) of the suspensions were removed at 0, 30, 90, and 120 min. After centrifugation, the concentration of nitrite in the supernatant was determined using a colorimetric nonenzymatic NO assay kit (Oxford Biomedical Research). The 0.1-mL aliquot was diluted with 0.9 mL of sterile water and sonicated with two 10-s pulses (60 W), using a Branson Sonifier 450 (Branson Ultrasonics), to lyse the AMs. The sonicate was then serially diluted, and 0.1 mL was plated in each of duplicate trypticase soy agar plates. Plates were incubated at 37°C in a nonhumidified incubator, and bacterial colonies were counted the next day. Results are expressed as bacterial killing = 100 - (N/N₀ × 100), where N is the number of colonies counted at each time point and N₀ is the number of colonies counted at time zero.

AMs (5 x 10⁶) were incubated either alone or with a 1:1 ratio of S. aureus at 37°C in the presence or absence of 200 pM GM-CSF (Biosource International) in a 1.0-mL total volume of HBSS, in accordance with the standard killing assay procedures. All conditions were duplicated for mRNA assays. At 90 min, AMs were separated from the cocultures by ficoll-hypaque density centrifugation, and the cell pellets were quick-frozen. For mRNA collection, cell pellets were resuspended in Nuclisens lysis buffer (Organon Teknika) and processed using the Nuclisens mRNA Isolation Kit. Total RNA (0.5 μg) was reverse transcribed for 50 min at 42°C, using 5 μM oligonucleotide primer (Boehringer Mannheim) and 200 U of reverse transcriptase (Gibco BRL–Invitrogen). For amplification of cDNA, the following gene-specific primers, based on the human hepatocyte iNOS cDNA sequence, were used: sense, 5′-TCCGAGGGCAACAGCACATTCA-3′, and antisense, 5′-GGGTTGGGCGTGTTGATGT-3′. Each polymerase chain reaction (PCR) was conducted in a total volume of 50 μL with 2 μL of cDNA, 0.5 μM sense and antisense primer, 50 μM dNTP, and 2 U of Taq DNA polymerase (Boehringer Mannheim). Conditions for PCR
amplification were 94°C for 30 s, 54°C for 60 s, and 72°C for 1.5 min for a total of 35 cycles. Electrophoresis of the PCR products was performed on 1.5% agarose gels containing 0.5 μg/mL ethidium bromide. A PCR including all components except reverse transcriptase was included as a negative control in each set of PCR experiments.

Mean data from each smoking group were compared, using analysis of variance with post hoc multiple-comparison testing. Within a given smoking group, different treatment conditions were compared using a paired Student’s t test. P ≤ .05 was considered to be statistically significant.

**Results.** AMs were recovered from 48 subjects (31 men and 17 women), including 14 NS (mean age ± SEM, 30.1 ± 5.8 years), 12 TS (37.0 ± 8.7 years), 12 MS (37.2 ± 9.3 years), and 10 CS (46.5 ± 5.5 years). Smokers were, on average, current heavy smokers of tobacco (18.2 ± 13.7 pack-years), marijuana (116.4 ± 111 joint-years), or cocaine (1.5 ± 1.5 g/week).

The use of tobacco by TS varied from 7 to 30 cigarettes/day. Recent marijuana use varied between 0.3 and 5 g/week. None of the CS were MS and none of the MS were TS at that time. However, 3 of 10 CS admitted to limited tobacco smoking. Cell pellets recovered from these subjects were routinely >85% AMs, with <2% neutrophils, as was reported in a related study [7].

Cocultures of AMs and *S. aureus* were evaluated at 30, 90, and 120 min of incubation for the release of nitrite and at the same time points for reductions in *S. aureus* colony counts. Nitrite concentrations increased over time in cocultures performed with AMs from either NS or TS (figure 1A and 1B, bar graphs), in parallel with an increase in antibacterial activity (figure 1A and 1B, line graphs). The role of NO as a mediator of bacterial killing was confirmed by the addition of NGMMA. NGMMA significantly diminished nitrite levels and concurrently reduced killing of *S. aureus* (P < .05). Interestingly, the addition of NGMMA had a smaller impact on cells recovered from TS (P = .05) than it did on cells recovered from NS (P ≤ .004). Under these conditions, residual antibacterial activity in the AMs from TS may result from other antimicrobial mechanisms, such as the presence of reactive oxygen species [8]. Because cytokines enhance the production of NO when added to murine macrophages in vitro [1], we evaluated GM-CSF (figure 1) and IFN-γ (data not shown) for similar effects when added to human AMs. In the case of AMs recovered from NS and TS, no significant cytokine-induced effects were noted either on nitrite production or on bacterial killing.

Nitrite production was significantly diminished (P < .01) in cocultures from MS and CS, compared with nitrite levels produced by AMs from NS and TS, at the same time points (figure 1, bar graphs). In addition, the presence of NGMMA in cocultures of MS and CS AMs had no significant effect on either nitrite production (P > .63) or antimicrobial activity (P > .23).

Collectively, these results suggest that AMs recovered from the lungs of MS and CS cannot generate NO and that this deficiency is responsible for the poor antimicrobial activity of AMs from MS and CS. However, the addition of cytokines to cultures containing AMs from MS or CS significantly improved responsiveness to *S. aureus* (P < .01). As shown in figure 1C and 1D, both killing activity and production of nitrite were restored to near-normal levels by the addition of GM-CSF (identical results were obtained by the addition of IFN-γ; data not shown). The role of NO in this cytokine responsiveness was confirmed by addition of NGMMA, which reduced bacterial killing to levels observed in cells that had never been exposed to cytokines (figure 1C and 1D). The impact of NGMMA on bacterial killing was more profound among cytokine-stimulated cells recovered from MS and CS than among cells recovered from NS and TS, which suggests that cells from these substance abusers were also deficient in production of other antibacterial mediators.

Production of NO in macrophages is catalyzed by iNOS. Resting AMs from NS failed to express detectable levels of iNOS mRNA, but iNOS mRNA expression was detected after 90 min of coculture with *S. aureus* (figure 2). In contrast, iNOS mRNA was detected in AMs from MS or CS only when cells were simultaneously exposed to *S. aureus* and stimulated with either...
GM-CSF or IFN-γ. This pattern of expression correlated with biochemical evidence of NO and NO-dependent bacterial killing.

**Discussion.** NO is an important signaling and effector molecule that mediates a diverse number of physiologic and pathophysiologic processes [1, 2]. High-output iNOS activity in response to stimulation by LPS, cytokines, and microbial challenge was initially characterized and subsequently verified in rodent macrophages [1]. NO clearly plays a critical role as a cytotoxic effector molecule in rodent macrophages. In contrast, the degree to which human mononuclear phagocytes produce and use NO has generated more controversy than consensus [3, 4].

In a previous report, we hypothesized that NO could be one of the mechanisms by which AMs kill *S. aureus*, a bacteria known for its ability to induce NO in murine models [6, 9]. AMs are the predominant lung leukocyte and act as the lung’s resident phagocytic defense against both bacteria and fungi [5]. Moreover, NO is synthesized in the lung and participates in a number of regulatory and protective functions [10]. Despite this evidence, no study to date has proved that a direct connection exists among iNOS gene expression, NO protein production, and NO-dependent antimicrobial activity in normal human AMs. On the basis of the results presented here, we are able to confirm that NO plays an essential role in the capacity of normal human AMs to respond to *S. aureus*, a common respiratory pathogen. The induction of NO was rapid, independent of priming with proinflammatory cytokines, blocked by NGMMA, and correlated with expression of iNOS mRNA.

In contrast to these results for cells from NS or TS, exposure of AMs from MS or CS to *S. aureus* failed to induce the expression of iNOS mRNA or to result in detectable NO production. The functional consequence of this inability to induce NO was an inability to effectively kill *S. aureus*. These findings are consistent with evidence that both cocaine and Δ^2^-tetrahydrocannabinol, the principal active ingredient in marijuana, can inhibit NO production in murine macrophages [11, 12]. Moreover, Lefkowitz et al. [11] demonstrated that the intraperitoneal administration of cocaine to mice not only impaired the ability of murine peritoneal macrophages to produce NO but also diminished the ability of these macrophages to kill tumor cells. The ability of cocaine and marijuana to affect NO synthesis may lead to biological effects in addition to modulation of macrophage function. For example, Mo et al. [13] have suggested that cocaine exerts its peripheral vasoconstrictor effect at least in part by inhibiting production of vasodilator NO, and Molina-Holgado et al. [14] reported that cannabinoids inhibit LPS-induced NO release in astrocyte cultures.

Although exposure to *S. aureus* alone did not result in significant NO production in AMs from MS and CS, we found that ex vivo treatment of these cells with inflammatory cytokines during culture with *S. aureus* restored NO production and antibacterial activity. We hypothesize that chronic in vivo exposure of AMs to inhaled crack cocaine or marijuana impairs the ability of these cells to produce proinflammatory cytokines in response to infectious stimuli. This conclusion is supported by data from our previously published report; specifically, AMs from MS were unable to secrete high levels of inflammatory cytokines when stimulated with LPS [6]. Although in the same report we noted that cytokine production by AMs from CS was highly variable, others have reported that cocaine prevents activated lymphocytes from producing IFN-γ [15]. In vivo cytokine dysregulation by AMs or by pulmonary lymphocytes may have significant consequences for immune regulation and host defense in the lungs of MS and CS.

In summary, our results support the conclusion that NO is an important mediator of antibacterial effects in normal human AMs. Additionally, in MS and CS, alterations in NO production and impaired AM antibacterial function indicate that marijuana and cocaine adversely affect host immunity, cytokine production, and host antibacterial defense. These findings support epidemiological reports and case studies associating the use of marijuana and cocaine with an increase in opportunistic infections, susceptibility to human immunodeficiency virus infection, and possibly progression of AIDS.

**Acknowledgment**

We thank Wendy Aft for preparation of the manuscript.

**References**