

Induced production of nitric oxide, tumor necrosis factor, and interleukin-6 in RAW 264.7 macrophages by streptomycetes from indoor air of moldy houses.

INDOOR BIOAEROSOLS are important health factors, and they can cause asthma, bronchitis, repetitive respiratory tract infection, and rhinitis.[1, 2] The tip of the iceberg is seen in moldy houses in which a variety of fungi and bacteria, especially actinomycetes, are grown; this growth occurs from the excess moisture in structures after accidental leakage or from gradual condensation of water.[3] Investigators have not described satisfactorily the most important causative microbes among the mixed population. In addition, almost nothing is known about the cellular mechanisms, even if the clinical entity associated with exposure has been described and the exposure levels to the most prevalent bacteria and fungi present in moldy houses have been characterized.[4,5] We hypothesized that a likely contributing factor in the etiological mechanism of respiratory symptoms could be an inflammatory response toward specific organic materials in the microbes. This mechanism would include production of inflammatory mediators, such as cytokines and nitric oxide (NO), in immunologically active cells.

Cluzel et al.[6] reported that cytokines are well-known mediators, which play an important physiological role in the functions of the immune system. Activated macrophages secrete a variety of biologically active substances (e.g., eicosanoids, oxygen radicals, cytokines [including tumor necrosis factor alpha {TNF[Alpha]} and interleukin-6 {IL-6}]). Moreover, it is now evident that the highly reactive radical, nitric oxide, is an important mediator in nonspecific host defense against microbes and tumors.[7,8] Recently, investigators have focused much interest on the possible role of NO as an immune defense molecule.[9] Researchers have suggested that NO is produced by activated macrophages via a nonspecific immune response directed against invading microorganisms. The biological source of NO is L-arginine, which is converted to L-Citrulline and NO in macrophages by inducible NO synthase (iNOS).[10] Under normal steady-state conditions, iNOS is not detectable, but it is induced within a few hours after stimulation by lipopolysaccharide (LPS) and various cytokines (e.g., interferon-gamma [INF[Gamma]], TNF[Alpha]).[10] Thus, macrophage-derived NO and cytokines function as cytotoxic molecules, which can kill invading microorganisms,[11] but they also have important roles in the functions of the immune system and in the pathophysiology of inflammatory diseases[12] and asthma.[13]

In this study, we investigated whether any of the microbial strains typically present in the indoor air of moldy houses (i.e., *Candida* sp., *Stachybotrys* sp., and *Streptomyces* sp.), or strains belonging to the normal flora of indoor air (i.e., *Penicillium* sp., *Cladosporium* sp., and *Aspergillus* sp.), can induce the production of proinflammatory cytokines TNF[Alpha] and IL-6 or activate iNOS with subsequent NO production in RAW 264.7 macrophage cell line.

Material and Method

Materials. We obtained mouse macrophage cell line RAW 264.7 (RAW) from American Type Tissue Collection (Rockville, Maryland). We grew it on six well plates at 37 [degrees] c (5% [co.SUB.2]) IN rpmi MEDIUM 1640 (GIBCO LAB [Grand Island, New York]), which was supplemented with 10% fetal bovine serum (Hyclone Lab [Logan, Utah]), 1% L-glutamine, and 1% PNS antibiotic mixture (both from Gibco Lab). We obtained sulfanilamide, naphthylethylenediamine di hydrochloride, and 5-bromo-

4-chloro-3-idolylphosphate/nitrobluetetrazolium (BCIP/NBT) from Sigma Chemical Co. (St. Louis, Missouri). In addition, we obtained rabbit monoclonal 4195 antibody against mouse macrophage iNOS from Burroughs Wellcome (North Carolina) and antirabbit IgG from Jackson Immune Research Lab (West Grove, Pennsylvania).

Isolation, identification, and preparation of fungi and bacteria. We used five strains of fungi (i.e., *Candida* sp., *Aspergillus* sp., *Cladosporium* sp., *Penicillium* sp., and *Stachybotrys* sp.) and two strains of mesophilic gram-positive bacteria (i.e., actinomycetes A4 and A91, representing the genus *Streptomyces*). We isolated the strains from indoor samples of mold problem houses, as described by Hyvarinen et al. in 1993.[3] We used light microscopy[14,15] to identify the four strains of fungi (*Aspergillus* sp., *Cladosporium* sp., *Penicillium* sp., and *Stachybotrys* sp.) morphologically. One of the fungal strains was identified as a yeast, *Candida albicans*, by use of the API ID32C test kit. The actinomycete strains were identified in Deutsche Sammlung von Micro-organismen und Zellkulturen GmbH as *Streptomyces anulatus* (A91) and *Streptomyces californicus* M).

We cultured the fungal strains on malt extract agar (MEA; Blakeslee) and the strains of actinomycetes on Trypton yeast-glucose agar (Bacto Plate Count agar, Oxoid). The cultures were incubated in the dark at 20-23 [degrees] C for 7 d. The spore suspensions from these cultures were prepared in a phosphate buffered saline (PBS) medium that contained 0.0001% of Triton X-100.

Treatment of RAW 264.7 cells. We incubated the RAW 264.7 macrophages for 24 h in a fresh medium containing 0, 1 x [10.sup.5], 5 x [10.sup.5], 1 x [10.sup.6], 5 x [10.sup.6], or 1 x [10.sup.7] spores/[10.sup.6] cells of *Candida* sp., *Aspergillus* sp., *Cladosporium* sp., *Penicillium* sp., *Stachybotrys* sp., or *Streptomyces* sp. A4 or A91. We studied the effects of spores on cell viability, nitrite production, expression of iNOS, and production of TNF[Alpha] and IL-6.

Nitrite analysis. Nitrite produces a chromophore with the Griess reagent, with an absorbance maximum at 543 nm, which can then be quantitated spectrophotometrically[16] with an automated colorimetric procedure. Briefly, we added 50 [micro]l of cell culture medium to each well of a 96-well plate. We added 50 Jai of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2% phosphoric acid) and left the plate shaking for 10 min at room temperature. We used a microplate reader (Labsystems iEMS Reader MF) to measure the [OD.sub.540]nm. To calculate nitrite concentrations, we compared them with standard solutions of sodium nitrite produced in the culture medium.

Western blot analysis. The cells were washed with cold PBS, lysed in lysis buffer (50 mM Tris-hydrochloric acid (Tris-HCl), 1 mM ethylenediaminetetra acid (EDTA), 10 [micro]mol PMSF, and 10 [micro]l/ml leupeptin), and resuspended on ice for 10 min. The cell suspension was lysed further via ultrasound sonicator, and it was centrifuged at 1 400 rpm for 15 min. At the completion of this procedure, 4x sample buffer was added to the supernatant and heated at 95 [degrees] C for 10 min. Lysates (20-[micro]g protein) were subjected to electrophoresis via 7.5% SDS-PAGE, and proteins were transferred electrophoretically to a nitrocellulose filter. The filters were incubated overnight at 4 [degrees] C in blocking buffer (50 mM Tris-HCl, 150 mM sodium chloride [NaCl], 1 mg/ml PEG 2000, 3% BSA). The filters were then incubated for 2 h at room temperature in a washing buffer (50 mM Tri-HCl, 150 mM NaCl, 1 mg/ml PEG 2000, 1 mg/ml BSA) that contained a dilution of 1:500 of rabbit monoclonal antibody 4195 obtained against the N-terminal fragment of mouse macrophage iNOS.[17] Three 10-min washes in washing buffer were completed, after which the filters were incubated

for 1 h at room temperature in a washing buffer containing a 1:2 000 dilution of alkaline-phosphatase-conjugated anti-rabbit IgG. Three 10-min washes in a washing buffer were completed and were followed by exposure of the filters to alkaline phosphatase developing buffer (100 mM NaCl, 100 mM Tris-Base, 5 mM magnesium chloride [MgCl], pH = 9.5) for 2 min; the filters were developed with BCIP/NBT.

Analysis of TNF[Alpha] and IL-6. We used 100 [micro]l/well of antibody solution in 0.1 M sodium carbonate buffer (0.1 M Na[HCO.sup.3], pH adjusted to 9.6 with 0.1 M [Na.sub.2][CO.sub.3]) to coat the microtiter strips with a monoclonal antibody for IL-6 (1 [micro]g/ml) or TNF[Alpha] (2 [micro]g/ml). The strips were shaken at 200 rpm for 2 h at room temperature. Three washings were completed, after which we blocked nonspecific binding by incubating the wells with 200 [micro]l of 1% BSA in 50 mM Tris-HCl buffer containing 150 mM NaCl (pH = 7.5) at room temperature for 1 h; the strips were again washed three times. The samples and standards, diluted in the [DELFIA.sup.R] assay buffer, were added to the strips (200 [micro]l/well) and incubated overnight at +4 [degrees] C. The strips were washed three times, and the biotinylated second antibody (250 ng/ml of IL-6 and 500 ng/ml of TNF[Alpha] was added to the wells in 200 [micro]l of [DELFIA.sup.R] assay buffer. The strips were incubated for 1 h at room temperature, after which they were washed once, and 200 [micro]l of Europium-labelled streptavidin (100 ng/ml) in [DELFIA.sup.K] assay buffer was dispensed to the wells. The strips were incubated at room temperature at 30 rpm shaking and were washed six times. Europium was rendered fluorescent by releasing it from the streptavidin with [DELFIA.sup.K] enhancement solution (200 [micro]l/well) and by shaking the strips gently for 5 min. After 10-15 min of equilibration, a LKB Wallac 1230 Arcus fluorometer[18] used the spline smoothed algorithm of the RIA Calc software to measure fluorescence.

Cell viability. We calculated the percentage of macrophages alive, after dying the cells with Trypan Blue solution, to measure culture viability.

Statistical analysis. We used one-way analysis of variance and Duncan's multiple-range test to analyze the data statistically. The accepted level of statistical significance was p [is less than] .05.

Results

Nitrite production and expression of iNOS. Nitric oxide production in RAW 264.7 macrophages was assayed in the culture medium as the stable NO oxidation product, nitrite, and it was significantly and close dependently increased at 24 h, by all the tested doses of *Streptomyces anulatus* (A91) and *Streptomyces californicus* (M). However, only the two highest doses of *Stachybotrys* sp., *Candida albicans* sp., and *Cladosporium* sp. slightly increased the nitrite levels in the culture medium, whereas *Aspergillus* sp. and *Penicillium* sp. did not affect NO production (compared with controls [Fig. 1]).

[Figure 1 ILLUSTRATION OMITTED]

To rule out NO production induced by endotoxin contamination in the two strains of actinomycetes (*Streptomyces* sp. A4 and A91), we also used a spectrophotometer method to test spore samples with the *Limulus* Amebocyte Lysate test. Both strains were free of endotoxin contamination (i.e., [is less than] 0.005 ng/[10.sup.6] cells).

To definitely demonstrate the different ability of the tested microorganisms to induced NO production in RAW 264.7 macrophages, we also analyzed expression of iNOS. Consistent with the nitrite levels in the culture medium, Western Blot analysis (with antibody against iNOS [1 30 kDa])

revealed that the expression of iNOS was induced only by *Streptomyces* A91 and A4, but not by any of the tested fungal strains (Fig. 2)

[Figure 2 ILLUSTRATION OMITTED]

Production of TNF[Alpha] and IL-6. The RAW 264.7 macrophages stimulated by *Streptomyces* A91 or A4 produced dose-dependent high amounts of TNF[Alpha] at 24 h. In addition, the production of TNF[Alpha] was increased by the highest dose of *Penicillium* sp. and *Cladosporium* sp., compared with controls (Fig. 3A).

[Figure 3A ILLUSTRATION OMITTED]

Incubation of the cells with the graded concentrations of spores of *Streptomyces* A4 or A91 induced a massive dose-dependent accumulation of IL-6 in the culture medium of RAW 264.7 macrophages. All the other microorganisms tested had only a minor effect on IL-6 production, compared with controls (Fig. 3B).

[Figure 3B ILLUSTRATION OMITTED]

Cell viability. We explored the viability of RAW 264.7 macrophages after they were stimulated for 24 h with the tested microorganisms to determine whether the alterations in endogenous NO, TNF[Alpha], or IL-6 production were associated with a loss of cell viability. Only *Stachybotrys* sp. induced a significant dose-dependent decrease in cell viability (i.e., down to 8%) in 24 h, whereas the viability among control cells was 97%. The other strains of fungi and both strains of actinomycetes (*Streptomyces* A4 and A91) either did not appreciably affect cell viability in the culture or decreased viability only slightly at the highest dose (Fig. 4).

[Figure 4 ILLUSTRATION OMITTED]

Discussion

The results of the present study indicate that gram positive bacteria *Streptomyces californicus* (M) and *Streptomyces anulatus* (A91), isolated from the indoor air of moldy buildings, stimulate macrophages, which produce significant amounts of TNF[Alpha] and IL6, and induce the expression of iNOS with subsequent production of NO. However, under the present experimental situation, NO-producing macrophages did not induce cytotoxicity. Interestingly, the other microorganisms tested, also typical to moldy buildings, were without any effect or, at the most, caused only a slight increase in the production of inflammatory mediators.

Streptomyces-induced production of NO in macrophages is of particular interest because recently Barnes et al.[19] reported that NO was produced by a variety of cells in the airways, suggesting that respiratory tract infections may induce iNOS expression.[20] Consistent with this notion, a marked increase in the concentration of exhaled NO was described in asthmatic patients[21,22]; perhaps this resulted from iNOS induction in airway epithelial cells and inflammatory cells (e.g., macrophages). Sustained production of high amounts of NO by iNOS may exert proinflammatory effects, including vasodilatation, edema, cytotoxicity, and induction of cytokine-dependent processes.[10]

In this study, we showed that gram-positive bacteria *Streptomyces* sp. A4 and A91 induced the expression of iNOS and subsequent production of NO in the same manner as was reported by Moncada et al.[23] and Hirvonen et al.[26] for exposure to interferon-gamma ([INF.sub.[Gamma]]) and endotoxin LPS, which is an important factor in the cytotoxicity induced by gram-negative bacteria. *Streptomyces* sp. A4 and A91 induced

expression of iNOS, without prior priming of the macrophages, and this is contrary to the general assumption that iNOS-inducing agents do not act alone but, rather, in combination with [INF.sub.[Gamma]].[24] 14 Moreover, neither of the strains of *Streptomyces* sp. was cytotoxic to NO-producing macrophages themselves, whereas in earlier studies by others[25] and us,[26] it was suggested that the life span of macrophages, stimulated by LPS and/or [iNF.sub.[Gamma]], correlated negatively with their NO production. Only *Stachybotrys* sp. induced a significant dose-dependent decrease in cell viability, but it was not related to NO production of the cells; therefore, perhaps the cellular mechanisms behind the health effects induced by *Stachybotrys* sp. are different from those induced by *Streptomyces* sp. These results are very interesting, because researchers have speculated that NO is one of the cytotoxic agents by which activated macrophages kill bacteria, tumor cells, and a variety of other pathogens--even normal tissue cells during autoimmune reactions.[27] Therefore, whereas NO plays an important immunological, defensive role in the killing of foreign organisms, in acute inflammation its excessive production may cause tissue damage.[28]

During our search of the plausible cascade of events that lead to iNOS expression and NO production in macrophages stimulated by gram-positive bacteria A4 and A91 *Streptomyces* sp., we also studied the production of proinflammatory cytokines TNF[Alpha] and IL-6. Moncada et al.[23] reported that these cytokines are released by other iNOS expression-inducing agents (e.g., LPS). Both of the strains we tested induced significant release of TNF[Alpha] and IL-6 in cell culture medium, thereby suggesting that some components of gram-positive bacteria may also be effective inducers of these mediators. All the other microorganisms that we isolated from moldy buildings were without effect or, at most, caused only a slight increase in IL-6 and TNF[Alpha] production. Increased levels of TNF[Alpha] were associated with elevated levels of IL-6 in macrophages activated by *Streptomyces* sp. This finding is supported by the results of Bauman et al.,[29] who speculated that TNF[Alpha] is important in the initiation of events that cause the release of other cytokines (e.g., IL-6). Moreover, these results are consistent with the assumptions that (a) cytokines are powerful stimulators of the iNOS pathway in many cell types,[30-32] and (b) lysis of target cells by activated macrophages can be explained by the actions of cytokines and NO.

The present findings also shed new light on previous results from our laboratory (i.e., A4 and A91 *Streptomyces* sp. induced a marked increase in the production of reactive oxygen metabolites [ROM] in human polymorphonuclear leukocytes [PMNLs][33]). Reactive oxygen metabolites may significantly increase the cytotoxicity of NO, because NO readily reacts with superoxide to generate a stable peroxy nitrite anion, which, once protonated, decomposes to form an extremely reactive hydroxyl radical.[34] Both of these radicals are strong oxidants, which may be involved in tissue damage and in the cytotoxicity attributable to NO.[34] Investigators have assumed that the reaction of NO with superoxide anion plays a pivotal role in acute and chronic lung disease, because proxynitrite induces lipid peroxidation and interferes with several enzymatic pathways.[35,36] Moreover, given that IL-6 primes respiratory burst in neutrophils and monocytes,[37] our present observation of massive production of IL-6 upon stimulation of macrophages by gram-positive bacteria is consistent with the finding of Ruotsalainen et al.,[33] who reported increased production of ROM in PMNLs.

Therefore, it appears that spores of *Streptomyces* sp., which are present in the indoor air of moldy buildings and are sufficiently small (1 [micro]m) to reach the alveoli, can be added to the list of stimuli that induce production of cytokines and NO in murine macrophages. If such production occurs in vivo, cytokines and NO may indeed play a role in the responses evoked by exposure to these microbes.

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