Mycobacterium terrae Isolated from Indoor Air of a Moisture-Damaged Building Induces Sustained Biphasic Inflammatory Response in Mouse Lungs

Juha Jussila,[1] Hannu Komulainen,[1] Kati Huttunen,[1] Marjut Roponen,[1] Eila Iivanainen,[2] Pirjo Torkko,[2,3] Veli-Matti Kosma,[4] Jukka Pelkonen,[5,6] and Maija-Riitta Hirvonen[1]

[1]Laboratory of Toxicology, and [2] Laboratory of Environmental Microbiology, National Public Health Institute, Kuopio, Finland; [3]Department of Clinical Microbiology, Kuopio University Hospital, Kuopio, Finland; [4]Department of Pathology and Forensic Medicine, University of Kuopio and Kuopio University Hospital, Kuopio, Finland; [5]Department of Clinical Microbiology, University of Kuopio, Kuopio, Finland; [6]Department of Pediatrics, Kuopio University Hospital, Kuopio, Finland

Occupants in moisture-damaged buildings suffer frequently from respiratory symptoms. This may be partly due to the presence of abnormal microbial growth or the altered microbial flora in the damaged buildings. However, the specific effects of the microbes on respiratory health and the way they provoke clinical manifestations are poorly understood. In the present study, we exposed mice via intratracheal instillation to a single dose of *Mycobacterium terrae* isolated from the indoor air of a moisture-damaged building (1×10^7, 5×10^7, or 1×10^8 microbes). Inflammation and toxicity in lungs were evaluated 2 hr later. The time course of the effects was assessed with the dose of 1×10^8 bacterial cells for up to 28 days. *M. terrae* caused a sustained biphasic inflammation in mouse lungs. The characteristic features for the first phase, which lasted from 6 hr to 3 days, were elevated proinflammatory cytokine [i.e., tumor necrosis factor α (TNFα) and interleukin-6 (IL-6)] levels in the bronchoalveolar lavage fluid (BALF). TNFα was produced in the lungs more intensively than was IL-6. Neutrophils were the most abundant cells in the airways during the first phase, although their numbers in BALF remained elevated up to 21 days. The characteristics of the second phase, which lasted from 7 to 28 days, were elevated TNFα levels in BALF, expression of inducible nitric oxide synthase in BAL cells, and recruitment of mononuclear cells such as lymphocytes and macrophages into the airways. Moreover, total protein, albumin, and lactate dehydrogenase concentrations were elevated in both phases in BALF. The bacteria were detected in lungs up to 28 days. In summary, these observations indicate that *M. terrae* is capable of provoking a sustained, biphasic inflammation in mouse lungs and can cause a moderate degree of cytotoxicity. Thus, *M. terrae* can be considered a species with potential to adversely affect the health of the occupants of moisture-damaged buildings. *Key words:* bronchoalveolar lavage, cytokines, inflammation, intratracheal instillation, lung, moisture-damaged building, *Mycobacterium terrae*, nitric oxide. *Environ Health Perspect* 110:1119–1125 (2002). [Online 19 September 2002]

http://ehpnet1.niehs.nih.gov/docs/2002/110p1119-1125jussila/abstract.html

Microbial growth in moisture-damaged buildings has been associated with adverse health effects, such as frequent respiratory infections, rhinitis, eye irritation, and asthma (Brunekreef 1992; Koskinen et al. 1999; Pirhonen et al. 1996; Platt et al. 1989; Spengler et al. 1994; Strachan 1988; Taskinen et al. 1997; Waegemaekers et al. 1989). Even serious effects, such as idiopathic pulmonary hemorrhage in infants, have been suspected to be caused by excessive microbial growth in moisture-damaged buildings (Dearborn et al. 1999; Flappan et al. 1999; Hagmann 2000). Lungs represent a very important route of microbial exposure in the occupants of moisture-damaged buildings. However, the responsible microbes and the specific mechanisms of the effects on the respiratory tract leading to clinical symptoms are still unknown. This is particularly true for environmental mycobacteria growing in moisture-damaged buildings.

Mycobacteria are present in the building materials of moisture-damaged buildings (Andersson et al. 1997), and they may spread into the indoor air, especially during demolition work. *Mycobacterium terrae* is an environmental, slow-growing mycobacterium, usually considered to be nonpathogenic to humans (Goodfellow and Magee 1998; Shimizu et al. 1999). However, some cases of mycobacterial infections in lungs caused by this microbe have been diagnosed among both immunocompromised patients and patients with normal immunologic status (Krisher et al. 1988; Kuze et al. 1983; Palmero et al. 1989; Peters and Morice 1991; Spence and Ferris 1996). For example, *M. terrae* has provoked lymphadenitis in a nonimmunocompromised host (Shimizu et al. 1999). We have previously shown that *M. terrae,* isolated from indoor air of a moisture-damaged building, is exceptionally potent in increasing the production of the inflammatory mediators tumor necrosis factor α (TNFα), interleukin-6 (IL-6), and nitric oxide in mouse macrophages *in vitro* (Huttunen et al. 2000). IL-6 and TNFα are proinflammatory cytokines and endogenous pyrogens (Janeway and Travers 1997), and NO is an important immunologic component of host defense as well (Clancy and Abramson 1995; Hibbs et al. 1987; Jorens et al. 1993; Moncada et al. 1991). NO possesses microbicidal activity and participates in nonspecific immune responses (Jorens et al. 1993), but excessive NO production is involved in several pathologic conditions, such as asthma (Barnes and Kharitonov 1997) and some autoimmune diseases (Kolb and Kolb-Bachofen 1992). Macrophages and epithelial cells in lungs are able to produce large amounts of NO via inducible NO synthase (iNOS) after presentation of an appropriate stimulus (Asano et al. 1994; Moncada et al. 1991).

To study the pulmonary effects of *M. terrae*, isolated originally from indoor air of a moisture-damaged building, we instilled the bacteria as a single intratracheal dose into mouse lungs. Both the time course and the dose response in inflammatory and cytotoxicity markers were evaluated.

Materials and Methods

Exposures. *M. terrae* was recovered from the indoor air of a moldy building during demolition work (Rautiala et al. 1996). The isolate, encoded as BA26 in our previous *in vitro* studies (Huttunen et al. 2000), was identified by gas liquid chromatography of the fatty acids, fatty alcohols, and mycolic acid cleavage products and by its growth and biochemical characteristics. Moreover, the result was negative when the isolate was tested with a commercial DNA probe specific for *Mycobacterium avium* complex (AccuProbe; Gen-Probe Inc., San Diego, CA, USA), as described previously (Torkko et al. 1998). The microbe was cultured on Mycobacteria 7H11 agar supplemented with oleic acid-albumin-dextrose-catalase enrichment (Difco Laboratories, Detroit, MI,

Address correspondence to J. Jussila, Laboratory of Toxicology, National Public Health Institute, P.O. Box 95, FIN-70701 Kuopio, Finland. Telephone: 358 17 201320; Fax: 358 17 201265. E-mail: Juha.Jussila@ktl.fi

We thank L. Heikkinen, H. Martikainen, M. Ojainväli, A. Rönkkö, A. Seppä, and I. Väänänen for their excellent technical assistance. We also thank A. Naukkarinen for consultation on mycobacterial staining and E. MacDonald for reading and commenting on the manuscript.

This study was supported by the Finnish Research Programme on Environmental Health, Academy of Finland.

Received 14 January 2002; accepted 29 March 2002.

USA) at 30°C for 5 weeks. Then the bacterial cells were washed, suspended in Hank's balanced salt solution (HBSS) (Gibco, Paisley, UK), and sonicated in a water bath (Ultrasonic, Lahti, Finland) for 15 min. The microbe concentration was counted with an epifluorescence microscope by using the acridine orange staining method adjusted for mycobacteria (Hobbie et al. 1977; Katila and Mäntyjärvi 1982). Before dosing, the cell suspension was sonicated in a water-bath sonicator on ice for 40 min to ensure a single cell suspension.

Animals. Specific pathogen-free male NIH/S mice (8–9 weeks old) were obtained from the breeding colony of the National Public Health Institute, Division of Environmental Health (Kuopio, Finland). They were transferred from the barrier unit to a conventional animal room and housed singly in metal cages on aspen wood chips (FinnTapvei, Kaavi, Finland) 1 week before the experiment. Animals received water and food (R36 Maintenance Diet for rats and mice, Lactamin, Stockholm, Sweden) *ad libitum*. The mice were on a 12-hr light/dark cycle (lights on 700 hr–1900 hr) at 21°C and 27–38% relative humidity. The experimental protocols were approved by the Research Animal Committee of the University of Kuopio and the Provincial State Office.

Experimental design. The dose dependence of the effects induced by the microbe was investigated by exposing mice (10 animals/group) to a single dose of *M. terrae* (1×10^7, 5×10^7, or 1×10^8 microbes/animal) or HBSS (carrier control; Gibco) for 24 hr by intratracheal instillation (50 µL/animal) under 6% sevoflurane (Sevorane; Abbot, Abbott Park, IL, USA) anesthesia. The time course of the effects was studied at 6 and 24 hr and at 3, 7, 14, 21, and 28 days after the exposure (10 animals/group) to a single dose of 1×10^8 cells of *M. terrae* or HBSS.

Exposure to microbes. The bacteria were instilled into mouse lungs as described previously (Jussila et al. 2001). In brief, an anesthetized mouse was laid in a 66° upward posture and the incisors were placed on a wire. The dosing was performed under visual control by using a cold-light source (KL 1500 electronic; Schott, Mainz, Germany) placed against the throat. The nostrils were blocked, the tongue was extended, and the dose was delivered onto the vocal folds with a Finn pipette tip.

Sample collection. At the indicated exposure times, the mice were anesthetized with pentobarbital (100 mg/kg) and exsanguinated by cardiac puncture. Blood was collected and serum separated (Capiject T-MG; Terumo, Elkton, MD, USA) for cytokine analyses. Lungs (filled with 10% phosphate-buffered formalin), liver, spleen, and lymph nodes (mediastinal, axillary, brachial, and both superficial and deep cervical nodes) of three or four mice of each group were preserved in 10% phosphate-buffered formalin for histopathologic evaluation. The tracheas of the remaining animals (six or seven per group) were cannulated with polyethylene tubing. At first, lungs were lavaged with two portions of sterile HBSS (without Ca^{2+} and Mg^{2+}, containing 10 mM D-glucose, 37°C, 30 mL/kg), three times with each, and these two portions were combined. Thereafter, to obtain a greater number of cells, the lungs were further lavaged with six portions of HBSS as before, and these portions were combined together. Lavage fluids of these two sets were kept on ice. Cytospin was performed with the first set of lavages (200 µL, 500 rpm for 10 min; Megafuge; Heraus Instruments, Hanau, Germany). Slides were fixed in methanol and stained with May-Grünwald–Giemsa dye. Depending on the number of cells on the slide, 16 or 24 fields were counted to define the cell differentials. Cells were separated from the first two lavages by centrifugation (540 × *g* for 8 min), and the supernatant was centrifuged once more (13,700 × *g* for 6 min at 4°C) to discard all red blood cells and cell debris. Lactate dehydrogenase (LDH) concentrations were analyzed in fresh supernatants. The rest of the supernatants were frozen (–80°C) for analyses of TNFα, IL-6, albumin, and total protein.

Cells of the first two lavages were resuspended in 200 µL HBSS (without Ca^{2+} and Mg^{2+}, containing 10 mM D-glucose), and the total cell number of each sample [cells per milliliter bronchoalveolar lavage fluid (BALF)] was counted by the Trypan blue exclusion method. The rest of the cell suspension was centrifuged (540 × *g* for 8 min), and after discarding the media, red blood cells were hemolyzed from the cell pellet by hypotonic shock. The cells were incubated for 30 sec with 0.2% NaCl solution, and the suspension was adjusted to be isotonic. After centrifugation (540 × *g* for 8 min), supernatant samples were collected for hemoglobin assay. Cells of the other set of lavages were centrifuged (540 × *g* for 8 min at 4°C), the buffer discarded, and red blood cells hemolyzed as described above. The cells were combined with the surplus cells from the first set of lavages, pelleted (540 × *g* for 8 min at 4°C), and frozen for later Western blot analyses of iNOS.

Analysis of IL-6 and TNFα. Cytokines were analyzed from BALF and serum using the enzyme-linked immunosorbent assay (ELISA) method. Antibody pairs were obtained from R&D Systems (Minneapolis, MN, USA), and analyses were performed according to the manufacturer's instructions. The concentrations of monoclonal capture antibody were 1 µg/mL phosphate-buffered saline (PBS) for IL-6 and 0.8 µg/mL PBS for TNFα; concentrations of biotinylated second antibody were 0.2 µg/mL for IL-6 and 0.3 µg/mL for TNFα. We used 96-well microtiter plates (Maxisorb; Nunc, Naperville, IL, USA). The standards were diluted in diluent [0.1% bovine serum albumin (BSA), 0.05% Tween-20 in Tris-buffered saline, pH 7.3 (20 mM Trizma base, 150 mM NaCl)] for the analyses of BALF samples, and in NIH/S mouse serum collected from animals of our stock for serum analyses. Horseradish peroxidase (HRP)-conjugated streptavidin was used as a concentration of 1:200. Tetramethyl benzidine (TMB) substrate solution (TMB Single Solution; Zymed, San Francisco, CA, USA) was incubated with samples and standards for 20 min. The absorbances were measured by ELISA reader (iEMS Reader MF; Labsystems, Helsinki, Finland) at the wavelength of 450 nm.

Analysis of LDH, total protein, albumin, and hemoglobin. We analyzed the LDH concentration in BALF using the Cytotoxicity Detection Kit (Boehringer Mannheim, GmbH, Mannheim, Germany) with minor modifications. Briefly, standards and BALF samples (diluted 1:4 to lavage buffer) were pipetted to 96-well MicroWell plates (Nunc, Roskilde, Denmark) in duplicate, and the mixture of the catalyst and the dye solution was added into the wells. Reactions were terminated by addition of stop solution (1 M HCl) after 25-min incubation in the dark. The absorbances were measured by ELISA reader at the wavelength of 492 nm.

Total protein concentration in BALF was determined by using the modified Lowry method, DC Protein Assay (Bio-Rad, Hercules, CA, USA). The BSA standards (diluted to lavage buffer; Sigma, St. Louis, MO, USA) and BALF samples were pipetted to MicroWell plates in duplicate. An alkaline copper tartrate solution and a dilute Folin reagent were added, the plate was mixed, and after 15 min of incubation, the absorbances were measured by ELISA reader at the wavelength of 690 nm.

The albumin concentration in BALF was analyzed by using the ELISA method. Antibody pairs were obtained from Bethyl Laboratories (Montgomery, TX, USA), and analyses were done according to the manufacturer's instructions. The concentration of the monoclonal capture antibody was 10 µg/mL coating buffer (0.05 M sodium carbonate, pH 9.6), and HRP-conjugated second antibody was diluted 1:80,000 in diluent [1% BSA, 50 mM Tris (pH 8.0), 0.15 M NaCl, 0.05% Tween-20]. We used Maxisorb 96-well microtiter plates (Nunc). BALF samples (1:900) and the standards were diluted in diluent. TMB substrate solution (TMB Single Solution) was incubated with samples and standards for 4 min. The absorbances were measured by ELISA reader (iEMS Reader MF) at the wavelength of 450 nm.

Hemoglobin concentrations in the supernatants of hemolyzed cell pellets were analyzed by using the modified Stadie method (Procedure No. 525; Sigma). Briefly, 250 µL Drabkin's reagent and human methemoglobin standards (diluted to Drabkin's reagent) were pipetted to MicroWell plates in duplicate, and 10-µL samples were added to the wells in duplicate. After mixing and 15 min of incubation, the absorbances were measured by ELISA reader at the wavelength of 540 nm. Hemoglobin concentrations are expressed as micrograms per milliliter BALF.

Western blot analysis of iNOS. Cells were lysed in lysis buffer [20 mM Tris HCl (pH 8.0), 2 mM EDTA, 3% Triton X-100, 100 mM NaCl, 1 mM Na_3VO_4, 10 µg/mL aprotinin, 10 mg/mL leupeptin, 1 mM phenyl methyl sulfonyl fluoride] using a 26-gauge needle and a syringe (1 mL). We added 4 × sample buffer [124 mM Tris HCl (pH 6.8), 140 mM sodium dodecyl sulfate (SDS), 2,700 mM glycerol (20%), 0.3 mM bromophenol blue, 100 mM mercaptoethanol] 1:4 and then heated the samples to 95°C for 7 min. When cells were lysed, the samples (> 53 µg protein) and markers (Bio-Rad) were subjected to electrophoresis [7.5% Tris HCl gels (Criterion, Bio-Rad); running buffer: 25 mM Tris, 190 mM glycine, 0.1% SDS; 150 V for 50 min]. Proteins were transferred electrophoretically to a PVDF membrane (Immun-Blot, Bio-Rad; transfer buffer: 25 mM Tris, 190 mM glycine, 20% methanol, 0.05% SDS; 100 V for 1 hr). After blocking with 5% BSA (Sigma), the membranes were incubated in primary antibody solution [0.1% rabbit anti-iNOS polyclonal antibody (BD Transduction Laboratories, Lexington, KY, USA) in 5% BSA] for 1 hr. After 6 × 5 min washings in washing buffer (10 mM Tris, 100 mM NaCl, 0.1% Tween-20; pH 7.5), the membranes were incubated in alkaline phosphatase (AP)-conjugated second antibody solution [0.1% AP-goat anti-rabbit immunoglobulin G (Zymax, Zymed) in 5% BSA] for 1 hr. The membranes were washed six more times and exposed to AP developing buffer (100 mM NaCl, 100 mM Tris base and 5 mM MgCl) for 1 min. Finally, the membranes were developed using BCIP/NBT (330 µg/mL nitro blue tetrazolium, 165 µg/mL bromochloroindolyl phosphate disodium) in developing buffer, and the reaction was stopped by rinsing the membranes in tap water.

Analysis of histopathologic changes and mycobacterial infiltration. Tissue samples from lungs, lymph nodes, spleen, and liver of three or four nonlavaged mice from each group were fixed and stored in 10% buffered formalin. Samples were then trimmed, dehydrated, embedded in paraffin, cut into 5-µm sections, and stained with standard hematoxylin and eosin. We used a light microscope to evaluate histopathologic changes. To evaluate infiltration of *M. terrae* especially in lungs, sections including left lung and spleen tissue from three animals in each group in the time-course experiment were stained with standard Ziehl-Neelsen or auramine-rhodamine. Infiltration was subjectively graded as none, scattered, moderate, strong, or very strong from Ziehl-Neelsen–stained sections under a light microscope, and identification of the mycobacteria from the slides was confirmed from auramine-rhodamine-stained sections under a fluorescence microscope. The clearly strongest occurrence of the mycobacterial cells observed in this study was graded as very strong. Scattered infiltration was indicated by slight, dispersed mycobacterial cell occurrence in the alveolar macrophages or other cells. Samples between these two grades were divided into two more grades, moderate and strong, according to the occurrence of the mycobacterial cells in the lungs.

Statistical analysis. In the dose–response experiment, we analyzed the normally distributed data with equal variances between groups using analysis of variance and Dunnett's test: exposed groups were compared with the carrier control group. Otherwise, we performed Kruskall-Wallis (SPSS, version 7.5.1; SPSS Inc., Chicago, IL, USA) and Dunn's tests (Zar 1996). In the time-course experiment, we analyzed biochemical data at each time point using the two-tailed Mann-Whitney *U*-test (SPSS, version 7.5.1). The difference was considered significant at $p < 0.05$.

Results

Production of proinflammatory cytokines. To evaluate the ability of *M. terrae* to induce proinflammatory cytokine production in mouse lungs, we measured the concentrations of TNFα and IL-6 in BALF. In the dose–response experiment, neither the TNFα nor IL-6 concentrations changed statistically significantly, although the IL-6 level was almost 3-fold with the highest dose (1×10^8 cells) when compared with carrier controls at 24 hr after the instillation (Table 1).

The time course of these cytokines in BALF indicated that the peak concentrations had been attained earlier than 24 hr after the instillation (Figure 1). Six hours after the instillation, *M. terrae* (1×10^8 cells) increased TNFα concentration by 10-fold. The TNFα level had decreased very steeply by 24 hr, but it remained significantly elevated up to 14 days and 40% above the control values on days 21 and 28 after the instillation ($p = 0.055$ at both time points). The IL-6 concentration in BALF was five times higher in the *M. terrae* group than in the control group at 6 hr, and 3-fold higher at 24 hr, but it had leveled off by 7 days after the instillation.

To evaluate the possibility that the *M. terrae* exposure in lungs might also affect systemic cytokine levels, we analyzed TNFα and IL-6 concentrations in serum. At these doses, *M. terrae* did not alter the cytokine concentrations compared with controls (data not shown). The cytokine levels were mostly below the detection limit.

Expression of iNOS. Instillation of *M. terrae* induced the expression of NO-producing iNOS protein in BAL cells. The protein was detectable from 7 to 28 days after the instillation of the bacterium (Figure 2).

Cells in BALF. The cell profile and numbers in alveoli and BALF reflected the process in lungs induced by *M. terrae*. As shown in Figure 3A, only the highest dose of the bacterial cells increased the total cell number in BALF by 75% at 24 hr after the instillation, even though the increase was not statistically significant. A similar pattern was evident in macrophages and neutrophils in BALF. The lymphocyte and eosinophil numbers did not change at 24 hr.

Interestingly, the time course of the cellular response seemed to consist of two phases in *M. terrae*-treated animals. The total cell

Table 1. Components of BALF 24 hr after instillation of the graded doses of *M. terrae*.

	TNFα (pg/mL)	IL-6 (pg/mL)	Albumin (µg/mL)	Total protein (µg/mL)	LDH (mU/mL)	Hemoglobin (µg/mL)
Carrier control	40 ± 9	7 ± 1	145 ± 21	176 ± 41	179 ± 29	26 ± 18
M. terrae 1×10^7	56 ± 26	9 ± 3	185 ± 27	173 ± 32	215 ± 50	7 ± 5
M. terrae 5×10^7	36 ± 5	8 ± 1	168 ± 20	149 ± 08	237 ± 53	ND
M. terrae 1×10^8	59 ± 12	21 ± 8	220 ± 67	217 ± 41	322 ± 53	37 ± 14

ND, not detected. Values shown are mean ± SE of six or seven animals per group.

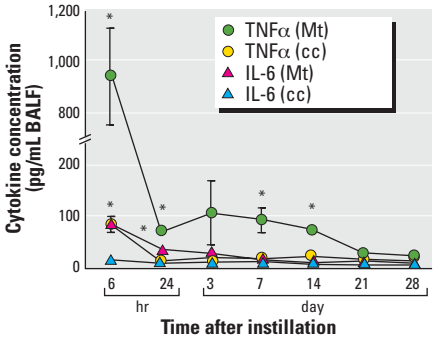

Figure 1. Concentrations of TNFα and IL-6 in BALF at seven different time points after the instillation of *M. terrae* (1×10^8; $n = 5$–6). Abbreviations: cc, carrier control; Mt, *M. terrae*. HBSS was used as the carrier control. Each point represents mean ± SE.
*Statistically significantly different from the carrier control ($p < 0.05$).

number in BALF was increased at 6 hr, reached the maximum at 24 hr after the instillation of *M. terrae* (Figure 3B), and decreased by 7 days. The increase in the total cell number in this phase was mainly due to neutrophils (Figure 3B,C). Thereafter, the total cell number increased again, to a constant level that was approximately four times higher than in the control group. The neutrophil concentration was significantly increased at 6 hr, reached the highest level at 24 hr, but decreased slowly to near the control level by 28 days. Macrophages and lymphocytes were predominantly responsible for the increased total cell concentration at the second phase of the cellular response. The number of lymphocytes and macrophages increased significantly in BALF from day 7 to the end of the experiment (Figure 3C). Eosinophils were not observed in BALF in either experiment.

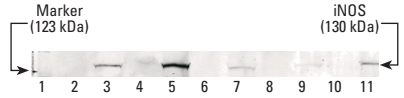

Figure 2. Western blot for iNOS in cells obtained by BAL from mouse lungs at indicated times after the instillation of 1 × 10^8 bacteria. Abbreviations: cc, carrier control; pc, positive control; Mt, *M. terrae*. More than 53 μg of protein was loaded in each lane. Lane 1, marker; lane 2, cc 3 days; lane 3, Mt 7 days; lane 4, cc 7 days; lane 5, Mt 14 days; lane 6, cc 14 days; lane 7, Mt 21 days; lane 8, cc 21 days; lane 9, Mt 28 days; lane 10, cc 28 days; lane 11, pc.

Red blood cells in BALF may indicate increased epithelial permeability and/or tissue damage. *M. terrae* did not increase the hemoglobin concentration in BALF in a statistically significant manner (Table 1).

Albumin, total protein, and LDH. Vascular leakage and cytotoxicity were assessed further by measuring albumin, total protein, and LDH concentrations from BALF. The mean albumin concentration in BALF was increased slightly by all doses of *M. terrae* at 24 hr, most notably with the highest dose (Table 1). The data from the time-course experiment indicated that the albumin level remained elevated up to 28 days after the instillation of the bacteria (Figure 4A). The highest albumin levels were detected at 14 and 21 days after the dosing.

Only the highest dose of *M. terrae* increased total protein in BALF. This effect was more evident in the time-course experiment (Figure 4B), for which the total protein level was increased throughout the experiment. The highest level was reached on day 14. The LDH concentrations in BALF were above the control level up to 28 days during the exposure to the highest dose of *M. terrae* (Table 1, Figure 4C).

Interestingly, albumin, total protein, and LDH responses all displayed the similar two-peak pattern as the total cell number: the first increase was detected before 7 days, and the second was observable from 14 days onward. The responses were present throughout the experiment (up to 28 days).

Histopathologic changes in lungs and clearance of the mycobacterium from the lungs. At necropsy, hyperemia was seen in lungs from 7 to 21 days after the instillation of *M. terrae* (1 × 10^8). The frequency of animals with hyperemia and severity of the change increased up to 3 weeks. Moreover, lungs appeared patchy and edemic, especially at 14 and 21 days. Macroscopic changes had almost disappeared by 28 days.

In histopathology, a slight focal increase in neutrophils was observed in alveoli and partly in bronchiolar lumen 6 hr after the instillation of 1 × 10^8 mycobacterial cells. Neutrophils increased focally in a dose-dependent manner. Also, several larger neutrophil-rich areas were

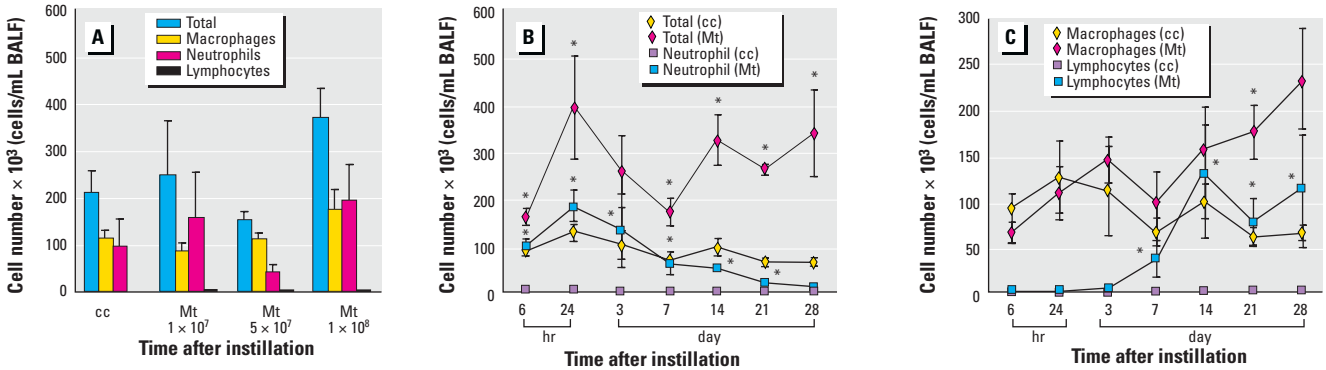

Figure 3. Inflammatory cells in BALF at (*A*) 24 hr after the instillation of the graded doses of *M. terrae* (1 × 10^7, 5 × 10^7, and 1 × 10^8; n = 6–7 animals) and at (*B* and *C*) seven different time points after the instillation of the bacterial cells (1 × 10^8; n = 3–6). Abbreviations: cc, carrier control; Mt, *M. terrae*. Values shown are mean ± SE.
*Statistically significantly different from the carrier control ($p < 0.05$).

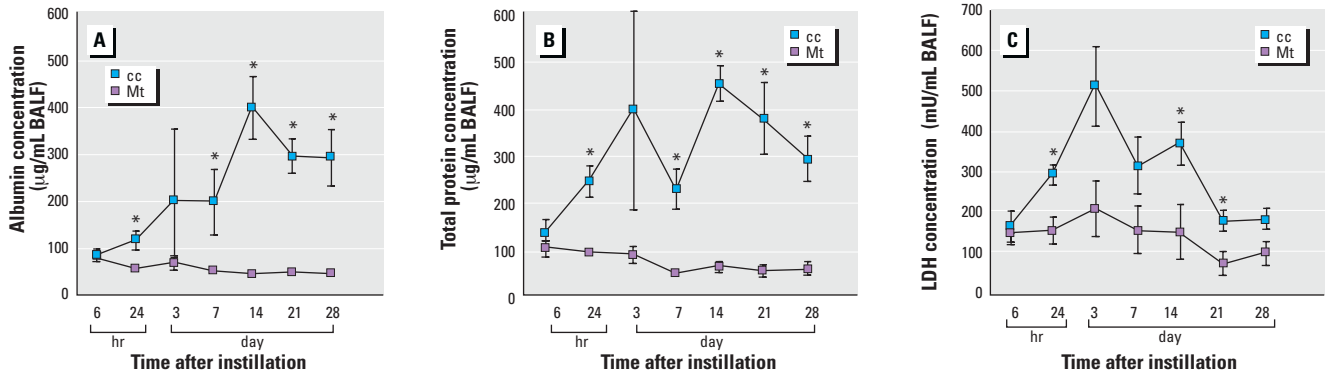

Figure 4. (*A*) Albumin, (*B*) total protein, and (*C*) LDH concentrations in BALF at seven different time points after the instillation of *M. terrae* (1 × 10^8; n = 5–6). Abbreviations: cc, carrier control; Mt, *M. terrae*. Values shown indicate mean ± SE.
*Statistically significantly different from the carrier control ($p < 0.05$).

seen in the lungs at 24 hr (Figure 5). Macrophages and other mononuclear cells were increasingly present in alveolar and partly in bronchiolar spaces at 3 days, and their occurrence in alveoli was increased up to 28 days. Mononuclear cells such as lymphocytes and macrophages were typically seen perivascularly and peribronchiolarly; even large lesions were frequently observed, especially at days 14 and 21. Histopathologic findings correlated well with the cell profiles of BALF. Moreover, a reactive hyperplastic change was observed in lymph nodes associated with lungs from 14 to 28 days. Spleens and livers seemed to be normal in the microscopic examination.

Mycobacterial cells were present in the lungs throughout the experiment, even on day 28 (Figures 5, 6). The numbers seemed to be highest at 24 hr (Figure 6), which was presumably due to the fact that all of the mycobacteria were not phagocytized at 6 hr, and they might have been partly lost during the processing of the samples. The elimination phase seemed to begin after 14 days.

Discussion

Our results show that a single dose of *M. terrae*, originally isolated from a moisture-damaged building, can induce a sustained, biphasic inflammation in mouse lungs, which lasts at least for 28 days. This effect was verified both biochemically in BALF and histopathologically. The present data demonstrate that *M. terrae* can cause a prolonged inflammation in the lungs, and via that pathway it may affect health, even though it can rarely cause severe mycobacterial diseases in humans (Peters and Morice 1991; Spence and Ferris 1996).

Production of proinflammatory cytokines (i.e., TNFα and IL-6) was increased in mouse lungs in a time-dependent manner after exposure to *M. terrae*. This is consistent with our recent *in vitro* findings demonstrating that *M. terrae* induces dose- and time-dependent increases in TNFα and IL-6 production in mouse RAW264.7 macrophages *in vitro* (Huttunen et al. 2000, 2001). The acute phase of TNFα production was over by 24 hr, but increased TNFα production in lungs was evident for up to 2 weeks. Mycobacteria contain several components, such as different types of cell envelope glycolipid lipoarabinomannans (LAMs), which can increase TNFα production (Chatterjee and Khoo 1998; Marshall et al. 1997). This is of interest because TNFα plays an important role in both protective and pathologic immune responses against mycobacterial infections (Marshall et al. 1997). IL-6 production in mouse lungs induced by *M. terrae* was more transient and weaker than was TNFα production. This effect is in line with our *in vitro* findings with mouse RAW264.7 macrophages (Huttunen et al. 2001). The correlation between the *in vitro* and *in vivo* data for *M. terrae* supports the view that macrophages are involved in the production of these proinflammatory mediators in the lungs. In addition, neutrophils and lymphocytes are commonly known sources of these cytokines (Barnes et al. 1998; Jablonska et al. 1999).

TNFα is classically considered to be one of the mediators triggering NO production via iNOS (Cunha et al. 1994), and nitrogen radicals have been suggested to be essential in depletion of intracellular mycobacteria (Denis 1991; Flesch and Kaufmann 1991). Different mycobacterial strains possess varying abilities to affect TNFα and NO synthesis. For example, the two mycobacterial species *M. smegmatis* and *M. vaccae* have been shown to increase TNFα production of mouse macrophages more than does *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) *in vitro* (Marshall et al. 1997). The strains causing higher TNFα production did not apparently induce NO production, in contrast to *M. bovis* BCG. In the present study, *M. terrae* caused an acute and high TNFα production in mouse lungs. However, iNOS expression was not detected until after 7 days, although the induction of the expression of iNOS can occur within a few hours. Hence, *M. terrae* may induce expression of iNOS in mouse lungs via a TNFα-independent pathway, or high TNFα may even suppress the induction of iNOS expression during the infection. In our recent *in vitro* study, the exact same strain of *M. terrae* induced iNOS expression in mouse RAW264.7 macrophages at 24 hr (Huttunen et al. 2001). However, the regulation of the iNOS expression *in vivo* may be more complex than *in vitro*. Inducible NOS response was sustained because the protein was detected from lavaged cells up to 28 days. So far, it is not known which cells were responsible for the iNOS expression in the

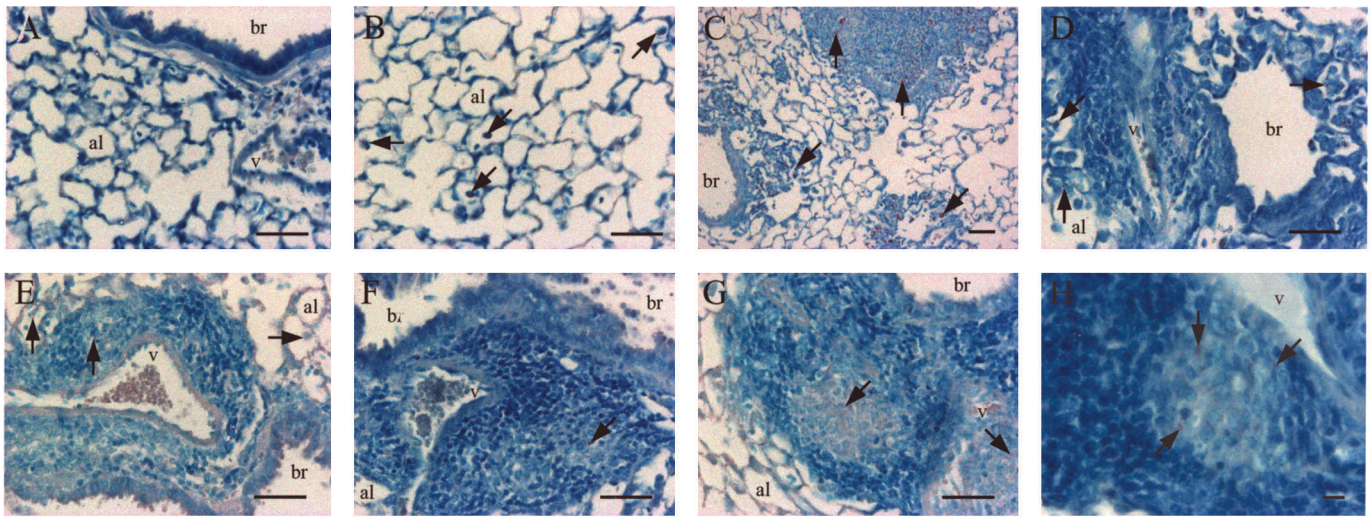

Figure 5. Histopathologic appearance of mouse lungs at different time points after the instillation of *M. terrae* (1×10^8 bacterial cells). Abbreviations: al, alveolar structures; br, bronchiole; v, blood vessel. (*A*) Carrier control-exposed lungs 6 hr after instillation; intact alveolar structures (al), a blood vessel (v), and part of a bronchiole (br) are shown. (*B*) *M. terrae*-exposed lungs 6 hr after instillation; alveolar structures are visible with a few mycobacterium-infected alveolar macrophages (some of them indicated by arrows; the mycobacteria were stained red). (*C*) Intense neutrophil response was observed 24 hr after *M. terrae* instillation; neutrophil infiltrations are clearly visible near or inside the bronchioles (br; lower arrows), and a large neutrophil-rich area is visible in upper part of the photograph (upper arrows). Phagocytized mycobacterial cells were most abundantly seen at this time point (*D* and *E*). Perivascular mononuclear cell infiltrations near the infected cells (arrows) in alveolar spaces are visible 3 days (*D*) and 7 days (*E*) after the instillation. (*F*) Large peribronchiolar and perivascular mononuclear cell infiltrations with granuloma-like structure (arrow) are apparent at 14 days. (*G* and *H*) Peribronchiolitis and perivasculitis are visible also 21 days (*G*) and 28 days (*H*) after dosing. Moreover, granulomas with the mycobacteria inside (arrows) are apparent at both late time points. The mycobacterial cells are visible throughout the lesion (*H*). Arrows indicate some of the mycobacteria-infected cells. Scale bars in A–G = 50 μm; scale bar in H = 10 μm.

lavaged cells and, accordingly, to what extent it reflects changes in cell populations over time. The data suggest that NO production was increased in the lungs at the later phase of the inflammatory response. This is of special interest because high levels of NO, produced by iNOS, may cause vasodilatation, edema, and cytotoxicity (Barnes et al. 1998; Clancy and Abramson 1995). For example, NO is thought to be an important factor in acute lung injury induced by the bacterial endotoxin lipopolysaccharide (Kristof et al. 1998). A similar time frame for NO production has also been detected during *M. bovis* BCG exposure, which induced NO synthesis in mouse pleural cells, presumably via iNOS at 1 and 2 weeks after intrathoracic injection (Moura et al. 1999).

In this study, *M. terrae* caused transient IL-6 production in mouse lungs. IL-6 has been reported to be involved in lymphocyte activation, growth, and differentiation; to increase secretion of immunoglobulins; and to be one factor that can initiate an acute phase response (Barnes et al. 1998; Van Snick 1990). TNFα can also induce IL-6 production in some circumstances (VanHeyningen et al. 1997). Moreover, mycobacterial LAM can also increase the production of IL-6 (Chatterjee and Khoo 1998). The role of IL-6 is not clear in mycobacterial infections. It has been suggested to decrease mycobacterial growth at low levels and increase it at higher levels (VanHeyningen et al. 1997). Even though IL-6 seems to be crucial for controlling intracellular infections, constitutive release of this cytokine is able to impair the ability of macrophages to induce T-cell stimulation (Kopf et al. 1994; VanHeyningen et al. 1997). T-cell–mediated immunity is essential in the host defense against mycobacterial diseases (Holland 1996).

TNFα and NO have been shown to be important mediators in recruiting neutrophils

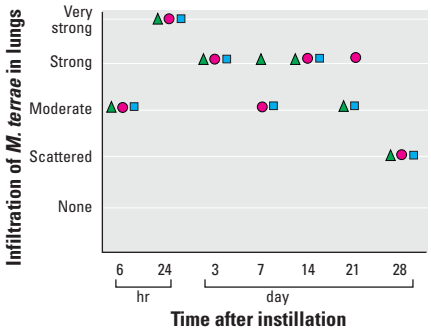

Figure 6. Clearance of *M. terrae* from mouse lungs after the instillation of 1×10^8 mycobacteria. The bacteria were stained with standard Ziehl-Neelsen, and slides were evaluated under a light microscope as described in "Materials and Methods" (some examples are shown in Figure 5). Each symbol represents a single animal. Mycobacteria were not found in control animals (data not shown).

into thoracic cavity of the mouse during *M. bovis* BCG exposure (Menezes-de-Lima et al. 1997) and might contribute to attracting neutrophils into the airways also during the *M. terrae* exposure. In this study, inflammatory cell response seemed to consist of two phases. In the first phase, neutrophils represented over 60% of the lavaged cells, the rest being mainly macrophages. This phase was distinct at 24 hr, verified also in histopathology, and represents an acute inflammation in lungs. Mononuclear cells, such as lymphocytes and macrophages, constituted the second phase, chronic inflammation, manifested from 14 days onward, this being observed both in lavaged cells and with histopathologic analysis. A biphasic cellular response was also observed after intrathoracic injection of *M. bovis* BCG in C57Bl/6 mouse (Menezes-de-Lima et al. 1997). The sustained increase in alveolar macrophages caused by *M. terrae* may be due to the observed slow clearance of the mycobacteria in the lungs and subsequent attraction of macrophages by the microbial cells as long as they exist in the bronchiolar and alveolar spaces. Scattered mycobacteria were seen in the lungs even at the end of the surveillance period to maintain the cell response as well as the biochemical changes.

The rapid neutrophil response has been shown to control fast-growing intracellular bacteria such as *Listeria monocytogenes* and *Salmonella typhimurium* but not slow-growing *Mycobacterium tuberculosis* or *M. bovis* BCG in intravenously infected mice (Seiler et al. 2000). Even though neutrophils may not be crucial for defending against invading *M. terrae*, the sustained neutrophil response observed in this study suggests that they participate in controlling the slow-growing *M. terrae* infection in mouse lungs, when mice have been exposed via their airways. In a similar manner, neutrophils have been reported to play a role also in controlling slow-growing *M. bovis* BCG lung infection in mice when bacteria were delivered by intratracheal instillation (Fulton et al. 2000). Neutrophils can produce chemokines and other inflammatory mediators that enhance accumulation of monocytes into infected lung areas (Cassatella 1995), and at least human neutrophils have been reported to be able to phagocytose and kill slow-growing mycobacteria (Jones et al. 1990). However, *M. terrae* caused faster increase in neutrophils, and the response settled down earlier than during *M. bovis* BCG exposure. Moreover, the route of exposure may be an important factor when inflammatory responses of lungs are evaluated.

Acute pulmonary inflammation is known to increase epithelial permeability, which increases albumin and other protein influx into alveolar spaces (Li et al. 1995, 1999). In concordance with this, in this study both albumin and total protein increased transiently in BALF, after the cell influxes during *M. terrae* infection. The biphasic nature of inflammatory responses induced by *M. terrae* was seen also in these parameters. The sustained increase of vascular leakage from 7 days up to the end of this study (28 days) was apparent. Because hemoglobin in BALF did not increase, erythrocytes did not excessively flow into alveolar spaces; that is, capillary destruction was not evident. Also, the histopathology did not reveal any gross toxicity besides inflammation-associated changes in lungs. The sustained LDH response in BALF might predominantly represent dying inflammatory cells in the lungs during a prolonged inflammatory process.

In the case of particles, overloading of alveolar macrophages is a phenomenon that may cause nonspecific inflammatory responses, even though the mouse may not be a particularly sensitive species in this respect (Donaldson 2000; Morrow 1992). Previously we used this mouse model to study the inflammatory responses induced by the spores of *Streptomyces californicus*, a gram-positive bacterium frequently isolated from moisture-damaged buildings (Jussila et al. 2001). Both the spores of *S. californicus* and bacterial cells of *M. terrae* are spherical particles, and their diameters are approximately the same size (~1 μm). Volumetric particle loads of the doses in the present study were below the suspected dose level which can cause nonspecific responses due to overloading of the alveolar macrophages when calculated as described previously (Jussila et al. 2001).

In summary, *M. terrae*, originally isolated from indoor air of a moisture-damaged building, provoked a sustained, biphasic inflammatory reaction in mouse lungs and caused moderate cytotoxicity. The results suggest that the first phase, acute inflammation, is mediated via TNFα, and the second, chronic phase, via NO produced by iNOS. The long-lasting response can be explained by slow eradication of the mycobacteria from the mouse lungs. The bacteria were detectable at least for 28 days after the dosing. Hence, *M. terrae* may well be among the microbial species causing respiratory symptoms and other immunologic disturbances in the occupants of moisture-damaged buildings. Even short, intermittent exposures may suffice to maintain the inflammation.

References

Andersson MA, Nikulin M, Köljalg U, Andersson MC, Rainey F, Reijula K, et al. 1997. Bacteria, molds, and toxins in water-damaged building materials. Appl Environ Microbiol 63:387–393.

Asano K, Chee CBE, Gaston B, Lilly CM, Gerard C, Drazen JM, et al. 1994. Constitutive and inducible nitric oxide synthase gene expression, regulation, and activity in human lung epithelial cells. Proc Natl Acad Sci USA 91:10089–10093.

Barnes PJ, Chung KF, Page CP. 1998. Inflammatory mediators of asthma: an update. Pharmacol Rev 50:515–596.

Barnes PJ, Kharitonov SA. 1997. Exhaled nitric oxide in monitoring asthma. Drugs Today 10:715–727.

Brunekreef B. 1992. Damp housing and adult respiratory symptoms. Allergy 47:498–502.

Cassatella MA. 1995. The production of cytokines by polymorphonuclear neutrophils. Immunol Today 16:21–26.

Chatterjee D, Khoo KH. 1998. Mycobacterial lipoarabinomannan: an extraordinary lipoheteroglycan with profound physiological effects. Glycobiology 8:113–120.

Clancy RM, Abramson SB. 1995. Nitric oxide: a novel mediator of inflammation. Proc Soc Exp Biol Med 210:93–101.

Cunha FQ, Assreuy J, Moss DW, Rees D, Leal LM, Moncada S, et al. 1994. Differential induction of nitric oxide synthase in various organs of the mouse during endotoxaemia: role of TNF-alpha and IL-1-beta. Immunology 81:211–215.

Dearborn DG, Yike I, Sorenson WG, Miller MJ, Etzel RA. 1999. Overview of investigations into pulmonary hemorrhage among infants in Cleveland, Ohio. Environ Health Perspect 107(suppl 3):495–499.

Denis M. 1991. Interferon-gamma-treated murine macrophages inhibit growth of tubercle bacilli via the generation of reactive nitrogen intermediates. Cell Immunol 132:150–157.

Donaldson K. 2000. Nonneoplastic lung responses induced in experimental animals by exposure to poorly soluble nonfibrous particles. Inhal Toxicol 12:121–139.

Flappan SM, Portnoy J, Jones P, Barnes C. 1999. Infant pulmonary hemorrhage in a suburban home with water damage and mold (*Stachybotrys atra*). Environ Health Perspect 107:927–930.

Flesch IE, Kaufmann SH. 1991. Mechanisms involved in mycobacterial growth inhibition by gamma interferon-activated bone marrow macrophages: role of reactive nitrogen intermediates. Infect Immun 59:3213–3218.

Fulton SA, Martin TD, Redline RW, Boom WH. 2000. Pulmonary immune responses during primary *Mycobacterium bovis*-Calmette-Guerin bacillus infection in C57Bl/6 mice. Am J Respir Cell Mol Biol 22:333–343.

Goodfellow M, Magee JG. 1998. Taxonomy of mycobacteria. In: Mycobacteria: Vol 1. Basic Aspects (Gangadharam PRJ, Jenkins PA, eds). New York: Chapman & Hall, 1–71.

Hagmann M. 2000. A mold's toxic legacy revisited. Science 288:243–244.

Hibbs JB Jr, Taintor RR, Vavrin Z. 1987. Macrophage cytotoxicity: role of L-arginine deiminase activity and imino nitrogen oxidation to nitrite. Science 235:473–476.

Hobbie JE, Daley RJ, Jasper S. 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl Environ Microbiol 33:1225–1228.

Holland SM. 1996. Host defence against nontuberculous mycobacterial infections. Semin Respir Infect 11:217–230.

Huttunen K, Jussila J, Hirvonen MR, Iivanainen E, Katila ML. 2001. Comparison of mycobacteria-induced cytotoxicity and inflammatory responses in human and mouse cell lines. Inhal Toxicol 13:977–991.

Huttunen K, Ruotsalainen M, Iivanainen E, Torkko P, Katila ML, Hirvonen MR. 2000. Inflammatory responses in RAW264.7 macrophages caused by mycobacteria isolated from moldy houses. Environ Toxicol Pharmacol 8:237–244.

Jablonska E, Jablonski J, Holownia A. 1999. Role of neutrophils in release of some cytokines and their soluble receptors. Immunol Lett 70:191–197.

Janeway CA, Travers P. 1997. The immune system in health and disease: host defence against infection. In: Immuno Biology: The Immune System in Health and Disease (Janeway CA, Travers P, eds). 3rd ed. Singapore:Stamford Press, 9:1–9:52.

Jones GS, Amirault HJ, Andersen BR. 1990. Killing of *Mycobacterium tuberculosis* by neutrophils: a nonoxidative process. J Infect Dis 162:700–704.

Jorens PG, Vermeire PA, Herman AG. 1993. L-Arginine-dependent nitric oxide synthase: a new metabolic pathway in the lung and airways. Eur Respir J 6:258–266.

Jussila J, Komulainen H, Huttunen K, Roponen M, Hälinen A, Hyvärinen A, et al. 2001. Inflammatory responses in mice after intratracheal instillation of spores of *Streptomyces californicus* isolated from indoor air of a moldy building. Toxicol Appl Pharmacol 171:61–69.

Katila ML, Mäntyjärvi RA. 1982. Acridine orange staining of smears for demonstration of *Mycobacterium tuberculosis*. Eur J Clin Microbiol 1:351–353.

Kolb H, Kolb-Bachofen V. 1992. Nitric oxide: a pathogenic factor in autoimmunity. Immunol Today 13:157–160.

Kopf M, Baumann H, Freer M, Freudenberg M, Lamers M, Kishimoto T, et al. 1994. Impaired immune and acute-phase responses in interleukin-6 deficient mice. Nature 368:339–342.

Koskinen OM, Husman TM, Meklin TM, Nevalainen AI. 1999. The relationship between moisture or mould observations in houses and the state of health of their occupants. Eur Respir J 14:1363–1367.

Krisher KK, Kallay MC, Nolte FS. 1988. Primary pulmonary infection caused by *Mycobacterium terrae* complex. Diagn Microbial Infect Dis 11:171–175.

Kristof AS, Goldberg P, Laubach V, Hussain SNA. 1998. Role of inducible nitric oxide synthase in endotoxin-induced acute lung injury. Am J Respir Crit Care Med 158:1883–1889.

Kuze F, Mitsuoka A, Chiba W, Shimizu Y, Ito M, Teramatsu T, et al. 1983. Chronic pulmonary infection caused by *Mycobacterium terrae* complex. Am Rev Respir Dis 128:561–565.

Li XY, Brown D, Smith S, MacNee W, Donaldson K. 1999. Short-term inflammatory responses following intratracheal instillation of fine and ultrafine carbon black in rats. Inhal Toxicol 11:709–731.

Li XY, Donaldson K, Brown D, MacNee W. 1995. The role of tumor necrosis factor in increased airspace epithelial permeability in acute lung inflammation. Am J Respir Cell Mol Biol 13:185–195.

Marshall BG, Chambers MA, Wangoo A, Shaw RJ, Young DB. 1997. Production of tumor necrosis factor and nitric oxide by macrophages infected with live and dead mycobacteria and their suppression by an interleukin-10-secreting recombinant. Infect Immun 65:1931–1935.

Menezes-de-Lima O Jr, Werneck-Barroso E, Cordeiro RS, Henriques MG. 1997. Effects of inhibitors of inflammatory mediators and cytokines on eosinophil and neutrophil accumulation induced by *Mycobacterium bovis* bacillus Calmette-Guerin in mouse pleurisy. J Leukoc Biol 62:778–785.

Moncada S, Palmer RMJ, Higgs EA. 1991. Nitric oxide: physiology, pathophysiology and pharmacology. Pharmacol Rev 43:109–142.

Morrow PE. 1992. Dust overloading of the lungs: update and appraisal. Toxicol Appl Pharmacol 113:1–12.

Moura AC, Werneck-Barroso E, Rosas EC, Henriques MG, Cordeiro RS. 1999. Opposite cellular accumulation and nitric oxide production *in vivo* after pleural immunization with *M. leprae* or *M. bovis* BCG. Int J Mol Med 3:69–74.

Palmero DJ, Teres RI, Eiguchi K. 1989. Pulmonary disease due to *Mycobacterium terrae*. Tubercle 70:301–303.

Peters EJ, Morice R. 1991. Miliary pulmonary infection caused by *Mycobacterium terrae* in an autologous bone marrow transplant patient. Chest 100:1449–1450.

Pirhonen I, Nevalainen A, Husman T, Pekkanen J. 1996. Home dampness, moulds and their influence on respiratory infections and symptoms in adults in Finland. Eur Respir J 9:2618–2622.

Platt SD, Martin CJ, Hunt SM, Lewis CW. 1989. Damp housing, mould growth, and symptomatic health state. Br Med J 24:1673–1678.

Rautiala S, Reponen T, Hyvärinen A, Nevalainen A, Husman T, Vehviläinen A, et al. 1996. Exposure to airborne microbes during the repair of moldy buildings. Am Ind Hyg Assoc J 57:279–284.

Seiler P, Aichele P, Raupach B, Odermatt B, Steinhoff U, Kaufmann SHE. 2000. Rapid neutrophil response controls fast-replicating intracellular bacteria but not slow-replicating *Mycobacterium tuberculosis*. J Infect Dis 181:671–680.

Shimizu T, Furumoto H, Takahashi T, Yasuno H, Nishioka K, Muto M. 1999. Lymphadenitis due to *Mycobacterium terrae* in an immunocompetent patient. Dermatology 198:97–98.

Spence TH, Ferris VM. 1996. Spontaneous resolution of a lung mass due to infection with *Mycobacterium terrae*. South Med J 89:414–416.

Spengler J, Neas L, Nakai S, Dockery D, Speizer F, Ware J, et al. 1994. Respiratory symptoms and housing characteristics. Indoor Air 4:72–82.

Strachan DP. 1988. Damp housing and childhood asthma: validation of reporting of symptoms. Br Med J 297:1223–1226.

Taskinen T, Meklin T, Nousiainen M, Husman T, Nevalainen A, Korppi M. 1997. Moisture and mould problems in schools and respiratory manifestations in schoolchildren: clinical and skin test findings. Acta Paediatr 86:1181–1187.

Torkko P, Suutari M, Suomalainen S, Paulin L, Larsson L, Katila ML. 1998. Separation among species of *Mycobacterium terrae* complex by lipid analysis: comparison with biochemical tests and 16S rRNA sequencing. J Clin Microbiol 36:499–505.

VanHeyningen TK, Collins HL, Russell DG. 1997. IL-6 produced by macrophages infected with *Mycobacterium* species suppresses T cell responses. J Immunol 158:330–337.

Van Snick J. 1990. Interleukin-6: an overview. Annu Rev Immunol 8:253–278.

Waegemaekers M, Wageningen N, Brunekreef B, Boleij JSM. 1989. Respiratory symptoms in damp homes. Allergy 44:192–198.

Zar JH. 1996. Multiple comparisons. In: Biostatistical Analysis (Zar JH, ed). 3rd ed.Upper Saddle River NJ:Prentice-Hall Inc., 211–234.