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ORIGINAL ARTICLE

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4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone modulation of cytokine release in U937 human macrophages

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Abstract The nicotine-derived *N*-nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), is one of the most abundant and potent carcinogens found in tobacco smoke. NNK induces lung tumors in rodents and is most likely involved in lung carcinogenesis in humans. Studies on the metabolism and carcinogenicity of NNK have been extensive. However, its effects on the immune system have not been investigated thoroughly. Considering that tobacco smoking partially suppresses the immune response in humans, and that immune surveillance plays a critical role in cancer development, we examined the effects of NNK on the production of selected cytokines. In a previous study, we observed an inhibition of NK cell activity and IgM secretory cell number in NNK-treated A/J mice [Rioux and Castonguay (1997) J Natl Cancer Inst 89: 874]. In this study, we demonstrate that U937 human macrophages activate NNK to alkylating intermediates by α-carbon hydroxylation and detoxify NNK by *N*-oxidation. We observed that NNK, following activation, induces the release of soluble tumor necrosis factor (TNF), but inhibits interleukin(IL)-10 synthesis. We also report that 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone, and nitroso(acetoxymethyl)methylamine, which generate the same alkylating intermediates as NNK, have similar effects on TNF and IL-10. This suggests that pyridyloxobutylating and methylating intermediates generated from NNK are potent modulators of the immune response. The levels of IL-6, granulocyte/macrophage-colony-stimulating factor and macrophage chemotactic protein 1 were also decreased in supernatants of NNK-treated U937 macrophages. In contrast, IL-2 synthesis in Jurkat cells was inhibited by NNK treatment. This is the first study demonstrating that NNK, via its alkylating intermediates, alters the cytokine synthesis profile in human cells. Modulation of cytokine synthesis by NNK might partially explain the immunosuppresion observed in smokers. Inhibition of immune functions, resulting from NNK activation to alkylating agents, may facilitate lung tumor development.

Key words Nitrosamine · Cytokines · Macrophages · Tobacco

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Introduction

Tobacco smoking alters the immune response by inhibiting functions of B and T lymphocytes, natural killer (NK) cells, neutrophils and macrophages (reviewed in [20, 38]). Serum levels of immunoglobulins (IgG, IgM and IgA) are reduced in smokers [20]. Treatment of macrophages in vitro with condensate of mainstream cigarette smoke decreases their functional capacities and enhances their production of tumor necrosis factor (TNF) [5]. In contrast, macrophages of healthy smokers have a depressed capacity to release TNF [41], while chronic exposure to tobacco smoke have no statistically significant effect on TNF production by murine alveolar macrophages [17], suggesting that the modulation of TNF synthesis by tobacco components is cell-specific. These imbalances of the immune system may predispose smokers to infections and cancers. The mechanism by which tobacco smoke suppresses the immune response has not been established and the smoke component(s) responsible for this immunosuppression has (have) not been identified.

Tobacco smoke contains at least 55 compounds known to induce tumors in laboratory animals, and tobacco smoking is a well-established risk factor for human lung cancer [19]. Among these carcinogens, one of the most potent and abundant is the nicotine-derived 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK),

which has a remarkable specificity for the lungs in rodents [15]. NNK is a pro-carcinogen activated by *P*-450 monooxygenases, cyclooxygenase-1 and -2 and lipoxygenases in lungs [15, 31, 32]. This activation, initiated by α-carbon hydroxylation of NNK, produces various reactive electrophilic intermediates resulting in methylation and pyridyloxobutylation of specific sites of DNA (reviewed in [15]; Fig. 1). 4-(Acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butonone (NNKOAc), an analog of NNK, and nitroso(acetoxymethyl)methylamine (NDMAOAc), an analogue of *N*-nitrosodimethylamine, generate the same pyridyloxobutylating and methylating agents respectively and selectively. We have previously observed that NNK treatment inhibits NK cell cytotoxicity and decreases the number of IgM-secreting cells, in A/J mice, suggesting that a sustained treatment with NNK is immunosuppressive [30]. A recent study by Goud and Kaplan confirmed our conclusion that NNK is an inhibitor of NK cell activity in mice [13]. In this study, the NK activity was restored by injection of interleukin-12 (IL-12), suggesting that NK cell cytotoxicity is modulated by cytokines [13]. The mechanism of NNK-mediated immunosuppression is unknown.

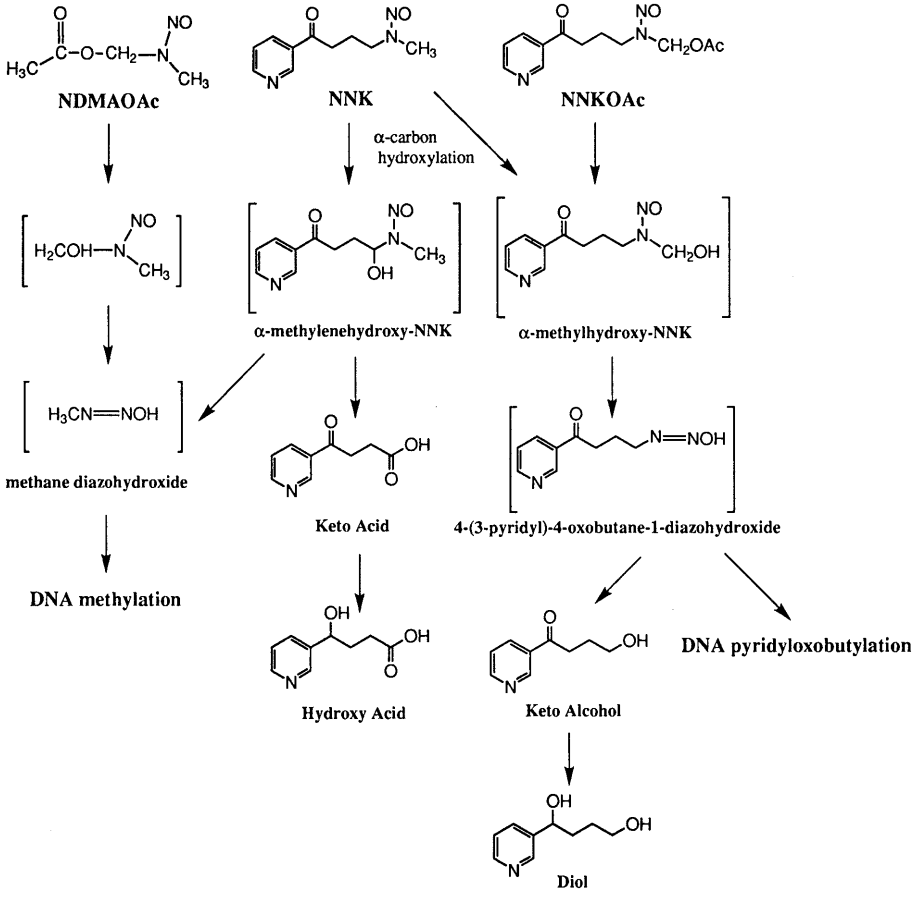
Human histiocytic U937 lymphoma cells, differentiated into macrophages, were selected for this study. Macrophages may serve as both positive and negative mediators of the immune system. Macrophages mediate direct antitumor cytotoxicity; they display tumor-associated antigens and stimulate antitumor lymphocytes, but they also demonstrate the ability to promote the growth of tumor cells (reviewed in [8]). Macrophages were also selected because of their ability to activate NNK to reactive intermediates, which generate mutations in target pulmonary cells, and initiate lung tumor development. Monocytes/macrophages secrete several cytokines regulated by nuclear factor (NF)-κB, such as granulocyte/macrophage-colony-stimulating factor (GM-CSF), IL-1, monocyte chemotactic protein (MCP)-1 and TNF [3]. We recently observed an activation of NF-κB in human macrophages treated with NNK [33]. NF-κB is induced rapidly by TNF and inhibited by IL-10 [3, 33, 42]. We hypothesized that NNK, via its alkylating intermediates, alters cytokine synthesis by human macrophages, thus favoring lung tumor development.

Fig. 1 Proposed bioactivation pathways of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (*NNK*), and hydrolysis of 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone (*NNKOAc*) and nitroso (acetoxymethyl) methylamine (*NDMAOAc*) to DNA-methylating or -pyridyloxobutylating intermediates

Materials and methods

Chemicals

NNK (99% pure by thin-layer chromatography) and [5-^{3}H]NNK (99% pure by high-pressure liquid chromatography, HPLC) were



supplied by Chemsyn Science Laboratory (Lenexa, Kas.). The synthesis of metabolites used as standards in HPLC analysis has been reported [16]. The synthesis of NNKOAc (more than 85% pure by analytical reverse-phase HPLC) has been described by Peterson et al. [29] and Cloutier and Castonguay [7]. NDMAOAc was purchased from LKT Laboratories (St. Paul, Minn.). Phorbol 12-myristate 13-acetate (PMA) was provided by Sigma-Aldrich (St. Louis, Mo.). RPMI medium, fetal bovine serum (FBS), and trypsine/EDTA were obtained from Life Technologies (Burlington, Canada).

Cell lines

The human histiocytic lymphoma cell line U937 (American Type Culture Collection) was routinely grown in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin (Life Technologies, Burlington, Canada) at 37 °C in a humidified 5% CO_2 atmosphere [39]. Cells in the logarithmic phase of growth were harvested, adjusted to 1×10^6 cells/ml in the presence of 10 nM PMA and incubated for 3 days to allow them to differentiate into adherent macrophages [1]. Adherent cells were incubated in fresh complete RPMI-1640 medium in the absence of PMA for 6 h, trypsinised and used for cytokine assays. The human leukemia cell line Jurkat (American Type Culture Collection) was routinely grown in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. Under all experimental conditions, viability was better than 95% as determined by trypan-blue dye exclusion.

NNK metabolism

Differentiated U937 cells (1×10^6), resuspended in 2 ml fresh RPMI medium supplemented with 10% FBS, were incubated in the presence of [^{3}H]NNK (20 μM, 0.5 μCi/ml, Chemsyn Science Laboratories). Controls consisting of [^{3}H]NNK added to the medium, without cells were included. Supernatants were collected after a 24-h incubation period at 37 °C. The medium was filtered and analyzed for [^{3}H]NNK metabolites by a reverse-phase HPLC system, using a ODS 2 column (5 μm, 4.6 × 250 mm; Waters Associates, Milford, Mass.). A 1-ml aliquot of medium and 7 μl standard NNK metabolites were co-injected and eluted with a pH 6.0 sodium acetate buffer and methanol mobile phase, as described previously [22]. The elution was monitored at 254 nm and 1-ml fractions were collected. Scintisafe Plus (5 ml; Fisher Scientific, Montreal, Canada) was added to each fraction and radioactivity was measured by liquid scintillation spectroscopy. The recovery of total radioactivity during HPLC analysis was more than 70%. Peaks with counts equivalent to less than twice the background were considered insignificant. A preliminary study demonstrated that NNK metabolism was below detection limits at 6 h and linear from 24 h to 72 h (r^2 value of 0.99 for both activation and detoxification of NNK).

IL-10 and TNF assays

PMA-differentiated U937 cells (5×10^5) were incubated for 18 h in 1 ml RPMI 1640 medium supplemented with 10% FBS, with or without NNK (250, 500 and 1000 μM), NNKOAc (50, 100, 200 μM) or NDMAOAc (50, 100, 200 μM). At these concentrations, these nitroso compounds had no effect on cell proliferation as determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (data not shown). NNK and NNKOAc were dissolved in sterile saline. Under these conditions, more than 90% of the NNKOAc was hydrolyzed into alkylating intermediates, as determined by HPLC analysis (data not shown). Media were collected, diluted 1:5 and 1:10 with RPMI for cells treated with NNK or diluted 1:2 and 1:5 for NNKOAc- or NDMAOAc-treated cells. The IL-10 level was measured in duplicate for each dilution, by the Quantikine immunoassay (R & D Systems, Minneapolis, Minn.). Levels of soluble TNF were measured, without dilution, using an immunoassay kit from Cayman Chemical (Ann Arbor, Mich.). Results are shown for 10^6 cells.

GM-CSF, IL-6, IL-12 and MCP-1 assays

PMA-differentiated U937 cells (1×10^6) were incubated for 18 h in 1 ml RPMI-1640 medium supplemented with 10% FBS, with or without NNK (250, 500 and 1000 μM). The medium was collected and the level of each cytokine was determined, without further dilution, with specific Quantikine immunoassay kits from R & D systems.

IL-2 assay

Jurkat cells (1×10^6), stimulated with 5 nM PMA, were incubated in 1 ml RPMI-1640 medium supplemented with 10% FBS in the presence or absence of NNK (250, 500 and 1000 μM) or NNKOAc (50, 100, 200 μM). The medium was collected after an 18 h incubation, and used without dilution or with 1:2 dilution. The level of IL-2 was measured in duplicate for each dilution, using an immunoassay kit (Cayman Chemical).

Statistical analysis

Statistical analyses were performed for untreated or carcinogen-treated cells with the SAS program. Analysis of variance with Dunnett's multiple-comparisons two-tailed test was used. Results are shown as means ± SD of at least three determinations. A P value less than 0.05 was selected to indicate statistical significance.

Results

We observed that U937 human macrophages metabolize NNK. NNK is bioactivated by hydroxylation of the carbons adjacent (α-carbon) to the *N*-nitroso group, producing the hydroxy acid, oxo acid, diol and oxo alcohol. NNK *N*-oxide and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butan-1-ol (NNAL) *N*-oxide result from pyridine *N*-oxidation, and are considered detoxification products. Reduction of the NNK carbonyl group produces NNAL, the two compounds have similar carcinogenic activities [15]. Blanks were conducted without cells. The data are expressed as the mean ± SD of three determinations. After a 24-h incubation period, the predominant metabolite of NNK formed in PMA-differentiated U937 cells was NNAL; 11.2 ± 3.0% of the total amount of [^{3}H]NNK was converted to this metabolite. U937 cells activated [^{3}H]NNK (5.2 ± 2.0%) by α-carbon hydroxylation to the oxo acid (3.2 ± 0.8%) and diol (2.0 ± 0.9%). U937 also detoxified 4.3 ± 0.6% of NNK and NNAL.

Synthesis of the cytokines selected in this study was modulated by NNK. TNF was induced by NNK, while other cytokines were inhibited (Fig. 2). The basal level of soluble TNF released by 10^6 U937 macrophages was 66 ± 8 pg/ml; it reached 103 ± 17 pg/ml at 500 μM NNK ($P < 0.05$) and doubled to 129 ± 22 pg/ml at 1000 μM ($P < 0.05$). The induction of TNF release in the medium was statistically higher following treatment with 1000 μM NNK than when 500 μM was used ($P < 0.05$). This induction was not observed at higher

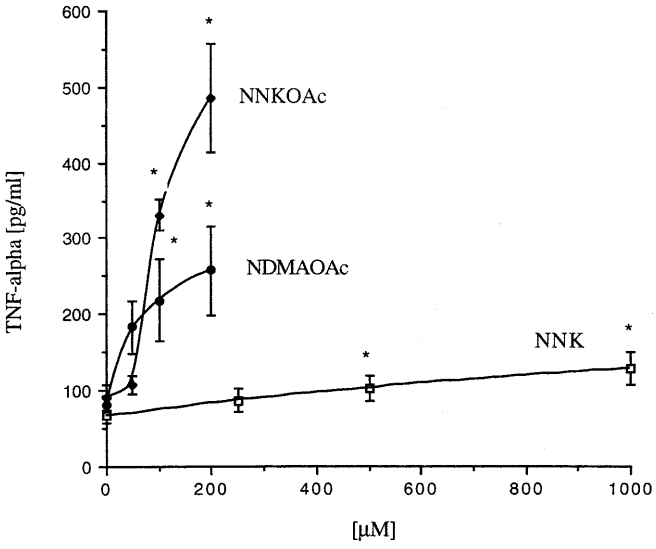


Fig. 2 Induction of tumor necrosis factor (*TNF*) synthesis by NNK (□), NNKOAc (◆) and NDMAOAc (●) in U937 human macrophages. TNF released in the culture medium by 1×10^6 cells, after an 18-h incubation was immunoassayed. Each point is the mean of three determinations ±SD. *Statistically different from untreated cells, $P < 0.05$. Cells treated with 500 μM and 1000 μM NNK are statistically different, $P < 0.05$

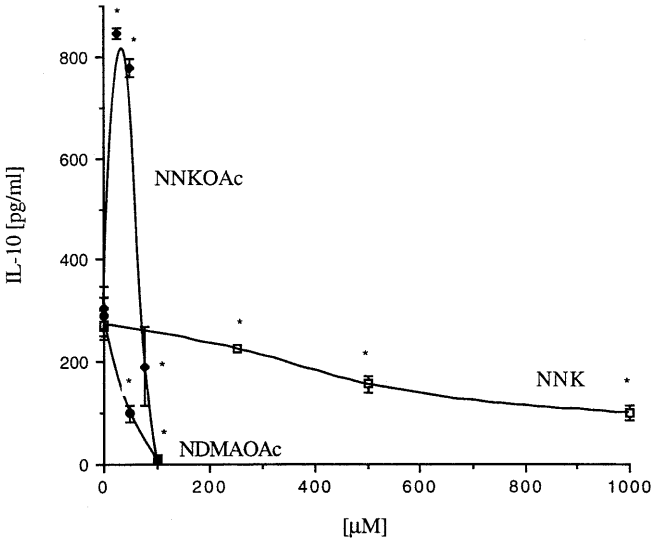


Fig. 3 Inhibition of interleukin-10 (*IL-10*) synthesis by NNK (□), NNKOAc (◆) and NDMAOAc (●) in U937 human macrophages. IL-10 released in the culture medium of 1×10^6 cells, after an 18-h incubation was measured by immunoassay. Each point corresponds to the mean of three determinations ±SD. *Statistically different from untreated cells, $P < 0.05$. For NNK or NDMAOAc, each dose is also statistically different, $P < 0.05$

concentrations of NNK; in fact, at 2 mM, the level of soluble TNF decreased (data not shown). NNKOAc and NDMAOAc, at a concentration of 200 μM, also increased the release of TNF, which reached 485 ± 71 pg/ml and 258 ± 58 pg/ml ($P < 0.05$) respectively.

IL-10 synthesis decreased from a mean of 273 ± 30 pg/ml in untreated macrophages to 100 ± 15 pg/ml in cells treated with 1000 μM NNK, corresponding to a 63% inhibition ($P < 0.05$, Fig. 3). NNK inhibition of IL-10 synthesis was dose-dependent, each dose being statistically different from the other ($P < 0.05$). Quite unexpectedly, IL-10 synthesis was induced in cells treated with a low concentration of NNKOAc (25 and 50 μM, $P < 0.05$), but was almost totally inhibited at a concentration of 100 μM ($P < 0.05$). At 200 μM NNKOAc, the level of IL-10 synthesis was below the detection limit of the immunoassay (less than 4 pg/ml). The IL-10 level was inhibited in a dose-dependent manner by NDMAOAc, being below the detection limit of 200 μM.

Release of IL-6 by U937 cells was 45% and 52% inhibited by 500 μM and 1000 μM NNK respectively ($P < 0.05$), while GM-CSF synthesis was inhibited by NNK in a dose-dependent manner, to reach 64% inhibition (from 3.1 ± 0.2 pg/ml to 1.1 ± 0.3 pg/ml) at a concentration of 1000 μM ($P < 0.05$) (Fig. 4a). At 500 μM and 1000 μM NNK, MCP-1 synthesis was decreased by 35% and 40% respectively ($P < 0.05$) (Fig. 4b). IL-12 was not detected in cell supernatants of untreated or NNK-treated U937 (data not shown).

Release of IL-2 in Jurkat cells medium was inhibited by both NNK and NNKOAc. The IL-2 level in untreated cells was 5.2 ± 0.4 pg/ml and decreased to 2.4 ± 0.4 pg/ml in cells treated with 1000 μM NNK ($P < 0.05$, 53% inhibition). The higher dose of NNK was statistically different from all others ($P < 0.05$). In NNKOAc-treated Jurkat cells, an inhibition of 44% was observed at 50 μM, which reached 74% at 200 μM ($P < 0.05$, Fig. 5). With NNKOAc, a trend was observed in the dose/response effect but did not reach statistical significance.

Table 1 Metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (*NNK*) in U937 human macrophages after a 24-h incubation period. Results are means of three determinations ± SE. *NNAL* 4-(methylnitrosamino)-1-(3-pyridyl)-1-butan-1-ol, *ND* not detected

Metabolite	Amount relative to total ^{3}H (%)
Hydroxy acid	ND
Oxo acid	3.2 ± 0.8
Diol	2.0 ± 0.9
Oxo alcohol	ND
NNAL *N*-oxide	4.3 ± 0.6
NNK *N*-oxide	ND
NNAL	11.2 ± 3.0
NNK	79.2 ± 1.7
α-Carbon hydroxylation[a] (%)	5.2 ± 2.0
N-oxidation[b] (%)	4.3 ± 0.6

[a] Hydroxy acid + oxo acid + diol + oxo alcohol
[b] NNAL *N*-oxide + NNK *N*-oxide

Discussion

Tobacco smoking suppresses the immune response in humans [20, 38]. In a previous study, we observed that

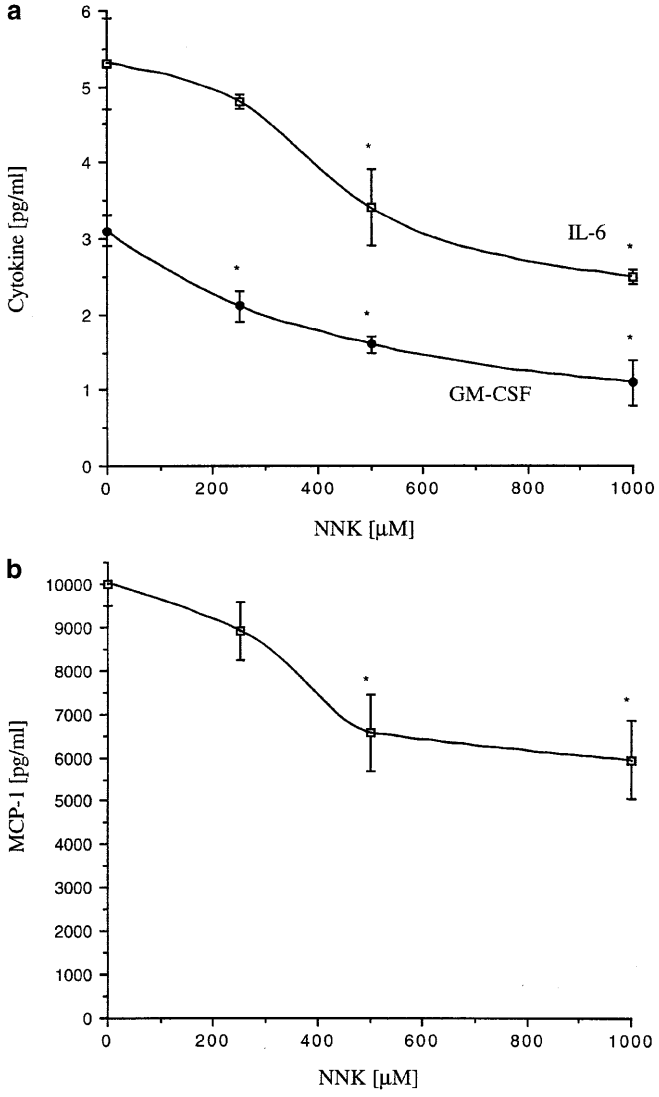


Fig. 4 **a** Inhibition of granulocyte/macrophage-colony-stimulating factor (*GM-CSF*) (●) and IL-6 release (□) by NNK in U937 human macrophages. *Statistically different from untreated cells, $P < 0.05$. For IL-6, the effect of the 250 μM NNK dose is different from the that of 500 and 1000 μM NNK, while for GM-CSF, the effects of all doses differ from each other, $P < 0.05$. **b** Inhibition of monocyte chemotactic protein 1 (*MCP-1*) synthesis by NNK. The level of each cytokine released in the culture medium by 1×10^6 cells was immunoassayed after an 18-h incubation with or without NNK. *Statistically different from untreated cells, $P < 0.05$. Each point corresponds to the mean of two (IL-6), or three determinations (GM-CSF and MCP-1) ± SD

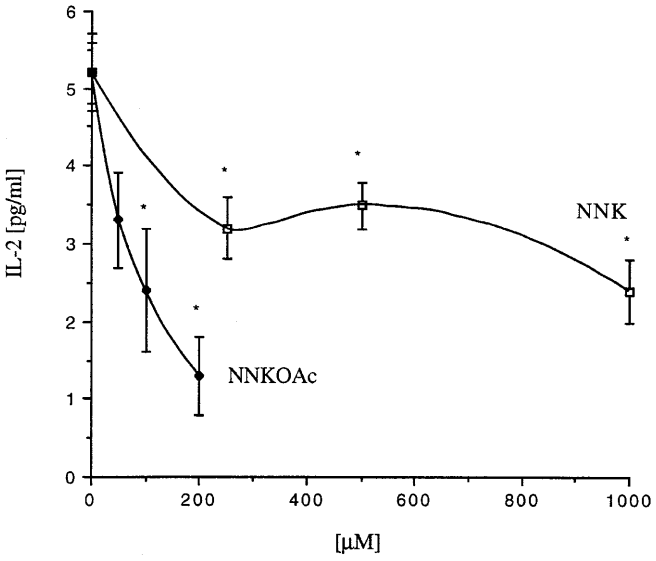


Fig. 5 Inhibition of IL-2 synthesis in Jurkat cells. Cells (1×10^6) were incubated 18-h with NNK (□) or NNKOAc (◆), and medium was collected for immunoassay of IL-2. Mean of 3 determinations ± SD. *Statistically different from untreated cells, $P < 0.05$

the tobacco-specific carcinogen, NNK, activates NF-κB in U937 human macrophages [33]. NF-κB has been invoked as an important transcription factor in the expression of cytokine genes and various cell-surface receptors (reviewed in [2, 3]). In the present study, we demonstrated that NNK induces TNF release and inhibits IL-10 synthesis in U937 cells. Production of IL-2, IL-6, GM-CSF and MCP-1 was also decreased in NNK-treated cells. This is the first study demonstrating that NNK, via its alkylating intermediates, unbalances the cytokine synthesis profile of human cells. Mechanisms other than regulation by NF-κB might also be involved in the modulation of cytokine synthesis by NNK. These data also suggest that NNK is one of the tobacco components responsible for the immunosuppression observed in smokers, and that, in addition to its mutagenic potential, NNK might favor tumor growth by suppressing the host immunity.

The average intake of NNK in smokers is 28 nmol/day or 410 μmol in 40 years [15]. In this study, a high concentration of NNK had to be used to measure the effects of this carcinogen in human cells. Even if high doses of NNK are required, low concentrations of reactive metabolic intermediates are expected to be produced. Furthermore, cells were exposed to NNK for only 18 h, while smokers are exposed to this carcinogen for years.

In this study, we observed that NNK induces soluble TNF release by U937 cells (Fig. 2). TNF is an inducer of NF-κB activation in U937 [14], suggesting that this increase in TNF level could have led to the previously observed induction of NF-κB activation by NNK [33]. Janssen et al. observed that oxidants, such as reactive oxygen species, and TNF cooperate to activate NF-κB [21]. NNK bioactivation leads to the production of reactive oxygen species [23], and we have previously demonstrated that oxygen radical scavengers partially decrease NF-κB activation by NNK in U937 [33]. Altogether, these data suggest that TNF induction and reactive oxygen species generated during NNK activation could cooperate to activate NF-κB. The induction of TNF by NNK also correlates with the previously observed induction of prostaglandin E_2 synthesis by cyclooxygenase in U937 [33]. Arias-Negrette et al. showed that TNF induces cyclooxygenase-2 expression in interferon-γ-primed U937 cells [1]. TNF is a pro-

inflammatory cytokine that has been associated with the pathogenesis of several pulmonary disorders and modulates cellular differentiation, proliferation and immunoregulation [40], suggesting that an increase of this cytokine might favor lung tumor development. Nitrosamines other than NNK can activate TNF; dimethylnitrosamine raises circulating TNF in mice and induces TNF synthesis in peripheral macrophages [25, 34]. The presence of NNK in smokeless tobacco and tobacco smoke [6, 15], and the induction of TNF by NNK in U937 cells provide some basis for the observation by Seyedroudbari and Khan, and Braun et al. that TNF is induced in macrophages treated with cigarette smoke condensate or smokeless tobacco extract [5, 35].

In this study, we observed that human U937 macrophages activate NNK by α-carbon hydroxylation and detoxify NNK by pyridine *N*-oxidation. Smith et al. recently demonstrated that freshly isolated human lung cells, including macrophages, activate and detoxify NNK with a similar metabolite distribution [37]. The NNK metabolite(s) responsible for the induction of TNF synthesis had not been identified. We hypothesized that the mutagenic alkylating intermediates generated by either of the two pathways of α-carbon hydroxylation of NNK are implicated. Hydroxylation of the methylene carbon adjacent to the *N*-nitroso group produces α-methylenehydroxy-NNK, an unstable intermediate which spontaneously decomposes to an oxo aldehyde and methane diazohydroxide, an agent methylating DNA (Fig. 1, reviewed in [15]). The second pathway of NNK activation is initiated by methyl hydroxylation and generates an oxo alcohol and 4-(3-pyridyl)-4-oxobutane-1-diazohydroxide, which pyridyloxobutylates DNA (Fig. 1) [15]. To assess the implication of these two alkylating intermediates in TNF induction, we used NNKOAc and NDMAOAc, which respectively generate the same DNA-pyridyloxobutylating and -methylating agents as NNK, exclusively and with high yield (Fig. 1) [15]. Here we observed that both NNKOAc and NDMAOAc induce soluble TNF production by U937 cells and, as expected, we observed that, on a weight basis, those analogs are more potent modulators of TNF production than NNK (Fig. 2). The higher rate of formation of alkylating agents from NNKOAc and NDMAOAc compared to NNK could explain the higher induction of TNF by these agents (Fig. 2). Approximately 5% of the initial amount of NNK is activated by α-carbon hydroxylation, while 90% of the NNKOAc is hydrolyzed into DNA-pyridyloxobutylating agents. We concluded that pyridyloxobutylating and methylating metabolites generated during bioactivation of NNK are potent inducers of TNF.

IL-10 is a potent anti-inflammatory molecule that inhibits the synthesis of proinflammatory cytokines, such as TNF, and prostaglandins in monocytes/macrophages [27]. IL-10 is also known to prevent the activation of NF-κB and the induction of TNF by lipopolysaccharide [36]. Here we observed that IL-10 synthesis was inhibited by NNK in U937 (Fig. 3). This result was expected since we had previously observed an activation of NF-κB and the induction of prostaglandin synthesis in NNK-treated U937 [33]. To determine the role of alkylating intermediates derived from NNK as potent inhibitors of IL-10 synthesis, we exposed U937 to NNKOAc and NDMAOAc. NDMAOAc strongly inhibited IL-10 production, while NNKOAc had a stimulatory effect at low concentrations and an inhibitory effect at high concentrations (Fig. 3). NNKOAc was the only drug to show a biphasic effect on IL-10, such a biphasic effect being quite common; for instance, Morisset et al. reported a biphasic regulation of IL-10 expression by TNF in bovine chondrocytes [28]. In addition to TNF induction, IL-10 inhibition further supports a role for NNK via its alkylating intermediates in the immunosuppression observed in smokers. Inhibition of IL-10 leads us to propose that other cytokines might be affected by the activation of NNK.

The biochemical pathways regulating cytokine production are complex: numerous transcription factors are implicated and multiple loops regulate cytokine production. Autocrine and paracrine interactions regulate cytokine synthesis within a single cell type. For instance, Lehmann showed that recombinant GM-CSF induces IL-10 expression in U937, and Evens et al. demonstrated that IL-6 expression was regulated by GM-CSF and TNF in murine macrophages [9, 24]. In this study, NNK treatment inhibited both GM-CSF and IL-6 release. GM-CSF is a pleiotropic cytokine that stimulates the proliferation, maturation and function of hematopoietic cells [10]. A major function of IL-6 is to stimulate B cell differentiation and antibody secretion [18]. The inhibition of IL-6 production by NNK could be one factor leading to the decrease in the number of IgM-secretory cells previously observed in A/J mice [30]. The inhibition of GM-CSF and IL-6 production by NNK might facilitate lung tumor development. MCP-1 is a CC chemokine that mediates monocyte and T lymphocyte infiltration in tissues [4]. MCP-1 release from U937 cells was inhibited by NNK treatment, suggesting that NNK inhibits recruitment and infiltration of immune cells at the tumor site and could disturb the activity of other immune cell types.

IL-2 is the primary growth factor for T lymphocytes and is a stimulator of natural killer cell activity [12]. NF-κB is involved in the regulation of IL-2 in T cells and IL-2 receptor α chain in macrophages [3]. IL-2 synthesis is also regulated by prostaglandin E_2 levels in T cells [26]. We previously observed that NNK induces prostaglandin E_2 synthesis in U937 [33]. We used Jurkat cells to determine whether NNK affects IL-2 release. Again, NNK and NNKOAc treatments were suppressive. NNK is not the only tobacco component to inhibit IL-2 synthesis; benzo[*a*]pyrene inhibits its synthesis in mouse splenocytes while *p*-benzoquinone, a component of cigarette tar, inhibits IL-2 release from human peripheral blood monocytes [11, 26]. Altogether, these

data suggest that NNK is one of the agents modulating the immune response of smokers and that NNK treatment might affect cells other than macrophages. NNK has a broad range of action on the immune system.

In conclusion, NNK modified the synthesis level of various cytokines to various degrees. These data, added to the previously observed inhibition of NK cell activity and its effect on the number of IgM-secretory cells in mice [30], suggest that NNK inhibits the host immune response. An imbalance in cytokine synthesis in human cells caused by the tobacco-specific carcinogen, NNK, which is present in tobacco smoke, might partially explain the immunosuppression observed in smokers. This inhibition of the host immunity might accelerate lung tumor development. In addition to its well-documented mutagenic potential, the immunomodulatory capacity of NNK could contribute to the high potency of this carcinogen.

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References

1. Arias-Negrete S, Keller K, Chadee K (1995) Proinflammatory cytokines regulate cyclooxygenase-2 mRNA expression in human macrophages. Biochem Biophys Res Commun 208: 582
2. Baeurle PA, Baltimore D (1996) NF-κB: ten years after. Cell 37: 13–20
3. Baeuerle PA, Henkel T (1994) Function and activation of NF-κB in the immune system. Annu Rev Immunol 12: 141
4. Baggiolini M, Dahinden CA (1994) CC chemokines in allergic inflammation. Immunol Today 15: 127
5. Braun KM, Cornish T, Valm A, Cundiff J, Pauly JL, Fan S (1998) Immunotoxicology of cigarette smoke condensates: suppression of macrophages responsiveness to interferon gamma. Toxicol Appl Pharmacol 149: 136
6. Brunnemann KD, Prokopczyk B, Djordjevic MV, Hoffmann D (1996) Formation and analysis of tobacco-specific *N*-nitrosamines. Crit Rev Toxicol 26: 121
7. Cloutier JF, Castonguay A (1998) Modulation of DNA repair by various inhibitors of DNA synthesis following 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) induced DNA damage. Chem Biol Interact 110: 7
8. Elgert KD, Alleva DG, Mullins DW (1998) Tumor-induced immune dysfunction: the macrophage connection. J Leukoc Biol 64: 275
9. Evens R, Schultz LD, Dranoff G, Fuller JA, Kambar SJ (1998) CSF-1 regulation of IL-6 gene expression by murine macrophages: a pivotal role for GM-CSF. J Leukoc Biol 64: 810
10. Gasson JC, Baldwin GC, Sakamoto KM, DiPersion JF (1990) The biology of human granulocyte-macrophage colony-stimulating factor (GM-CSF). Prog Clin Biol Res 352: 375
11. Geiselhart LA, Christian T, Minnear F, Freed BM (1997) The cigarette tar component *p*-benzoquinone blocks T-lymphocyte activation by inhibiting interleukine-2 production, but not CD25, ICAM-1, or LFA-1 expression. Toxicol Appl Pharmacol 143: 30
12. Gesbert F, Delespine-Carmagnat M, Bertoglio J (1998) Recent advances in the understanding of interleukin-2 signal transduction. J Clin Immunol 18: 307
13. Goud SN, Kaplan AM (1999) Inhibition of natural killer cell activity in mice treated with tobacco specific carcinogen NNK. J Toxicol Environ Health 56: 131
14. Griffin GE, Leung K, Folks TM, Kunkel S, Nabel GJ (1989) Activation of HIV gene expression during monocyte differentiation by induction of NF-kappaB. Nature 339: 70
15. Hecht SS (1998) Biochemistry, biology, and carcinogencity of tobacco-specific *N*-nitrosamines. Chem Res Toxicol 11: 559
16. Hecht SS, Young GD, Chen CB (1980) Metabolism in the F344 rat of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, a tobacco-specific carcinogen. Cancer Res 40: 4144
17. Higashimoto Y, Fukuchi Y, Ishida K, Shimada Y, Ohata M, Funasako M, Shu C, Teramoto S, Matsuse T, Sudo E, Orimo H (1994) Effect of chronic tobacco smoke exposure on the function of alveolar macrophages in mice. Respiration 61: 23–27
18. Hirano T, Akira S, Taga T, Kishimoto T (1990) Biological and clinical aspects of interleukin 6. Immunol Today 11: 443
19. Hoffmann D, Rivenson A, Hecht SS (1996) The biological significance of tobacco-specific *N*-nitrosamines: smoking and adenocarcinoma of the lung. Crit Rev Toxicol 26: 199
20. Holt PG (1987) Immune and inflammatory function in cigarette smokers. Thorax 42: 241
21. Janssen-Heininger YM, Macara I, Mossman BT (1999) Cooperativity between oxidants and tumor necrosis factor in the activation of nuclear factor (NF)-kappaB. Requirement of ras/mitogen-activated protein kinases in the activation of nf-kappaB by oxidants. Am J Respir Cell Mol Biol 20: 942
22. Jorquera R, Castonguay A, Schuller HM (1992) Effects of pregnancy and ethanol treatment on the metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone by hamster liver and lung microsomes. Drug Metab Dispos 20: 510
23. Kim PM, Wells PG (1996) Genoprotection by UDP-glucuronosyltransferases in peroxidase-dependent, reactive oxygen species-mediated micronucleus initiation by the carcinogens 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and benzo[*a*]pyrene. Cancer Res 56: 1526
24. Lehmann MH (1998) Recombinant human granulocyte-macrophage colony-stimulating factor triggers interleukin-10 expression in the monocytic cell line U937. Mol Immunol 35: 479
25. Lockwood JF, Myers MJ, Rutherford MS, Shook LB (1991) Transcriptional changes in macrophage TNF-alpha expression following dimethylnitrosamine exposure in vivo. Immunopharmacology 22: 27
26. Lyte M, Blanton RH, Myers MJ, Bick PH (1987) Effect of in vivo administration of the carcinogen benzo(a)pyrene on interleukine-2 and interleukine-3 production. Int J Immunopharmacol 9: 307
27. Moore KW, O'Garra A, deWaal Malefyt R, Vieira P, Mosmann TR (1993) Interleukin-10. Annu Rev Immunol 11: 165
28. Morisset S, Patry C, Lora M, Brum-Fernandes AJ de (1998) Regulation of cyclooxygenase-2 expression in bovine chondrocytes in culture by IL-1, TNF, glucocorticoids and 17-estradiol. J Rheumatol 25: 1146
29. Peterson LA, Carmella SG, Hecht SS (1990) Investigation of metabolic precursors to hemoglobin and DNA adducts of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. Carcinogenesis 11: 1329
30. Rioux N, Castonguay A (1997) Recovery from 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induce immunosuppression in A/J mice by treatment with nonsteroidal anti-inflammatory drugs. J Natl Cancer Inst 89: 874
31. Rioux N, Castonguay A (1998) Prevention of NNK-induced lung tumorigenesis in A/J mice by acetylsalicylic acid and NS-398. Cancer Res 58: 5354
32. Rioux N, Castonguay A (1998) Inhibitors of lipoxygenase: a new class of cancer chemopreventive agents. Carcinogenesis 19: 1393
33. Rioux N, Castonguay A (2000) The induction of cyclooxygenase-1 by a tobacco carcinogen in U937 human macrophages is correlated to the activation of NF-κB. Carcinogenesis 21: 1745
34. Schook LB, Lockwood JF, Yang SD, Myers MJ (1992) Dimethylnitrosamine(DMN)-induced IL-1 beta, TNF-alpha, and

IL-6 inflammatory cytokine expression. Toxicol Appl Pharmacol 116: 110
35. Seyedroudbari SA, Khan MM (1998) In vitro effects of smokeless tobacco extract on tumor necrosis factor-alpha (TNF-alpha) and interleukin-1beta (IL-1beta) production, and on lymphocyte proliferation. Toxicon 36: 631
36. Shames BD, Selzman CH, Meldrum DR, Pulido EJ, Barton HA, Meng X, Harken AH, McIntyre RC Jr (1998) Interleukin-10 stabilizes inhibitory kappaB-alpha in human monocytes. Shock 10: 389
37. Smith GBJ, Castonguay A, Donnelly PJ, Reid K, Petsikas D, Massey TE (1999) Biotransformation of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in freshly isolated human lung cells. Carcinogenesis 20: 1809
38. Sopori ML, Kozak W (1998) Immunomodulatory effects of cigarette smoke. J Neuroimmunol 83: 148
39. Sundstrom C, Nilsson K (1976) Establishment and characterization of a human histiocytic lymphoma cell line (U937). Int J Cancer 17: 565
40. Vassalli P (1992) The pathophysiology of tumor necrosis factors. Annu Rev Immunol 10: 411
41. Vassyer M, Favatier F, Pinot F, Bachelet M, Polla BS (1998) Tobacco smoke induces coordinate activation of HSF and inhibition of NFkappaB in human monocytes: effects on TNFalpha release. Biochem Biophys Res Commun 9: 249–256
42. Wang P, Wu P, Siegel MI, Egan RW, Billah MM (1995) Interleukin (IL)-10 inhibits nuclear factor κB (NF-κB) activation in human monocytes. J Biol Chem 270: 9558