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**References**

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Involvement of Nicotinic Acetylcholine Receptors in Suppression of Antimicrobial Activity and Cytokine Responses of Alveolar Macrophages to *Legionella pneumophila* Infection by Nicotine

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Although nicotine is thought to be one of the major immunomodulatory components of cigarette smoking, how nicotine alters the host defense of the lung and, in particular, immune responses of alveolar macrophages, which are critical effector cells in the lung defense to infection, is poorly understood. Nicotinic acetylcholine receptors (nAChRs) are the receptor for nicotine and may be involved in the modulation of macrophage function by nicotine. In this study, therefore, nicotine-induced suppression of antimicrobial activity and cytokine responses of alveolar macrophages mediated by nAChRs to *Legionella pneumophila*, a causative agent for pneumonia, were examined. The murine MH-S alveolar macrophage cell line cells expressed the messages for α4 and β2 subunits of nAChRs, but not α7 subunits, determined by RT-PCR. The nicotine treatment of MH-S alveolar macrophages after infection with *L. pneumophila* significantly enhanced the replication of bacteria in the macrophages and selectively down-regulated the production of IL-6, IL-12, and TNF-α, but not IL-10, induced by infection. These effects were completely blocked by a nonselective antagonist, d-tubocurarine, for nAChRs, but not by a selective antagonist, α-bungarotoxin, for α7-nAChRs. Furthermore, the stimulation of nAChRs with another agonist, 1,1-dimethyl-4-phenylpiperazinium iodide, showed the same effects, which were blocked by the antagonist d-tubocurarine, on the bacterial replication and cytokine regulation with that of nicotine. Thus, the results revealed that nAChRs, the major exogenous ligands of which are nicotine, are involved in the regulation of macrophage immune function by nicotine and may contribute to the cigarette-induced risk factors for respiratory infections in smokers. *The Journal of Immunology*, 2001, 167: 6518–6524.
Materials and Methods

nAChR agonists and antagonists

nAChR agonists, nicotine hydrogen bitartrate and 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), and antagonists, α-bungarotoxin (α-BGTX) and β-tubocurarine (d-TC), were purchased from Sigma (St. Louis, MO), dissolved in pyrogen-free water, and sterilized by filtration with a membrane. All reagents were diluted for working concentrations with RPMI 1640 containing 10% heat-inactivated FCS (HyClone Laboratories, Logan, UT).

Bacteria

L. pneumophila M124, serogroup 1, was originally obtained from a case of fatal legionellosis (21). The bacteria were cultured on buffered charcoal yeast extract (BCYE) agar (BD Biosciences, San Diego, CA) for 3 days at 37°C. The bacterial suspensions were prepared in pyrogen-free saline, and the concentration of bacteria was determined by spectrophotometry.

Alveolar macrophages

The MH-S murine alveolar macrophage cell line purchased from the American Type Culture Collection (Manassas, VA) was used in this study. The cells were maintained in 10% FCS-RPMI 1640. The MH-S cells were adhered to 24-well tissue culture plates at a concentration of 5×10^3 cells/ml for 2 h in 5% CO_2 at 37°C. The resulting cell monolayers were washed with HBSS, supplied with 10% FCS-RPMI 1640 without antibiotics, and then used for experiments.

Macrophage infection

The macrophage monolayers were infected with L. pneumophila (infectivity ratio, 10 bacteria/cell) at 37°C in 5% CO_2 for 30 min, washed to remove nonphagocytized bacteria, and incubated in RPMI 1640 containing 10% FCS without antibiotics. The cultures were then incubated for up to 48 h at 37°C in 5% CO_2.

Macrophage treatment

The macrophage cultures infected with bacteria were treated with varying concentrations of nicotine (0.1–100 μM) or DMPP (0.1–100 μM) for up to 48 h at 37°C in 5% CO_2. In some experiments, macrophage cultures infected with bacteria were pretreated with either α-BGTX (100 nM) or d-TC (10 μM) 15 min before nAChR agonist treatment; then cell monolayers were incubated with either nicotine or DMPP for up to 48 h at 37°C in 5% CO_2. The concentrations of nAChR agonists and antagonist used were previously confirmed to be an appropriate concentration showing stimulation and blocking of nAChRs (22–25).

Macrophage viability assay

The effect of nAChR agonists and antagonists on MH-S cell viability was determined by the viable cell number count and tetrazolium colorimetric methods. In brief, 24 h after treatment of macrophages with agonists or antagonist, the cells were washed with HBSS and then detached with trypsin-EDTA (Sigma). The cells were resuspended with RPMI 1640 containing 10% FCS and pipetted to homogenize the cell suspensions. The number of viable cells was determined by trypan blue exclusion.

Viable bacteria in cell cultures (CFU assay)

The number of viable bacteria (CFU) in cell lysates was determined by standard plate counts on BCYE medium, as described previously (26). After incubation, the cell monolayers were lysed with 0.1% saponin, and the number of viable bacteria in the lysates was determined.

Antimicrobial activity

To evaluate direct in vitro anti-L. pneumophila activity of nicotine, a culture of L. pneumophila in bacterial medium with nicotine was performed. In brief, ACE yeast extract broth medium (27) with or without varying concentrations of nicotine was dispensed to culture flasks and then inoculated with L. pneumophila at the final concentration of 5×10^5 bacteria/ml. After incubation for 24 or 48 h at 37°C, the number of viable bacteria (CFU) in the culture broth was determined by standard plate counts on BCYE medium.

ELISA

The amount of IL-6, IL-10, IL-12 p40/p70, and TNF-α protein in the culture supernatants of macrophage cultures was determined by sandwich ELISA using matched Ab pairs and protein standard for ELISA (BD PharMingen, San Diego, CA; IL-6, IL-10, IL-12 p40/p70) and Duoset ELISA development system (R&D Systems, Minneapolis, MN; TNF-α). Concentrations were calculated from the standard curve performed for each cytokine protein.

RT-PCR

Total RNA was extracted from cells by the microspin technique with RNeasy Mini Kit (Qiagen, Valencia, CA) in accordance with the manufacturer’s manual. Reverse transcription (RT) of total RNA (1 μg) was performed with avian myeloblastosis virus transcriptase in a commercial reaction mixture (Reverse Transcription System; Promega). The resulting cDNA was subjected to PCR with primers for nAChR α6, α7, and β2 subunits. The primers used were designed from GenBank cDNA sequences using a website program, Primer 3 (http://www.path.cam.ac.uk/cgi-bin/primer3.cgi). The sequences of primer for nAChR α6 were 5′-ATC CTG ACA TCA CCT ACG CC-3′ (sense) and 5′-GTT GGT GTA TTA CGA CG-3′ (antisense). The sequences of primer for nAChR α7 were 5′-CAT TCA ACA CCA ACG TCT TG-3′ (sense) and 5′-TGA GCA CAC ACG GAA TGA GC-3′ (antisense). The sequences of primer for nAChR β2 were 5′-TGG ATT TCT GTG CTG GC-3′ (sense) and 5′-CAC GCT AGT GAC GAT GGA GA-3′ (antisense). The PCR was performed in a Minicycler (MJ Research, Watertown, MS) for 40 cycles, 57°C annealing temperature. PCR products were analyzed on an ethidium bromide-stained 2% agarose gel. The specificity of PCR was confirmed by oligonucleotide sequencing of PCR products (MWG, High Point, NC).

Statistical analysis

Statistical analysis was performed with a paired Student t test.

Results

Effect of nAChR agonists and antagonists on macrophage viability

To determine the toxicity of both nAChR agonists and antagonists on macrophages, the cells were incubated with various concentrations of either nAChR agonists (nicotine, 0.1–100 μg/ml; DMPP, 0.1–100 μM) or antagonists (α-BGTX, 1–100 nM; d-TC, 0.1–10 μM) for 24 h, and then the viability of macrophages was assessed.
by trypan blue dye exclusion and tetrazolium colorimetric assay. Nicotine did not show any cytotoxicity for the macrophages up to 10 μg/ml. However, 100 μg/ml nicotine treatment significantly reduced the macrophage viability (~70% reduction of viability compared with nontreated control group). In contrast, all concentrations of DMPP, α-BGTX, and d-TC tested did not affect the cell viability. These results were consistent in the two assay methods (data not shown).

Effect of nicotine on L. pneumophila growth
Because the growth of L. pneumophila in macrophages is dependent on the host’s macrophage activity, treatment of macrophages with nicotine may alter the growth of L. pneumophila in cells if nicotine has any immunomodulatory activity on macrophages. From the previous results, we used the concentration range of 0.1–10 μg nicotine/ml to evaluate the effect of nicotine on L. pneumophila growth in macrophages. As shown in Fig. 1, the treatment of macrophages with nicotine after infection with bacteria induced an enhancement of the growth of L. pneumophila in the cells in a dose-dependent manner at both 24 and 48 h after infection. A significant enhancement of L. pneumophila growth by nicotine occurred even with a concentration as low as 0.1 μg/ml at 48 h after infection. In contrast, it has been reported that nicotine has a direct antimicrobial activity against some bacterial and fungal pathogens (28). Therefore, to determine whether nicotine has a direct modulatory activity on L. pneumophila growth, the growth of L. pneumophila in liquid bacterial medium without host cells in the presence or absence of nicotine (0.1–100 μg/ml) was examined. However, nicotine did not alter the L. pneumophila growth in the bacterial medium regardless of the concentrations tested (data not shown). These results indicate that nicotine does not have a direct modulatory activity on L. pneumophila growth at the concentrations tested.

Effect of nicotine on macrophage cytokine production
Because nicotine enhanced the growth of L. pneumophila in macrophages without a direct effect on bacteria, it seemed likely that host cell modulation may be involved in the enhancement of L. pneumophila growth by nicotine. To examine such a possibility, the effect of nicotine on the production of macrophage cytokines, which are known to be involved in the regulation of bacterial growth in cells (29), was examined. As evident in Fig. 2, the treatment of macrophages with nicotine alone slightly induced macrophage IL-6, IL-10, IL-12, and TNF-α protein production, but this was minimal when compared with L. pneumophila-infected macrophages. In contrast, nicotine treatment markedly down-regulated the production of IL-6, IL-12, and TNF-α induced by L. pneumophila infection in a dose-dependent manner, even with a concentration as low as 0.1 μg/ml in the case of TNF-α. In contrast, the production of IL-10 induced by L. pneumophila infection was not affected by nicotine, even with a concentration as high as 10 μg/ml. These results indicate that nicotine may selectively alter the cytokine responses of macrophages against L. pneumophila infection and lead to an enhancement of bacteria growth in macrophages.

nAChRs mRNA expression in alveolar macrophages
It is widely accepted that nAChRs form a family of receptors and that they are differentially expressed in many tissues (30). Although the most frequent way to acquire nicotine is via tobacco smoking, the existence of nAChRs on lung tissues and cells has not yet been well investigated. If nAChRs exist on alveolar macrophages, the nicotine-induced immunomodulation of macrophages may be possibly mediated by nAChRs. To determine such a possibility, steady state levels of nAChR mRNA (α4, α7, and β2 subunits) in MH-S alveolar macrophages were analyzed by RT-PCR. NC, PCR products of MH-S cells without RT.
Effect of nAChR antagonists

FIGURE 4. Effect of nAChR antagonists on cytokine production of nicotine-treated or untreated macrophages in response to *L. pneumophila* infection. See the legend to Fig. 2. Macrophage cultures infected with bacteria and pretreated with or without 10 μM d-TC or 100 nM α-BGTX were incubated with or without 10 μg/ml nicotine. □, Non-*L. pneumophila* infection group; ■, *L. pneumophila* infection group. Results are expressed as means ± SD for three independent experiments. *p* < 0.05, significantly different from the control group.

### Table I. Effect of nAChR antagonists on *L. pneumophila* growth in nicotine-treated or untreated macrophages

<table>
<thead>
<tr>
<th>Treatment</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>d-TC (10 μM)</td>
<td>89 ± 15</td>
<td>95 ± 19</td>
</tr>
<tr>
<td>α-BGTX (100 nM)</td>
<td>113 ± 8</td>
<td>97 ± 11</td>
</tr>
<tr>
<td>Nicotine (10 μg/ml)</td>
<td>421 ± 91</td>
<td>432 ± 97</td>
</tr>
<tr>
<td>Nicotine + d-TC</td>
<td>113 ± 28</td>
<td>114 ± 24</td>
</tr>
<tr>
<td>Nicotine + α-BGTX</td>
<td>293 ± 67</td>
<td>322 ± 69</td>
</tr>
</tbody>
</table>

*Macrophage cultures infected with bacteria and treated with or without d-TC or α-BGTX were incubated with or without nicotine. The number of viable bacteria in macrophages was determined by the standard plate count method. Data are representative of three experiments. The numbers of viable bacteria (CFU) at 24 and 48 h after infection were 2.8 ± 0.6 × 10^7 and 1.4 ± 0.4 × 10^6, respectively.*

The growth of *L. pneumophila* enhanced by nicotine. The treatment of nAChR antagonists alone did not show any alteration of the bacterial growth in the macrophages.

### Involvement of nAChRs in nicotine-induced immunomodulation

When macrophages infected with bacteria were pretreated with d-TC, nicotine-induced suppression of IL-6, IL-12, and TNF-α production was readily restored to the control levels without the modulation of IL-10 production (Fig. 4). In contrast, pretreatment of macrophages with α-BGTX did not result in recovery of the nicotine-suppressed cytokine production. The treatment of nAChR antagonists alone did not alter the production of cytokines tested. Thus, the results with the nAChR antagonist treatment clearly indicate that the nicotine-induced suppression of alveolar macrophage activity, which is involved not only in antimicrobial activity but also cytokine responses against bacteria, is mediated, at least, by α4β2-nAChRs. The failure of blocking with the α7-nAChR antagonist α-BGTX on nicotine-induced modulation of macrophage cytokine production as well as antimicrobial activity was consistent with the results of no α7-nAChR message expression determined by RT-PCR.
nAChR agonist-induced modulation of macrophage function

To determine whether stimulation of nAChRs with other agonists causes modulation of macrophage function, the effect of another nAChR agonist DMPP was used for this purpose. When macrophages infected with the bacteria were treated with DMPP, a marked enhancement of bacterial growth in the cells was observed at 24 h after infection (Fig. 5), similar to the case with nicotine. Pretreatment of macrophages with antagonist d-TC completely abolished the effect of DMPP. A similar effect of DMPP and blocking with d-TC on cytokine production of the macrophages in response to bacterial infection was also evident (Fig. 6); i.e., the DMPP-treatment significantly down-regulated the production of IL-6, IL-12, and TNF-α induced by L. pneumophila infection. This selective inhibition on cytokine production by DMPP was completely blocked by d-TC treatment.

Discussion

Cigarette smoking is a significant risk factor in respiratory diseases including chronic obstructive lung disease and pneumonia. The bronchial alveolar lavage fluids obtained from cigarette smokers have increased numbers of alveolar macrophages and neutrophils (31, 32). Moreover, compared with nonsmokers, alveolar macrophages from smokers appear to be in an active state, exhibiting increased microsomal and lysosomal enzymes, elevated resting rates of glucose use, increased production of oxygen radicals and myeloperoxidase activity, and increased migration and chemotactic responsiveness (33). However, despite this increased activity, alveolar macrophages from smokers appear to be deficient in phagocytosis and bactericidal activity (34). Thus, it has been conjectured that cigarette smoking may cause a disruption of normal lung immune function against respiratory infections. However, little is known about the effect of cigarette components on antimicrobial activity and immune responses of alveolar macrophages. In this regard, the present study concerning the effect of nicotine on the susceptibility of alveolar macrophages to L. pneumophila infection revealed that nicotine suppresses cytokine responses of macrophages to infection by the bacteria and leads to an enhancement of bacterial replication in macrophages. The nicotine concentrations used in this study were higher than the level of nicotine reported in the plasma of heavy smokers (35). However, the concentrations of nicotine at the site of alveoli after smoking cigarettes, the nicotine yield of which is ~0.91 mg/cigarette (36), may be higher than the plasma level. Nevertheless, the findings constitute the first in vitro demonstration of how cigarette smoke components may contribute to risk factors for respiratory infections in smokers. In particular, the findings of the selective inhibition of cytokine productions, such as IL-6, IL-12, and TNF-α, by nicotine indicates how such immunomodulation may contribute to the susceptibility of cells to infections. TNF-α is required for the prompt resolution of pneumatic legionellosis and points to a direct role for TNF-α in the activation of phagocytes (19). The precise mechanism of nicotine-induced suppression of antimicrobial activity of macrophages is still unclear. However, our recent studies have
shown that epigallocatechin gallate, the major form of tea catechins, restores nicotine-suppressed TNF-α production as well as antimicrobial activity of macrophages (our unpublished data). Therefore, it seems likely that the impaired TNF-α production may be one of the major mechanisms responsible for the nicotine-induced impairment of antimicrobial activity against L. pneumophila infection. This hypothesis can be supported by previous reports that TNF-α is a strong activator for macrophages to induce anti-L. pneumophila activity (19, 37). In contrast, the role of the reduced IL-6 production in the nicotine-induced impaired antimicrobial activity is not clear. However, this may not have a direct role because of the absence of IL-6 modulatory effects on macrophages regarding anti-L. pneumophila activity (38).

IL-10 and IL-12 are key cytokines in the regulation of development of Th1/Th2 responses (18, 39, 40). Therefore, the participation of these two cytokines in the defense against L. pneumophila infection is critical (13, 17). The direct involvement of these cytokines in the regulation of bacterial growth in cells is less likely. Nevertheless, the suppression of IL-12 production, but not IL-10, by nicotine may cause an alteration of Th1 development by nicotine during infection, if this occurs in vivo. In this regard, it has been reported that the nicotine-derived N-nitrosamine directly modulates cytokine production, including inhibition of IL-2, IL-6, IL-10, GM-CSF, and monocyte chemoattractant protein-1 production in the U937 human macrophage cell line (41). It is also known that nicotine itself suppresses in vitro production of IL-2, TNF-α, and IFN-γ from human PBMC (10). Therefore, the present results are consistent with these previous reports regarding the inhibitory activity of nicotine and its derivative on cytokine production.

nAChRs form a family of receptors that are differentially expressed mainly in the central and peripheral nervous system, as well as on many other tissue cells. For instance, nAChRs are found in cochlea (8), ganglionic tissue (42), developing muscles (43), and lymphocytes and polymorphonuclear cells in the peripheral blood (7, 44). Although the function of peripheral nAChRs has not been well investigated, such localization suggests that peripheral nAChRs have nonsynaptic roles. The results of this study clearly support a possible nonsynaptic role of nAChRs in alveolar macrophages. Whether in muscle or neurons, nAChRs are allosteric activators of gamma-aminobutyric acid receptors that are assembled from five subunits according to defined combination rules. In mammals, nine homologous subunits (α2–α7 and β2–β4) have been identified thus far in the nervous system whereas the α9 subunit is present in sensory end organs (30).

Equilibrium binding studies have distinguished two main categories of nAChR pentamers on the basis of their high affinity for either nicotine or α-BGTX. The former are considered to be formed by α4 and β2 subunits containing nAChRs, and the latter are thought to be α7 subunit containing nAChRs (45, 46). Furthermore, α7-nAChRs exhibit low affinity for acetylcholine and nicotine and are rapidly desensitized, whereas α4β2-nAChRs are considered to form a high affinity receptor for acetylcholine and nicotine and are desensitized slowly (30). Although we attempted to determine the partial nAChR subunit mRNA expression in alveolar macrophages, the results of nAChR antagonist and agonist treatment and the determination of mRNA expression for nAChR α4 and β2 subunits in alveolar macrophages revealed the involvement of nAChR-mediated mechanism in the nicotine-induced suppression of antimicrobial activity and immune responses of macrophages. In addition, the results of this study indicated that nicotine-induced suppression of alveolar macrophage activity, which is involved in not only antimicrobial activity but also cytokine responses against bacteria, is mediated, at least, by α4β2-nAChRs.

Thus, the present study revealed that nicotine causes an alteration of immune responses of alveolar macrophages to bacterial infection by an nAChR-mediated mechanism.

Acknowledgments

We thank Drs. Susan Pross and Geoffrey Patton for helpful suggestions regarding the nicotine experiments.

References


