THE ROLE OF *Penicillium chrysogenum* Conidia IN SICK BUILDING SYNDROME AND AN ASTHMA-LIKE ANIMAL MODEL

by

JAMES DANNY COOLEY, B.S.

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Advisory Committee

David C. Straus, Chairperson
W. LaJean Chaffin
Joe A. Fralick
Abdul N. Hamood
James C. Hutson
Terrence M. Joys
Cynthia A. Jumper

Accepted

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ABSTRACT

Sick building syndrome (SBS) is a commonly used term for symptoms resulting from indoor air quality (IAQ) problems and has proven difficult to define. Complaints common to SBS include allergic rhinitis, difficulty in breathing, headaches, flu-like symptoms, and watering of the eyes. While fungal spores are now generally recognized as important causes of respiratory allergies, there are few studies showing which fungi and spores are associated with IAQ problems. Little is known about the role of fungal propagules in the pathogenesis of allergic diseases. These allergic reactions appear to result from the inhalation of fungal products, but the mechanism(s) responsible for these phenomena remain unclear.

In this study, we present evidence that *Penicillium* species, especially *Penicillium chrysogenum*, are strongly associated with the occurrence of SBS in public schools. In addition, our deposition, clearance, and retention studies demonstrated that a significant number of *P. chrysogenum* conidia that were introduced intranasally (IN) in a murine model were retained in the airways and remained viable for up to 36 h post-inoculation. Similar acute doses of viable conidia induced significant ($P<0.001$) increases in tumor necrosis factor α (TNF-α), while non-viable (NV) conidia did not.

In addition, the data demonstrated that mice inoculated intranasally (IN) weekly for 6 weeks with 10^4 *P. chrysogenum* conidia (average viability 25%) produced significantly increased amounts of total serum IgE ($P=0.017$), peripheral eosinophils

(P=0.001), and airway eosinophilia (P=0.01) along with an increase in the number of airway neutrophils (P=0.059). Mice inoculated IN with 10^4 NV conidia did not demonstrate significant changes in serum IgE, and peripheral or airway eosinophils. In addition, lung lavages from mice inoculated IN with 10^4 viable *P. chrysogenum* conidia demonstrated significant increases in interleukin 4 (IL-4) (P=0.015), and interleukin 5 (IL-5) (P=0.004).

These data suggest that long-term inhalation of viable *P. chrysogenum* propagules induces inflammatory responses, such as increases in serum IgE, IL-4, and IL-5, along with peripheral and airway eosinophilia and airway neutrophilia, which are mediators of allergic reactions. The results also suggest that viable *P. chrysogenum* conidia may be producing a substance that is necessary to induce these responses.

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CHAPTER I

INTRODUCTION

Diverse cultures and societies have, for many centuries, appreciated the importance of a clean and healthy environment (Bardana et al., 1988). Clearly, buildings and homes were constructed to protect humans against the elements, as well as the dangers of nature. Indeed, people of most ways of life spend the majority of their time indoors. However, it was not until well after the Arab oil embargo of 1973 that complaints of physical discomfort due to indoor air, with associated irritant symptoms, were reported. Reports of specific and chronic illnesses associated with buildings and building construction materials have appeared with increasing frequency in the medical and scientific literature (Brandt-Rauf et al., 1991; Lyles et al., 1991; Feder, 1985; Finnegan et al., 1984; Kreiss, 1989; Mishra et al., 1992; Sterling and Kobayashi, 1977). Such reports included a myriad of work-related symptoms, including lethargy, fatigue, mucous membrane and skin irritation, difficulty concentrating, asthma-like symptoms, and headaches (Burge, S. et al., 1987; Mishra et al., 1992; Norback et al., 1990). Reports of such symptoms have been described as arising in a wide variety of buildings, including office buildings (Bernstein et al., 1983; Burge, S. et al., 1987; Konopinski, 1983; Robertson et al., 1985), factories, schools (NIOSH, 1991; Norback et al., 1990), hospitals (Brandt-Rauf et al., 1991), and homes (EPA, 1978; Lundholm et al., 1990; Ju and Spengler, 1981; Reynolds et al., 1990; Sterling and Kobayashi, 1977). Much of the interest and research on this topic began in the Scandinavian countries and the United Kingdom. While many case studies have been anecdotal, there has been little evidence

for the identification of a causal agent. In 1983 at a World Health Organization (WHO) meeting in Geneva, a new symptom complex, "sick building syndrome" (SBS), was coined (WHO, 1983). SBS or tight building syndrome (TBS) is a vague term that denotes an excessive occurrence of certain symptoms in people who work together in a common building, the symptoms only occurring after the person comes to work and disappearing upon leaving the workplace (Bardana et al., 1988; Hodgson et al., 1991; Levin, 1989; Lyles et al., 1991). In the United States, there has been a marked increase in buildings under investigation by the National Institute for Occupational Safety and Health (NIOSH). As of 1991, over 100 buildings have been found to be associated with SBS. As with the European experiences, most complaints were vague. Although several important illnesses with objective signs and laboratory abnormalities, e.g., hypersensitivity pneumonitis (Banaszak et al., 1970), asthma, allergic rhinitis, infectious diseases (Fenstersheib et al., 1990; Fraser et al., 1977; Goldberg et al., 1989; McDade et al., 1977), and dermatitis, have been described, these diseases reflect only a small proportion compared to the complaints of workers with SBS. Indeed, even with humidifier-associated fever (Pickering et al., 1976; Rylander et al., 1978) and irrational syndromes, there is no clear picture of an inciting agent.

Since the 1960s, there has been a wide range of changes in houses and buildings that could have contributed to SBS, including increased carpeting, increased quantities of upholstered furniture, and possibly decreased standards of housekeeping. However, there are two changes that seem to be the most convincing, and they are interrelated. The first change that started as early as 1960 was the increase in indoor temperatures. As indoor temperatures rose, it became obvious that large quantities of energy were being wasted

because of ventilation. Indeed, throughout the 19th and early part of the 20th century, it was considered very important to maintain adequate ventilation, because of the risk of poisoning from coal gas.

In the United States after the Arab oil embargo of 1973, the campaign to reduce ventilation was federally funded, supposedly to conserve fuel and reduce heating cost. This movement resulted in the construction of tight, energy-efficient buildings. In fact, the results of non-operable, double-glazed windows and increased insulation were progressively higher indoor temperatures and very low exchange rates. Today it is not unusual for buildings to have air exchange rates as low as 0.2 air changes per hour (Woods and Rask, 1993). Thus, there have been very dramatic changes in temperature and ventilation. The effect of ventilation on indoor humidity depends on the outdoor conditions. Reduced ventilation will also allow the accumulation of small particles in the air. Thus, these tight, energy efficient structures foster the containment and concentration of indoor air pollutants. It is possible, therefore, that there has been a combination of improved conditions for mold growth combined with decreased ventilation, which allows for the accumulation of allergen containing particles in the indoor air. Whether these changes are the full explanation for the worldwide increase in allergic diseases in "developed" countries is not known, but they seem to be the most convincing of the explanations that have been proposed. Perhaps it is worth pointing out that over this same period another habit of humans has also accelerated; that is, the progressive increase in the amount of time spent indoors. Humans are spending a larger proportion of their time in buildings that are progressively becoming warmer and tighter. It is now estimated that the average individual spends 70-90% of his or her time in the indoor

environment, and for the elderly or the disabled, this percentage may be even greater (NRC Committee on Indoor Pollutants, 1981; Witek et al., 1984).

With the increasing awareness that poor indoor air quality may generate a variety of deleterious effects on human health, indoor air quality (IAQ) has become a major public health concern (Samet, 1990). As stated earlier, buildings are intended to provide relatively safe and comfortable environments in which individuals can live and work; however, it has become apparent that they do not always achieve this goal. It has been estimated that over 30% of the buildings in the developed countries have poor indoor air quality (Smith, 1990; WHO, 1984). The Occupational Safety and Health Administration (OSHA) has estimated that 30 to 70 million U.S. workers are affected by SBS (Bureau of National Affairs, 1992).

In the United States, many of the early studies showed that a number of the reported causes of SBS were due to undesirably high levels of known respiratory irritants such as nitrogen and sulfur dioxides, hydrocarbons, and particulates, known or suspected carcinogens such as asbestos, radon, formaldehyde, and tobacco smoke, or chemicals being released by new building materials (Konopinski, 1983; NAS, 1981; Sterling and Sterling, 1984). Many of these types of problems were relatively easy to mitigate. The air exchange (ventilation) was increased, the concentration of the offending irritant was decreased, and the numbers of IAQ complaints were significantly reduced. One of the best known investigations conducted in the U.S. comes from NIOSH in which investigators attempted to identify the single most likely cause of complaints (Melius et al., 1984). Fewer that 5% were thought to be related to mold and bioaerosols exposure, 2% to environmental tobacco smoke, 11% to pollutants entrained from the outside, and

17% from internally generated pollutants. In more than half of the cases, no cause except inadequate ventilation was identified. However, since no follow-up on the effectiveness of intervention was undertaken, the accuracy of these diagnoses remains unclear. Thus, increasing the ventilation became the underlying basis for mitigation of SBS in the U.S. This concept was reinforced when a reanalysis of six epidemiological studies (Woods, 1991) demonstrated that the prevalence of symptoms was consistently two to three times greater in buildings with mechanical ventilation and air conditioning with sealed windows, than in buildings with natural or simple mechanical ventilation with windows that would open. These studies also showed that an increase in symptoms was consistently associated with ventilation rates. A major problem with these studies was that many of these buildings were not experiencing SBS and the questionnaires employed could not accurately eludicate this fact. In a recent study, an important question regarding SBS was asked (Mikatavage et al., 1995). This question was "what is the background prevalence of SBS in modern, smoke-free office workplaces without the "sick" label?" In that study, it was demonstrated that the background prevalence estimate of SBS was in a range of 2.4 to 7.7% of workers.

However, several large series of studies in Europe have attempted to define the scope of the problem and categorize inciting factors. The Danish Town Hall study (Skov et al., 1987) was of particular importance because it failed to show a relationship between ambient carbon dioxide levels and worker complaints. Prior to this study, a CO_2 level of less than 1000 ppm was considered necessary to assure the comfort of the building occupants (Buring and Hennekens, 1991). This level is approximately three times higher than in outdoor air. The basic assumption was that levels greater than 1000 ppm

indicated inadequate ventilation and air exchange. As stated earlier, in the United States this was the basis for remediation of buildings experiencing SBS. It was thought that increasing the ventilation would decrease the contaminants, thus reducing the complaints. The Danish study demonstrated that a CO_2 concentration of 1000 ppm does not constitute a health hazard (Frank, 1990) and providing high ventilation rates sufficient to reduce CO_2 levels below 600 ppm does not guarantee acceptable IAQ in the presence of high production of indoor contaminants.

A number of European studies began to demonstrate an association between damp housing, increased respiratory disorders, and molds. In The Netherlands, studies found exposure to mold to be associated mainly with lower respiratory symptoms, cough and phlegm production, and wheezing (Brunekreef, 1992; Waegemaekers et al., 1989). In Sweden, a cross-sectional study of houses with problems with dampness and mold, and houses without dampness showed statistically significant associations between the exposure and upper and lower respiratory symptoms, eye irritation, headache, and tiredness (Holmberg, 1984). It was also demonstrated that the risk of prolonged cough after a respiratory infection was 2.3-fold higher for children living in homes with water damage, compared with those living in normal homes (Andrae et al., 1988). In a Finnish study examining preschool children, nasal congestion and excretion, persistent cough, phlegm, and wheezing were significantly associated with dampness and mold in the homes (Jaakkola et al., 1993).

In a Canadian study of almost 15,000 respondents, dampness and mold were significantly associated with several respiratory symptoms, eye irritation, and chronic

respiratory disease (Dales et al., 1991). In the examination of these studies, the most prevalent symptoms were irritation of the respiratory tract and the eyes.

However, in epidemiologic research, it is practically impossible to distinguish between the effects of various exposures, and thus the exposure is often defined as damp housing or as water-damaged buildings, than more specifically as fungi or microorganisms. In practical situations of risk assessment, it is necessary to quantify both the exposure and the effect, and still some amount of uncertainty will remain when causal relationships and the health risk are estimated. Moreover, the important factor in many of the epidemiologic studies was that there were strong associations of symptoms with water-damaged and damp buildings and fungi.

Moisture problems have been encountered with an increasing frequency both in family housing and in the workplaces, both in the U.S. and in Europe (Reijula, 1996). Water leaks and moist building materials inevitably lead to the growth of fungi and bacteria in these buildings. Several recent epidemiologic studies indicated that dampness and fungal problems are present in 20% to 50% of modern homes (Brunekreef, 1992; Dales et al., 1991; Jaakkola et al., 1993; Spengler et al., 1994; Verhoeff et al., 1995). It has become increasingly evident that bioaerosols, especially those that are fungal in nature, are of great importance in SBS. Recent studies have demonstrated that fungi are one of the causal factors in the relationship between home dampness and respiratory symptoms, and homes classified as damp tend to have higher levels of fungi than those not so classified (Platt et al., 1989; Verhoeff et al., 1992). In addition, poorly maintained heating, ventilation, and air-conditioning (HVAC) systems have been recognized as sources of microorganisms, including fungi (Pope et al., 1993; Spengler et al., 1992).

Fungi are well known as sources for allergens that cause allergic rhinitis, allergic asthma, and extrinsic allergic alveolitis (hypersensitivity pneumonitis) (Burge, 1989; Flannigan and Miller, 1994; Miller, 1992; Salvaggio and Aukrust, 1981; Tarlo et al., 1988). Up to 10% of the general population is skin test positive to fungal extracts (Barbee et al., 1976; Sears et al., 1989), and among patients with respiratory allergy, 2% to 80% are reported to be sensitized to fungi (Beaumont et al., 1985; Gravesen, 1979; Nordvall et al., 1990 ; Salvaggio and Aukrust, 1981). Patients often show multiple positive reactions to different fungal extracts. It is not yet clear whether these patients have independent sensitivities to many fungi or are sensitive to cross-reacting allergens produced by many fungi (Baldo, 1995).

Fungi also produce a variety of volatile organic compounds (VOC), including alcohols, aldehydes, and ketones, which are often evident as "moldy odors," and, when present in high concentrations, produce symptoms such as headache, eye, nose and throat irritation, or fatigue (Flannigan et al., 1991; Tobin et al., 1987). This became a popular explanation for SBS in that VOCs, all present at very low levels, could some how together, exert a toxic effect.

Fungi also produce toxic metabolites (e.g., mycotoxins). The best known are the aflatoxins, which are human carcinogens. Respiratory exposure to toxins produced by *Stachybotrys chartarum* has been a focus of attention with respect to nonallergic respiratory symptoms (Andersson et al., 1997; Croft et al., 1986; Johanning et al., 1996).

At present, no single environmental factor or group of factors has been established as the cause of SBS. Although fungal contamination in indoor environments has been shown to produce allergies in occupants of these buildings (Lehrer et al., 1983;

Licorish et al., 1985; Verhoeff et al., 1995), the role of fungi in SBS has become increasingly controversial. Numerous theories have been put forward (Mendell, 1993). Along with the VOC theory, a heightened neurogenic inflammatory response to low-level chemical exposures has also been suggested (Meggs, 1993), and other theories have focused on particulates (Salvaggio, 1994a) and physical factors (Levin, 1995). Inadequate ventilation is a factor in all of these theories. Investigators that are more skeptical have emphasized the roles of psychosocial factors, stress, and gender (Bachmann and Meyers, 1995; Salvaggio, 1994b; Stenberg et al., 1994).

One of the greatest areas of concern is the children that are forced to live and learn in "sick" buildings. Due to the fact that children spend most of the day indoors, and because dampness in buildings has increased over the last decade, a relationship exists between increased symptoms, dampness, and fungal spores (Dill and Niggemann, 1996; Garrett et al., 1998; Li and Hsu, 1997). In an office building that is experiencing SBS, the workers have an option, although sometimes limited, of leaving the building for other employment. Children in public schools do not always have that option. It has recently been shown that children attending school in buildings with dampness and fungal contamination suffer higher rates of respiratory infections than do children in schools without dampness and fungal contamination (Koskinen, 1995). This concern is evident in that many states, including Texas, have published voluntary guidelines for the IAQ in public schools (TDH, 1998).

The literature suggests that fungi may be one of the causative agents of allergic rhinitis, hypersensitivity pneumonitis and increased respiratory infections experienced by the occupants of buildings undergoing SBS. However, a major gap in the literature is

inadequately addressed microbial characterizations of indoor environments. Mishra et al. (1992, p. 288) state "SBS and BRI (building related illness) related to hypersensitivity to fungi or their metabolic products are probably the most difficult to define and least studied."

To address this gap in the literature, my research was divided into three phases. Phase 1 was to determine which fungal genera are consistently found in "sick buildings." Phase 2 was to determine the type of effects that these fungal conidia exert on alveolar macrophages. Phase 3 was to examine the conidia produced by these fungi to determine if they can induce allergic responses in experimental animals.

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CHAPTER II

CORRELATION BETWEEN THE PREVALENCE OF CERTAIN FUNGI AND SICK BUILDING SYNDROME

Introduction

Reports concerning buildings with air-related problems appeared with increasing frequency after the early 1970s, although this problem has certainly been with man for centuries (Hodgson, 1992; Spangler and Sexton, 1983). Sick building syndrome (SBS), a commonly used term for symptoms resulting from indoor air quality (IAQ) problems, was first recognized as an important problem affecting people in certain buildings in 1982. The first official study of SBS that examined more than one structure was published in 1984 (Finnigan et al., 1984). SBS has proven difficult to define and no single cause of this malady has been identified (Hodgson, 1992).

Complaints common to SBS include allergic rhinitis, difficulty in breathing, headaches, flu-like symptoms, and watering of the eyes (Mishra et al., 1992). Numerous studies have been carried out in an attempt to elucidate the cause of SBS (Feder, 1985; Finnigan et al., 1984). Early studies showed that many of the reported causes of SBS were undesirably high levels of known respiratory irritants such as nitrogen and sulfur dioxides, hydrocarbons and particulates (NAS, 1981), known or suspected carcinogens such as asbestos, radon, formaldehyde and tobacco smoke (Sterling and Arundel, 1984), or chemicals being released by new building materials.

While fungal spores are universal atmospheric components indoors and outdoors and are now generally recognized as important causes of respiratory allergies (Bernstein

et al., 1983; Burge, 1990; Dales et al., 1991; Huuskonen et al., 1984; Solomon, 1974; Solomon, 1975) there are few studies showing which fungi and spores are associated with IAQ problems (Roby and Sneller, 1979). This study was made possible due to our association with an IAQ company. The uniqueness of this study was that the sites were made available because the school officials contacted the IAQ company. This allowed us access to all the samples, data, questionnaires and occupant-generated complaints from schools that were experiencing IAQ problems.

While no one cause for the symptoms induced by IAQ problems is likely to exist, the presence of fungi in sick buildings is becoming consistently associated with this problem (Burrell, 1991; Lehrer et al., 1983; Miller, 1992; Mishra et al., 1992). Fungal contamination in indoor environments has been shown to produce allergies in occupants of these buildings (Salvaggio and Aukrust, 1981; Wanner et al., 1993). Even though the IAQ company's investigations were broad-ranged and in-depth, the presence of fungi was the primary focus of this study. In this study, we present evidence for the role of *Penicillium* species and *Stachybotrys* species in buildings experiencing IAQ problems.

Materials and Methods

Survey Procedures

The 22-month study examined forty-eight public schools that were experiencing IAQ problems. These sites were located in states along the United States Gulf of Mexico and the Atlantic seaboard. The sites were surveyed using the following criteria:

collection of building characterization data based on direct inspection and interviews with building occupants; building characterization including measurement of temperature and humidity, examination of heating, ventilation and air conditioning (HVAC) systems and physical examination of the building; particulates, CO_2, and chemical measurements; inspection of sites that had been wetted and/or exhibited mold growth; swab samples and air samples taken by an Andersen air sampler; and administration of a questionnaire to building occupants, and/or access to occupant-generated complaints (letters to school officials) and other complaint surveys conducted by other agencies such as public health departments.

The questionnaire that was administered by the IAQ company was designed to determine the areas or rooms in which the occupants had complaints about the IAQ. The questionnaire asked for such information as nature of complaint, symptom patterns, timing patterns, and observations about building conditions that might explain observed symptoms. The questionnaire's answers and comments, along with the occupant-generated complaints and surveys from other agencies, were placed into the following categories: (1) type of symptoms; (2) when do the symptoms start; (3) when do the symptoms go away; (4) when are the symptoms the worst; (5); preexisting symptoms (e.g., allergies, asthma, etc.); (6) discomfort complaints (noise, temperature, odors, etc.); and (7) complaint areas (rooms).

Non-complaint areas were also identified. The minimum criteria for designation as a non-complaint area were (1) all occupants, whose primary location was the non-

complaint area, had no IAQ complaints and (2) the HVAC system was separate from any complaint areas.

Microbiological Identification

Air samples were taken using a two-stage Bioaerosol Sampler (Model 2000 Andersen Samplers Inc., Atlanta, GA) at a calibrated flow rate of 28.4 liters/min for 5 min. During sampling, the Andersen samplers were placed approximately three feet above floor level. Sabouraud's Dextrose agar (SDA) pH 5.6 was used for air sampling and swab sampling. Plates were incubated at 22°C and 90% relative humidity (RH) for up to 14 days. The isolated fungi were identified using standard identification techniques (Frey et al., 1979; Larone, 1993; Rameriza, 1982; Samson and Pitt, 1990). CFU/m^3 of air were calculated using the formula:

CFU/m^3 = [number of CFU / ((number of minutes sampled)(1 ft^3/minute))] [35.3 ft^3/m^3].

The total fungal CFU/m^3 for each air sample was calculated and the ratio for each organism per sample was determined. The results were entered according to the area that was sampled [the outdoor air samples (OAS) areas, the indoor air samples (IAS) complaint areas or the IAS non-complaint areas] and the average CFU/m^3 and ratio, in terms of percentage, for each organism was determined for each area examined.

Using sterile swabs, samples were taken from areas of visible fungal growth, HVAC systems, wetted areas, standing water, dead air spaces and areas of dust accumulation. The swabs were either placed into sterile plastic bags for transport to the

laboratory or streaked undiluted onto SDA pH 5.6 plates. At the laboratory, the swab tip was placed into a sterile tube containing 10 ml of sterile phosphate buffer saline (PBS) and vigorously vortexed for one minute. Samples (100 µl) were pipetted onto SDA pH 5.6 plates and spread with sterile rods. The plates were incubated at 22°C and 90% relative humidity (RH) for up to 14 days. The fungi were identified and the fungal growth on the plates was estimated using the following criteria: 0 CFU, no growth; 1 to 5 CFU, very light growth; 6 to 10 CFU, light growth; 11 to 30 CFU, medium growth; 31 to 50 CFU, heavy growth; and, more than 50 CFU, very heavy growth.

Carbon Dioxide Measurements

The CO_2 content of the air, expressed in parts per million (ppm), was monitored using a Ventostat CO_2 Sensor (Model 1070/1071, Telaire Systems, Inc.).

Chemical Measurements

An independent laboratory was contracted by the IAQ company to perform sampling for various indoor air components such as formaldehyde, nitrogen dioxide, hydrogen sulfide, sulfur dioxide, and carbon monoxide. Formaldehyde samples were collected using a Sensidyne/Gastec (Sensidyne/Gastec, Goleta, CA) collector in association with colorimetric indicator tubes with a lower detection limit of 0.1 ppm. Nitrogen dioxide, hydrogen sulfide, sulfur dioxide, and carbon monoxide samples were collected using a MultiRAE PGM-50 gas-sampling device (Rae Systems, Inc., Sunnyvale, CA). The collector was calibrated prior to sampling for each particular component. Samples were taken outdoor and indoor in the complaint and non-

complaint areas. All sampling was performed in accordance to the manufacturers suggested use.

Particulates, Temperature and RH Measurements

Airborne particles were counted with an APC-1000 Airborne Particle Counter (Atcor Inc., San Jose, CA) which complies with FDA laser performance standard 21 CFR 1040.10 and 1040.11. The APC-1000 detects particles relative to four thresholds: greater than 0.3, 0.5, 1.0 and 5.0 microns. The APC-1000 also measures temperature and RH.

Building Remediation

Any building materials that showed physical deterioration were removed and replaced. Existing microbial contamination on intact and structurally sound surfaces were cleaned and treated with an approved disinfectant. The HVAC remediation consisted of the removal of visible surface contaminants and the cleaning of air-side surfaces of all internal air handling surfaces including, but not limited to, fans, coils, drain pans, filter racks, motors, dampers, and specific air ducts. Any damaged or delaminated insulation within the airducts being cleaned and sanitized was replaced. All work areas inside the air handlers, air ducts, and equipment rooms were isolated and kept under negative air pressure using high efficiency particulate arrestor (HEPA) filtered negative air machines to prevent migration of particulates. The cleaning was

conducted in accordance with the National Air Duct Cleaners Association (NADCA) Standard 1992-01 (NADCA, 1992). All work was done after hours and on weekends. All personnel involved in the remediation had the proper safety equipment and training and the Occupational Health and Safety Administration standards were observed. Air and swab samples were retaken within 60 days and after at least 6 months of completion of remediation.

Data Analysis

Data were analyzed by a computer program (Sigma Stat) employing the Mann-Whitney rank sums test (U test), the Kruskal-Wallis one-way ANOVA (H test), Spearman's product moment correlation, and Dunn's multiple comparisons and partial correlations (Freund and Simen, 1992).

Results

Complaints

Of the forty-eight public schools surveyed, forty were elementary schools (grades K-6). At most of these sites the school nurse distributed the IAQ company's questionnaire (Figure 2.1) to only the faculty and staff. There was a total of 622 occupants that reported IAQ symptoms or complaints which represented 28% of the total staff and faculty (students were not included). With the exception of nausea, there were no significant differences between the reported complaints and symptoms at the different sites. All of the sites were combined and the average incidence rates (IR) per 100 employees along with the 95% confidence interval (CI) are displayed in Table 2.1

Nasal drainage and congestion (IR=19.3, 95% CI ±1.3) and itchy and watering eyes (IR=14.0, 95% CI ±1.1) were always the most common complaints, although all of the symptoms, (with the exception of nausea) listed in Table 2.1 were reported at each site. Most of the occupants registering complaints stated that their symptoms were a result of either entering or working in the building and a majority stated that the symptoms usually went away during weekends and/or vacations and returned upon entering the building. More than half of the occupants that had IAQ complaints also complained of increased respiratory infections (such as tonsillitis, bronchitis, and some cases of pneumonia) (IR=14.3, 95% CI ±1.0). Over one-third of the occupants that registered complaints claimed that an increase in the relative humidity resulted in an increase in the severity of their symptoms (IR=12.0, 95% CI ±0.9).

At most schools, prior to the IAQ company's investigations, public health departments had conducted investigations and used questionnaires. These investigations consisted primarily of measuring the CO_2 in the buildings and increasing the ventilation rates, but with no success in reducing the IAQ complaints. The symptoms from the public health departments' questionnaires correlated to the symptoms obtained from the IAQ company's questionnaire. At all schools, many of the occupants that had IAQ complaints had submitted letters to the school officials, detailing their complaints and symptoms. The complaints and symptoms listed in these letters correlated to the complaints and symptoms obtained from the IAQ company's questionnaire.

After remediation of any building, questionnaires were made available to the faculty and staff. They were asked to respond if there were any complaints or symptoms related to the IAQ of the building. There were none or only a few complaints concerning the IAQ from the buildings that underwent remediation. Complaints following remediation were never registered by more than 3% of the faculty and staff at any school. The overall incidence rate was 2.5 per 100 employees with a 95% confidence interval of ±1.1. This represented a significant reduction ($p < 0.001$) in the number of IAQ complaints.

Carbon Dioxide, Chemical, and Particulate Measurements

Although CO_2 levels were higher indoors than the outdoor levels, there were no significant correlations between the indoor levels and the complaints or symptoms. All measured constituents were observed to be well within the normal, acceptable range of a school/office setting and there were no significant correlations between the indoor levels and complaints or symptoms. No correlations were observed between the outdoor particulate measurements, the indoor complaint areas and the non-complaint areas.

Fungi in Outdoor Air

Five fungal genera were consistently found in the outdoor air and comprised over 95% of the outdoor fungal ecology (Figure 2.2). These genera were *Cladosporium* (81.5%), *Penicillium* (5.2%), *Chrysosporium* (4.9%), *Alternaria* (2.8%) and *Aspergillus* (1.1%). The remaining fungi (*Fusarium*, *Epicoccum*, *Botrytis*, *Bipolaris*, *Acremonium*, *Drechslera*, *Rhizopus*, *Mucor*, and *Rhodotorula*) were present in very low numbers and

varied according to location and season. The predominant *Cladosporium* species isolated from the outdoor samples was *Cladosporium cladosporioides*. A variety of other *Cladosporium* species (such as *C. herbarum* and *C. sphaerospermum*) were also isolated, but these isolates were usually found in association with *C. cladosporioides*. The predominant *Penicillium* species isolated was *P. chrysogenum*. *Aspergillus niger* was the most commonly isolated *Aspergillus* species from outdoor air samples. With the exception of a few sites along the northern Atlantic coast, most of the buildings were in mild temperate zones, with little or no snowfall, and an average RH range of 30% to 60%. The rainfall in the survey areas was not abnormal, with the exception of states surrounding the southern region of the Gulf of Mexico, which were experiencing a drought during the survey period. The outdoor temperature was seasonal, varying from a low near 5°C to a high of 38°C.

Initial Indoor Air Samples

In most of the public schools (Figure 2.2), there were significant reductions in the CFU/m^3 of air of fungi in the IAS non-complaint areas compared to the OAS, but the fungal profiles were similar to outdoor air. In all of the buildings, the CFU/m^3 of air of *Cladosporium* species were significantly ($p < 0.05$) lower in the IAS non-complaint areas when compared to the OAS. In the IAS complaint areas, the CFU/m^3 of air of *Cladosporium* species were lower, but not always significantly lower, than the OAS. *Penicillium* species and *Aspergillus* species were the only fungi isolated from the IAS complaint areas that had higher CFU/m^3 of air when compared to the OAS and IAS non-complaint areas. All of the public schools had similar interior temperatures (23 C).

Most complaint sites showed very little HVAC maintenance as well as active water leaks.

At twenty public schools (Figure 2.3 and Table 2.2), there were significant increases ($p < 0.0001$) in the CFU/m^3 of *Penicillium* species in the air samples from IAS complaint areas when compared to the OAS and the IAS non-complaint areas. The swab samples (Table 2.3) from these sites had very heavy *Penicillium* species growth. The indoor relative humidity (IRH) in these complaint areas (IRH-C) had a mean of 50% (SD ± 12%) with a range of 23% to 67%. The IRH in non-complaint areas (IRH-NC) had a mean of 40% (SD± 10%) with a range of 30% to 48%. The outdoor RH (ORH) mean was 46% (SD± 20%) with a range of 22% to 81%. *P. chrysogenum* was the dominant fungal isolate in fourteen of these sites.

In the air samples from complaint areas at five public schools (Table 2.2), there were increases, although not significant ($p = 0.10$), in the number of CFU/m^3 of air of *Penicillium* species. These IRH-C had a mean of 64% (SD± 9%) with a range of 54% to 70%. These IRH-NC had a mean of 56% (SD± 3%) with a range of 54% to 58%. The ORH had a mean of 69% (SD± 15%) and ranged from 60% to 86%. The swab samples (Table 2.3) from these sites showed very heavy *Penicillium* species and heavy *Cladosporium* species growth that indicated possible fungal growth in the interior and a potential IAQ problem with *P. chrysogenum* as the most common isolate.

At eleven public schools (Table 2.2), *Stachybotrys atra*, which was not isolated from any air samples, was nonetheless isolated from swabs (Table 2.3) of the visible growth taken from under wetted carpets, interior painted gypsum board walls, or

especially behind vinyl coverings on gypsum board walls that had been wetted in indoor complaint areas. The IAS from these complaint areas were not significantly different from the IAS non-complaint areas and had profiles that were similar to the OAS. These IRH-C ranged from 58% to 66% with a mean of 62% (SD± 5%). The ORH ranged from 65% to a high of 90% with a mean of 83% (SD± 11%).

Many *Aspergillus* species (*A. glaucus, A. versicolor* and *A. flavus*) were isolated from interior air samples and swab samples in association with the *Penicillium* species, but *Aspergillus* species (*A. flavus*) were dominant in complaint areas at only one public school. The swab samples from the interior showed heavy to very heavy growth of *Aspergillus* species, along with medium to heavy growth of *Cladosporium* species and *Penicillium* species. The IRH at this site was 65% and the ORH was 75%.

In the remaining eleven public schools, the fungal ratios and CFU/m^3 of air (OSA and IAS) were not significantly different (Table 2.2). The swab samples (Table 2.3) from the interior of these sites showed heavy to very heavy growth of *Cladosporium* species and/or *Penicillium* species that indicated possible fungal growth in the interior. The IRH ranged from 56% to 64% with a mean of 60% (SD± 3%) and these ORH ranged from 57% to 62% with a mean of 60% (SD± 2%).

Post-Remediation Indoor Air Samples

Indoor air samples and swab samples were retaken within 60 days of completion of the remediation of a building and again at least 6 months after remediation. At all sites the fungal ratios (OAS compared to IAS) were very similar but the CFU/m^3 of air of fungi from the IAS were 50% to 90% less ($p < 0.05$) when compared to the OAS.

The IRH had a mean of 44% (SD 5%), with no site exceeding 57%. The ORH ranged from 37% to 87%. All swab samples showed very light to light growth of *Cladosporium* species and a mixture of other species.

Discussion

All of the IAQ complaints investigated were occupant generated. Since the validity of results from questionnaire studies may be altered by biases introduced by the observer or by the respondents (Samet, 1978), the results must be carefully weighed. Even when observer bias is reduced, the bias introduced by the respondent remains a potential source of systematic error (Samet, 1978). A major problem, however, is that it is often difficult to control independent variables because of the diversity of the study population, its motility, or a lack of personal exposure. The majority of occupants (90%) in the buildings that we investigated were teachers. Even though potential psychological disorders such as depression or anxiety were not directly addressed, we did, at most schools, observe a high job satisfaction and a genuine concern for the welfare of the students. Also, it is difficult to segregate the individual pollutant from copollutants and other confounders. However, tobacco smoke can be eliminated as a potential confounder since all of the schools prohibited tobacco use on the campuses. A causal relationship is rarely discernible even with strong statistical significance. Thus, at best, associations can be drawn only between the exposure and the effect.

Even though fungal contamination in indoor environments has been shown to produce allergies in occupants of these buildings (Lehrer et al., 1983; Licorish et al.,

1985; Miller, 1992; Verhoeff et al., 1995), the role of fungi in IAQ problems has become increasingly controversial. Our studies show that *Penicillium* species and *Stachybotrys* species are strongly associated with SBS. These data demonstrate that the *Penicillium* species, especially *P. chrysogenum*, can adapt to an environment in which man is most comfortable. Our studies also support earlier findings that *Penicillium* species has become an important indoor contaminant (Burge et al., 1989). This ubiquitous organism's optimal growth occurs between 10° and 25°C. It can grow over a wide range of water availability and has low water activity, although sporulation requires a higher water activity (Pitt, 1981). Although it is widely stated that relative humidity over 70% is needed for active fungal growth, the water activity of the substrate is actually the critical parameter (Gravesen, 1979). In the complaint areas where *Penicillium* species were the dominant species, we observed (with the exception of the HVAC system at the fan during cooling) that the range of the IRH was from 23% to 67%. *P. chrysogenum* is apparently capable of successfully competing with most conidial fungi over almost the entire range of water availability. Its conidia are small (1 to 5 µm) and are capable of entering the lower respiratory tract. It has been shown that bronchial challenges with *Penicillium* species conidia induced immediate- and delayed-type asthma in sensitized subjects (Licorish et al., 1985).

Stachybotrys species, some of which are capable of producing potent mycotoxins (Sorenson et al., 1987), require abnormally high RH or wetted surfaces to grow. This fungus has been associated with building-related illness and SBS (MMWR, 1997; Johanning et al., 1996). It is difficult to isolate *Stachybotrys* species from the air

and the presence of *Stachybotrys* may have been overlooked due to this phenomenon. Our findings suggest that when the fungal ecology of complaint air samples are similar to the outdoor air and non-complaint air samples, coupled with abnormally high interior RH and SBS symptoms, the possibility exists that a mycotoxin producing fungus such as *Stachybotrys*, may be hidden and growing in the interior of the building.

Spores of *Cladosporium* species probably occur more abundantly worldwide than any other spore type and are the dominant airborne spores in many areas, especially in temperate climates (Lacey, 1981; Solomon and Matthews, 1988). Similar to other studies, we observed *C. cladosporioides* growing inside buildings on a variety of building materials (Ahearn et al., 1991, 1992). Even though our observations demonstrated that *C. cladosporioides* was not associated with the IAS complaint areas, its presence indicated that the conditions favored fungal growth that could potentially allow fungal genera like *Penicillium* or *Aspergillus* to become the dominant organism.

The underlying factor for SBS is the modern, sealed building with its environment controlled by a HVAC system. HVAC systems probably contribute to the onset of SBS by allowing buildup of pollutants when the capacity of the HVAC system is inadequate or has been compromised. Our observation suggests that the initial stage of interior microbial growth began with water leaks that wetted a variety of building materials. If these wetted materials are not properly mitigated, fungal growth may occur. Eventually, the HVAC system becomes contaminated. Although an understanding of the pollutants or conditions directly responsible for SBS is essential to developing strategies for prevention, a thorough analysis of the HVAC system, along

with removing or properly repairing wetted areas, were often the key to mitigating the problem in a particular building.

After remediation, the average air change per hour (ACH) was 0.5 ACH. The air samples taken at 60 days and again at 6 months after remediation had at least a 50% reduction in the number of CFU/m^3 of air from the IAS when compared to the OAS. For particles with an aerodynamic size of 2.5 µm or greater, the current evidence suggests that 1.0 ACH results in indoor concentrations of about 30% to 80% of that outdoors (Costa and Amdur, 1996).

These findings demonstrate that remediation of the buildings removed interior fungal growth. With a significant reduction in IAQ occupant complaints, our data suggest that *Penicillium* (*P. chrysogenum*) spores and *Stachybotrys* species may be strongly associated with SBS.

Table 2.1. Incidence Rates and Confidence Intervals. Incidence rates (IR) per 100 employees and 95% confidence intervals (CI) of reported complaints and symptoms regarding IAQ at forty-eight U.S. public schools between 1994 and 1996.

Type of Symptom	**IR**	**CI**	**Type of Symptom**	**IR**	**CI**
Nasal Drainage & Congestion	19.8	±1.3	**Discomfort Complaints**		
Itchy/Watering Eyes	14.3	±1.1	Odors	5.2	±0.4
(Contact Problems)	(5.6)	±1.2	Temperature (Hot/Cold)	7.2	±0.1
Headaches	12.5	±0.6	Noise	0.8	±0.3
(Sinus)	(10.3)	±0.5	Ventilation	6.1	±0.3
(Severe)	(3.4)	±0.4			
Increased Airway	14.3	±1.0	**Onset of Symptoms**		
Cough	6.5	±0.6	Entering the Building	3.4	±0.9
Shortness of Breath	5.9	±0.4	Working in the	11.0	±1.7
Sneezing	6.8	±1.0	Start of School	11.3	±1.9
Dizziness	2.2	±0.5			
Fatigue	1.1	±0.3	**When do symptoms go away?**		
Flu-like Symptoms	1.8	±0.6	Never	3.5	±0.8
Nausea	1.8	±3.4	Leave Work	2.1	±0.6
Allergies	17.0	±1.0	Weekends	4.3	±0.9
Asthma	1.4	±0.3	Vacations	14.7	±2.5
Other Health Conditions	1.2	±0.5	Medications	4.4	±0.2
When are symptoms the worst?			**When are symptoms the worst?**		
High Humidity	12.0	±0.9	Monday	0.8	±0.3
Low Humidity	0.0	±0.0	Late in Week	0.8	±0.3
Spring	3.9	±0.8	No Pattern	1.1	±0.3
Summer	0.0	±0.0	Always	2.3	±0.6
Fall	2.7	±0.6			
Winter	4.5	±0.5	**Pre-remediation**		
Start of School	5.7	±2.0	IAQ complaints or symptoms	31.3	±6.8
Morning	3.4	±0.4	**Post-remediation**		
Afternoon	1.1	±0.3	IAQ complaints or symptoms	2.5	±1.1

Table 2.2. Air Samples. Fungal profiles (air), temperature (T°), and relative humidity (RH%) in 48 public schools in the United States that were experiencing IAQ problems.

	Cl[a]	%	*Pn*[b]	%	*Al*[c]	%	Ch[d]	%	As[e]	%	Fu[f]	%	Other[g]	%	Total	T°[h]	RH%
A.	20 Sites with IAS COMPLAINT AREAS *Penicillium* sp. P<0.0001																
	OUTDOOR AIR SAMPLES																
Average	620[i]	83	20	8	5	2	32	5	2	1	4	1	9	1	692	26	39.8
SD	754	13	30	12	8	3	73	7	5	2	10	2	20	3	766	5.8	9.8
Median	298	85	14	3	0	0	5	1	0	0	0	0	0	0	401	25	46.0
	IAS COMPLAINT AREAS																
Average	53	33	205	60	4	3	3	2	4	1	1	1	13	7	269	23	49.7
SD	51	20	223	19	9	6	5	3	15	4	2	3	36	17	228	1.0	12.1
Median	42	34	98	61	0	0	0	0	0	0	0	0	0	0	141	24	52.8
	IAS NON-COMPLAINT AREAS																
Average	81	69	6	10	2	2	4	6	1	3	1	1	7	10	101	25	39.9
SD	88	25	5	9	3	4	5	10	2	7	2	3	9	13	87	2.8	10.3
Median	39	73	7	8	0	0	0	0	0	0	0	0	5	5	67	24	42.0
B.	5 Sites with IAS COMPLAINT AREAS *Penicillium* sp. P=0.10																
	OUTDOOR AIR SAMPLES																
Average	584	81	25	7	5	1	18	5	9	2	2	0	16	5	658	23	68.6
SD	847	19	26	8	11	1	28	8	21	5	6	2	34	9	852	2.9	14.9
Median	294	91	14	3	0	0	14	2	0	0	0	0	0	0	336	22	64.0
	IAS COMPLAINT AREAS																
Average	91	60	40	22	3	2	4	3	44	8	2	1	11	8	187	22	63.8
SD	89	22	49	4	6	6	8	5	121	17	6	4	18	12	213	1.6	9.4
Median	77	72	21	21	0	0	0	0	0	0	0	0	0	0	107	22	69.0
	IAS NON-COMPLAINT AREAS																
Average	123	60	8	10	1	5	11	6	0	0	1	1	22	18	167	24	55.5
SD	109	29	6	10	3	11	13	7	0	0	3	2	19	13	101	2.1	2.9
Median	109	55	7	7	0	0	7	3	0	0	0	0	14	25	196	24	55.0
C.	11 Sites with IAS COMPLAINT AREAS *Stachybotrys* sp. Isolated																
	OUTDOOR AIR SAMPLES																
Average	569	88	15	3	7	1	10	2	0	0	24	4	49	9	633	26	82.5
SD	385	12	26	5	11	2	13	2	0	0	28	6	45	11	396	2.1	10.6
Median	392	94	0	0	0	0	0	0	0	0	21	4	42	5	420	25	83.0
	IAS COMPLAINT AREAS																
Average	208	78	7	5	4	4	4	4	5	5	0	0	22	21	233	23	61.8
SD	282	23	7	6	6	7	10	8	11	12	1	0	19	21	284	1.0	5.3
Median	112	92	4	4	0	0	0	0	0	0	0	0	17	13	123	23	60
	IAS NON-COMPLAINT AREAS																
Average	46	51	2	4	1	3	1	0	0	0	0	7	4	163	232	23	63.0
SD	5	69	2	5	1	4	1	0	0	0	0	10	6	231	269	1.1	1.6
Median	46	51	2	4	1	3	1	0	0	0	0	7	4	163	232	23	63
D.	11 Sites with IAS COMPLAINT AREAS Unknown Causes																
	OUTDOOR AIR SAMPLES																
Average	1115	85	152	5	3	1	58	4	0	0	0	0	88	9	1356	25	60.4
SD	859	12	439	10	7	1	62	4	0	0	0	0	88	8	1212	2.1	2.4
Median	908	89	12	2	0	0	32	3	0	0	0	0	63	6	1148	27	53
	IAS COMPLAINT AREAS																
Average	264	89	16	4	1	1	6	2	0	0	1	0	22	9	289	23	59.9
SD	261	11	21	4	2	2	9	2	1	1	3	1	28	10	281	0.8	3.4
Median	191	93	6	3	0	0	2	1	0	0	0	0	11	7	199	24	59
	IAS NON-COMPLAINT AREAS																
Average	162	83	4	4	8	1	2	2	0	0	0	0	29	14	195	23	60
SD	226	11	6	8	20	3	6	5	0	0	0	0	30	13	254	1.5	2.5
Median	84	82	0	0	0	0	0	0	0	0	0	0	25	13	109	23	60

[a] *Cladosporium* sp.

[b] *Penicillium* sp.

[c] *Alternaria* sp.

[d] *Chrysosporium* sp.

[e] *Aspergillus* sp.

[f] *Fusarium* sp.

[g] *Epicoccum* sp., *Botrytis* sp., *Bipolaris* sp., *Acremonium* sp., *Drechslera* sp., *Rhizopus* sp., *Mucor* sp., *Rhodotorula* sp. and other unknowns.

[h] Temperature in degrees Centigrade.

[i] All values are colony forming units per cubic meter of air.

Table 2.3. Swab Samples. Fungal profiles (swab) in 48 public schools in the United States that were experiencing IAQ problems.

	Cl[a]	*Pn*[a]	*Al*[a]	*Ch*[a]	*As*[a]	*Fu*[a]	*St*[b]	Others[a]
A.	**20 Sites with IAS COMPLAINT AREAS *Penicillium* sp. P<0.0001**							
	IAS COMPLAINT AREAS							
Average	3.8[c]	4.0	0.5	0.3	0.7	0.0	0.0	0.6
SD	1.7	1.5	1.4	0.0	1.8	0.0	0.0	1.4
Median	5.0	5.0	0.0	0.0	0.0	0.0	0.0	0.0
	IAS NON-COMPLAINT AREAS							
Average	2.4	1.6	0.0	0.0	0.0	0.0	0.0	0.9
SD	1.5	1.8	0.0	0.0	0.0	0.0	0.0	1.2
Median	2.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0
B.	**5 Sites with IAS COMPLAINT AREAS *Penicillium* sp. P=0.10**							
	IAS COMPLAINT AREAS							
Average	5.0	3.8	0.0	0.0	1.4	0.0	0.0	0.9
SD	0.0	1.8	0.0	0.0	2.0	0.0	0.0	1.9
Median	5.0	5.0	0.0	0.0	0.0	0.0	0.0	0.0
	IAS NON-COMPLAINT AREAS							
Average	2.3	1.3	0.0	0.0	0.0	0.0	0.0	0.3
SD	1.2	1.5	0.0	0.0	0.0	0.0	0.0	0.6
Median	3.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0
C.	**11 Sites with IAS COMPLAINT AREAS *Stachybotrys* sp. Isolated**							
	IAS COMPLAINT AREAS							
Average	2.6	1.3	0.0	0.9	0.0	0.1	3.1	0.1
SD	2.4	2.1	0.0	2.0	0.0	0.3	2.0	0.3
Median	3.5	0.0	0.0	0.0	0.0	0.0	4.0	0.0
	IAS NON-COMPLAINT AREAS							
Average	4.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SD	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Median	4.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0
D.	**11 Sites with IAS COMPLAINT AREAS Unkown Cause**							
	IAS COMPLAINT AREAS							
Average	3.7	2.6	0.0	0.0	0.5	0.5	0.0	0.5
SD	2.2	2.5	0.0	0.0	1.6	1.6	0.0	1.6
Median	5.0	3.0	0.0	0.0	0.0	0.0	0.0	0.0
	IAS NON-COMPLAINT AREAS							
Average	4.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SD	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Median	4.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0

[a] See Table 2.2 for legend

[b] *Stachybotrys atra*

[c] All values are arbitary and based on colony forming units (cfu). 0 = no growth (0 cfu); 1 = very light growth (1-5 cfu); 2 light growth (6-10 cfu); 3 = medium growth (11-30 cfu); 4 = heavy growth (31-50 cfu); and 5 = very heavy growth (· 50 cfu). These values were determined as described in Materials and Methods.

Occupant Complaint Profile

_____ Date:_____

_____

_____ File Number:_____

Occupant Name:_____ Work Location:_____
title

This form should be used if your complaint may be related to indoor air quality (IAQ). IAQ problems include concerns with temperature control, ventilation, and air pollutants. Your observations can help to identify and resolve the problem as quickly as possible.

Nature of complaint:_____

_____

_____

Potential causes:_____

_____

Symptom Patterns
Identify the symptoms in the order they occur. If the symptom pattern has changed over time, identify the patterns individually as (a), (b), (c), etc.

What kind of symptoms and how severe are the symptoms being experienced?

_____

_____

_____

Are you aware of others with similar symptoms or concerns in your work area? ❑ Yes ❑ No

Do you have any health conditions that make you particularly susceptible to environmental problems?

❑ contact lenses
❑ allergies
❑ chronic cardiovascular disease
❑ chronic respiratory disease
❑ chronic neurological problems
❑ undergoing chemotherapy or radiation therapy
❑ immune system suppressed by disease or other causes
❑ other_____

Timing Patterns
If the symptom pattern has changed over time, identify the patterns individually with the same nomenclature as above, i.e. (a), (b), (c), etc.

When did the symptoms start?_____

_____

When are they generally worse?

_____

Do they go away? If so, when?_____

_____

Figure 2.1. Occupant Survey Form. Example of the occupant survey used by the IAQ company.

Spatial Patterns

Please use room numbers and/or area locations identified on the official building map. If symptom profiles are different in various locations, link specific areas of the buildings with associated symptoms using the same nomenclature as above, i.e. (a), (b), (c), etc.

Where are you when you experience symptoms or discomfort?_____

_____

_____

When do you spend most of your time in the building?_____

_____

_____

Additional Information

Do you have any observations about building conditions that might need attention or might help explain your symptoms (e.g., temperature, humidity, drafts, stagnant air, odors, visible pollutants)?_____

_____

_____

_____

_____

Have you sought medical attention for your symptoms? ❑ Yes ❑ No

If yes, are medical records available? ❑ Yes ❑ No

If yes, what was your doctors diagnosis?_____

_____

_____

_____

_____

_____

Do you have any other comments? (attach on separate page if more room is needed)_____

_____

_____

_____

_____

_____

Question/Answer Request

If you have a specific question you would like answered concerning the building IAQ, please include your question below and QIC will respond.

_____

_____

_____

_____

_____

Occupant Record Keeping

It is important that you record the time and date and location within the building as accurately as possible, because that will help identify conditions (e.g., equipment operation) that may be associated with your problem. Also, please try to describe the severity of your symptoms (e.g., mild, severe) and their duration (the length of time that they persist). Any other observations that you think may help in identifying the cause of the problem should be noted in the "Comments" column. Please keep this record current and submit a current copy to the building manager each quarter. The following format should be used for this record.

Figure 2.1. Occupant Survey Form. Continued.

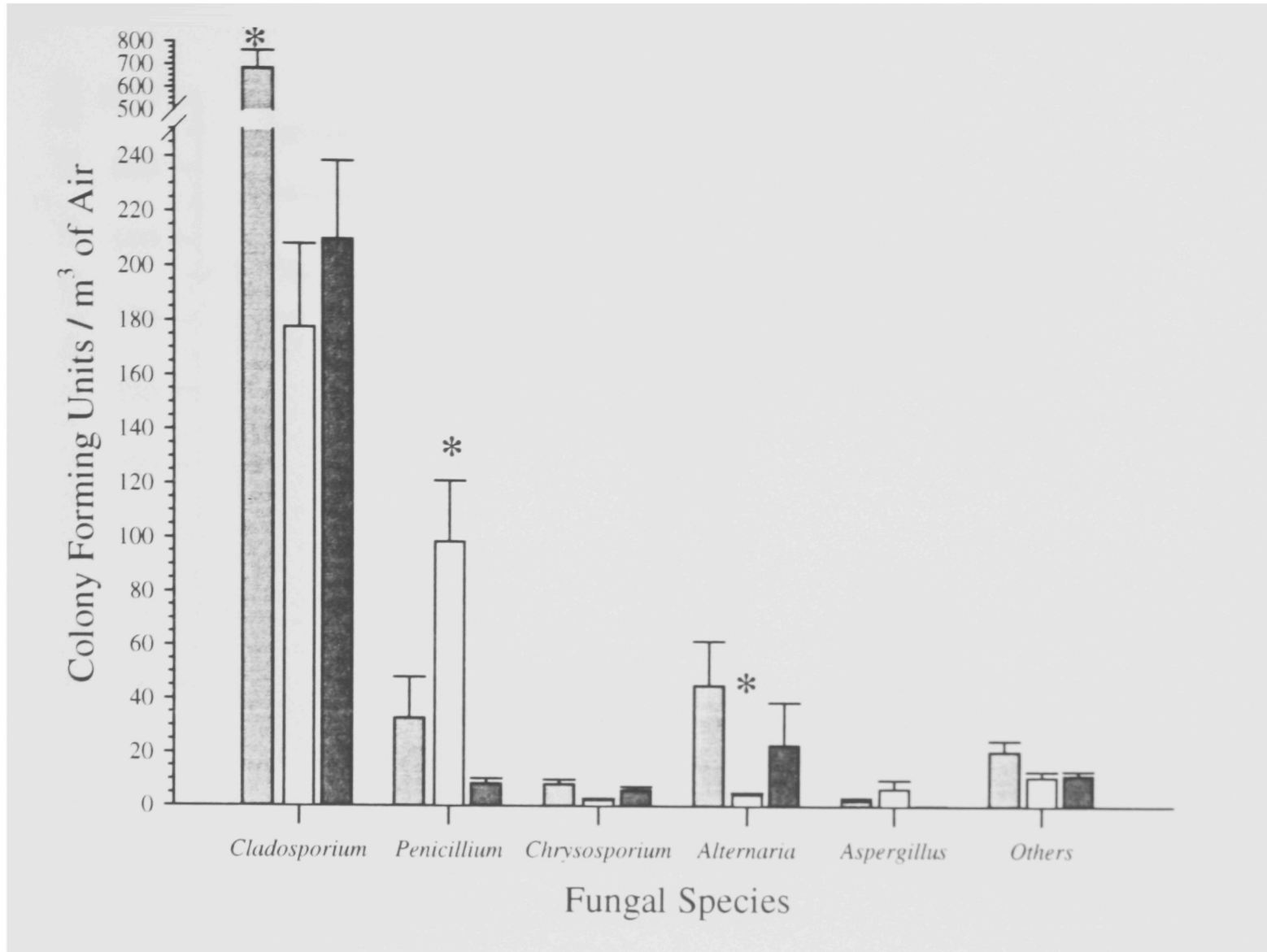

Figure 2.2. Bar Graph of all Air Samples. A bar graph of all of the air samples taken at the 48 public schools. The light grey bars represent outdoor air samples; the white bars represent indoor air samples from complaint areas; the dark grey bars represent indoor air samples from non-complaint areas. Air samples taken after remediation were similar to the outdoor air samples and the indoor air samples from non-complaint represented in this graph. The error bars represent standard error of means. The asterisk (*) represents $p < 0.05$.

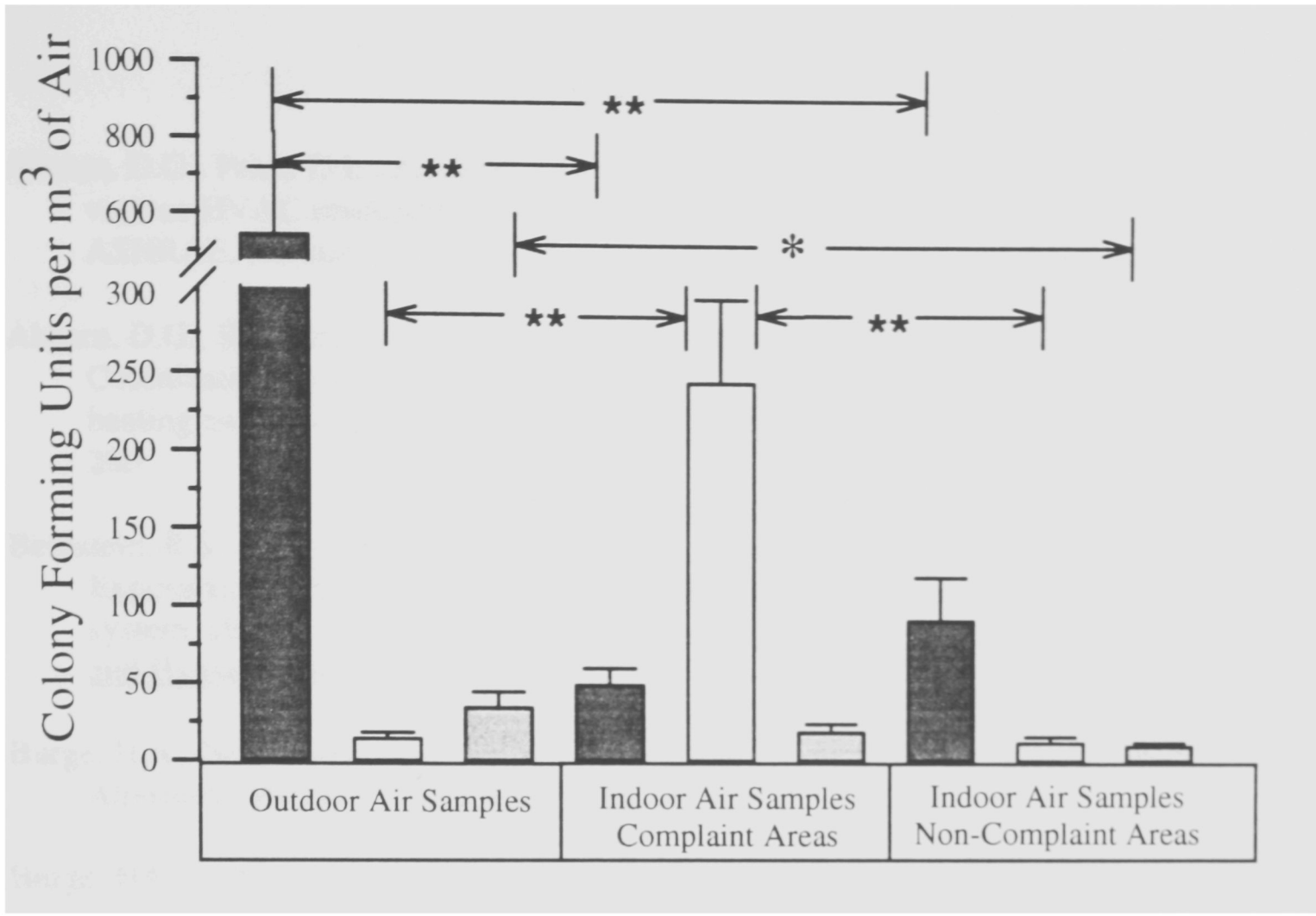

Figure 2.3. Bar Graph of 20 Schools with *Penicillium* species Dominant. The bar graph represents all of the air samples from 20 public schools where the *Penicillium* species was the dominant fungus in the indoor air samples taken in complaint areas. The dark grey bars represent the *Cladosporium* species; the solid white bars represent *Penicillium* species; the light grey bars represents all other fungi combined. The error bars represent the standard error of means. The single asterisk (*) represents $p < 0.05$ and the double asterisk (**) represents $p < 0.0001$.

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CHAPTER III

CONTINUALLY MEASURED FUNGAL PROFILES IN SICK BUILDING SYNDROME

Introduction

Sick building syndrome (SBS) is a commonly used term for symptoms resulting from indoor air quality (IAQ) problems. Complaints common to SBS include allergic rhinitis, headaches, flu-like symptoms, watering of eyes, and difficulty breathing (Ahearn et al., 1996). SBS has been recognized for a quarter of a century; the first official study of this phenomenon, which examined more than one structure, was published in 1984 (Finnigan et al., 1984).

SBS has proven difficult to understand, and no one cause has been described (Hodgson, 1992). Early researchers felt that most of the causes of SBS included higher than normal levels of known respiratory irritants. These included such compounds as hydrocarbons, nitrogen, and sulfur dioxides (NAS, 1981), chemicals being released by new buildings and their materials, or known or suspected carcinogens such as formaldehyde, asbestos, radon, and tobacco smoke (Sterling and Arundl, 1984).

It has recently come to the attention of the scientific community that fungi and their spores are associated with IAQ problems (Ahearn et al., 1997; Berstein et al., 1993; Boulet et al., 1997; Dales et al., 1991; Dill and Niggemann, 1996; Peat et al., 1993; Roby and Snelle, 1979; Senkpiel et al., 1996). In addition, fungal contamination in indoor environments has been shown to produce allergies in occupants of these buildings (Boulet et al., 1997; Dill and Niggemann, 1996; Peat et al., 1993; Senkpiel et al., 1996). We have demonstrated that there is a correlation between the prevalence of certain fungi

and SBS (Cooley et al., 1998). In that study, we showed that *Penicillium* and *Stachybotrys* species appear to be associated with SBS.

The above findings prompted us to initiate a study, in which we attempted to answer two important questions regarding IAQ. The first was, when taking an indoor air sample, is that sample an accurate reflection of the air in the building or is it just another "snapshot" that changes immediately after the "picture" is taken? The second question, a variation of the first, was, do fungal contaminated buildings stay "sick" over an extended period of time or do they "get better" and then become "sick" again.

Methods and Materials

Fungal Isolation and Identification

Measurements were made in September of 1997 in a multi-story hotel located in the Southwestern United States. The building had a history of staff and occupant complaints typical of IAQ problems, including eye irritation, irritation of the mucous membranes of the nose and throat, lethargy, and headache. On several occasions an IAQ company, called to investigate the building in response to these complaints, reported musty odors, fungal infestation in the heating, ventilation, and air conditioning (HVAC) system, and degradation of the facilities in many rooms by visible fungal growth, and they classified the structure as a "sick building." Additional follow-up studies showed the fungal infestation to be widespread throughout the building. Our research team was granted access to one room experiencing IAQ problems in the building for a 6h investigatory period. The room, a typical hotel room, showed visible fungal stains on the walls.

During the study, outside air temperature and relative humidity were 21°- 28°C and 62-74%, respectively. Inside air temperature and relative humidity were constant at 22°C and 69%, respectively. Air samples (non-aggressive) were drawn in the room, and ODA samples were taken simultaneously on an outside balcony, immediately adjacent to the room. The glass sliding door separating the balcony from the room was always kept closed during air sampling. Air samples were drawn for 5 min in triplicate by means of a two-stage bioaerosol sampler (Model 2000, Anderson Samplers Inc., Atlanta, GA). During sampling, the Anderson samplers were placed approximately 3ft above the floor level. Samples were drawn at a calibrated flow rate of 28.4 L/min for 5 min on Sabouraud dextrose agar (SDA), pH 5.6, at 1000, 1100, 1200, 1300, 1400, and 1500h inside and immediately outside the building. Burge and coworkers (Burge et al., 1977) have shown that, among the various fungal isolation media, SDA recovers the broadest range and the highest number of airborne fungal species. The agar plates were hand-carried to the laboratory. The plates were incubated for up to 14 days at 22°C and 90% relative humidity (RH). The isolated fungi were identified by standard methods (Frey et al., 1979; Larone, 1993; Rameriza, 1982; Samson and Pitt, 1990). CFU/m^3 were calculated with the formula:

$$CFU/m^3 = \{CFU/[(\text{min sampled})\ (ft^3/\text{min})]\}/[35.3\ ft^3 m^3].$$

The total fungal CFU/m^3 for each air sample was calculated and the ratio for each organism per sample determined. The results were entered according to the area that was sampled (the ODA or the IDA areas), and the average CFU/m^3 and the ratio, in terms of percentage, for each organism was determined for each area examined.

Statistics

Values for both the ODA and IDA were averaged for the six time points. A one-way analysis of variance (ANOVA) was used to test the significance of difference in the ODA and IDA measurements of *Cladosporium*, *Penicillium*, *Alternaria*, and other fungi. A P value of less than 0.05 was the minimal level of significance (Freund and Siwer, 1992). Fungal concentrations are reported as the mean $\pm$ SEM.

Results

The dominant fungi collected from the ODA were *Penicillium* species (multiple species including *Penicillium chrysogenum*), *Cladosporium* species, and *Alternaria* species (Figure 3.1). The concentration of *Cladosporium cladosporioides*, *Penicillium chrysogenum*, and *Alternaria* species in IDA were significantly different from the ODA concentrations of these same organisms. Although the concentrations varied with time, *Penicillium chrysogenum* were always the dominant organisms in the "sick" room, ranging from 150 to 567 cfu/m^3 (Figure 3.2). The three-plate range for the indoor air counts as a single collection was from 227 CFU to 535 CFU, whereas, the three-plate range for the indoor air counts at a single collection was from 156 CFU to 585 CFU. These values for *Penicillium chrysogenum* represented from 89% to 100% of the total fungi in the IDA.

In the ODA over the same time period, *Cladosporium* species were dominate (40.0- 70.6%) in four of the 6h samples (Figure 3.3), ranging from 47 to 378 cfu/m^3. In the two other ODA samples, *Penicillium* species were dominant (52.7- 79.6%), ranging

from 45 to 299 cfu/m^3. A comparison of the temporal variation of the ODA and IDA fungal profiles (Figures 3.2 and 3.3) shows that the ODA profiles vary with time and that changes occurring in the ODA have little effect on the fungal profile of the IDA.

Discussion

These data demonstrate that, while fungal profiles in ODA are continually changing, fungal profiles in a contaminated or "sick" building IDA are independent of fungal profiles in ODA. Moreover, "sick" buildings tend to stay "sick" for extended periods of time, in part because of a stasis in the IDA fungal profile. These results are in accord with our earlier study (Cooley et al., 1998) of public schools located in various areas throughout the United States.

Other workers have documented the importance of *Penicillium* species colonization of buildings and its relationship to occupant complaints. Ahearn and coworkers (Ahearn et al., 1997) demonstrated that the air-handling units and fiberglass duct liner of the HVAC system may be extensively colonized by *Penicillium* species and *Cladosporium* species, even in buildings without a history of water damage. Moreover, rooms supplied by an extensively colonized HVAC system may give rise not only to elevated concentrations of fungi, but fungal parts and metabolites, including volatile organics (Ahearn et al., 1996).

In conclusion, these results indicate that occupant complaints in sick buildings that we have studied have been associated with *Penicillium* species and that these sick buildings tend to remain sick (for at least 6h) despite changes in the outdoor fungal ecology profile. Moreover, these results indicate that single measurements of fungal

profiles in indoor air ("snapshots") may provide useful information in a building assessment.

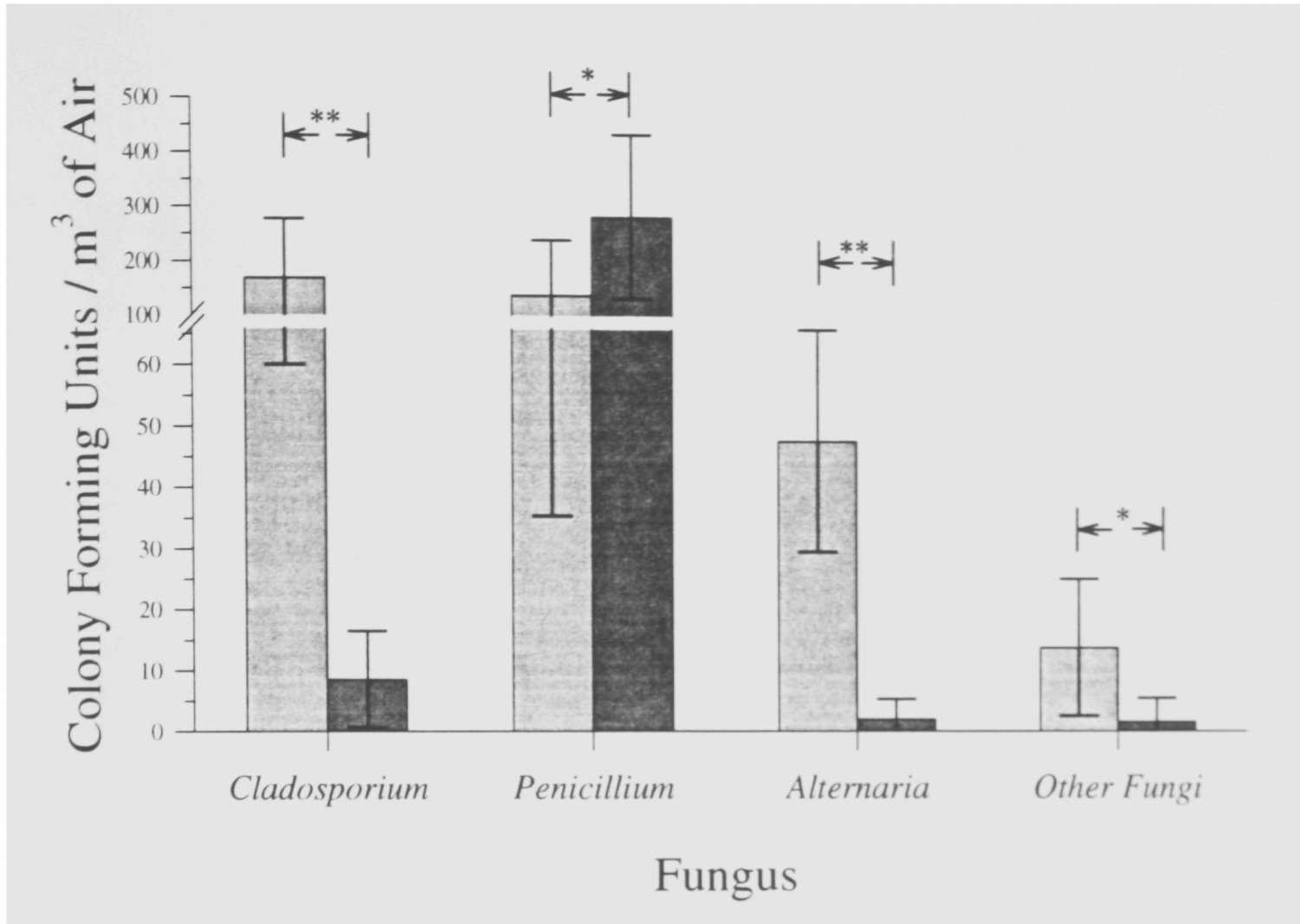

Figure 3.1. Total Indoor and Outdoor Air Fungal Concentrations. Fungal concentrations measured in indoor air and outdoor air. Each bar represents the average of all samples. The light grey bars represent the outdoor air samples and the dark grey bars represent the indoor samples. The single asterisk (*) represents P=0.01 and the double asterisk (**) represents P=0.001. Values are mean ± SEM.

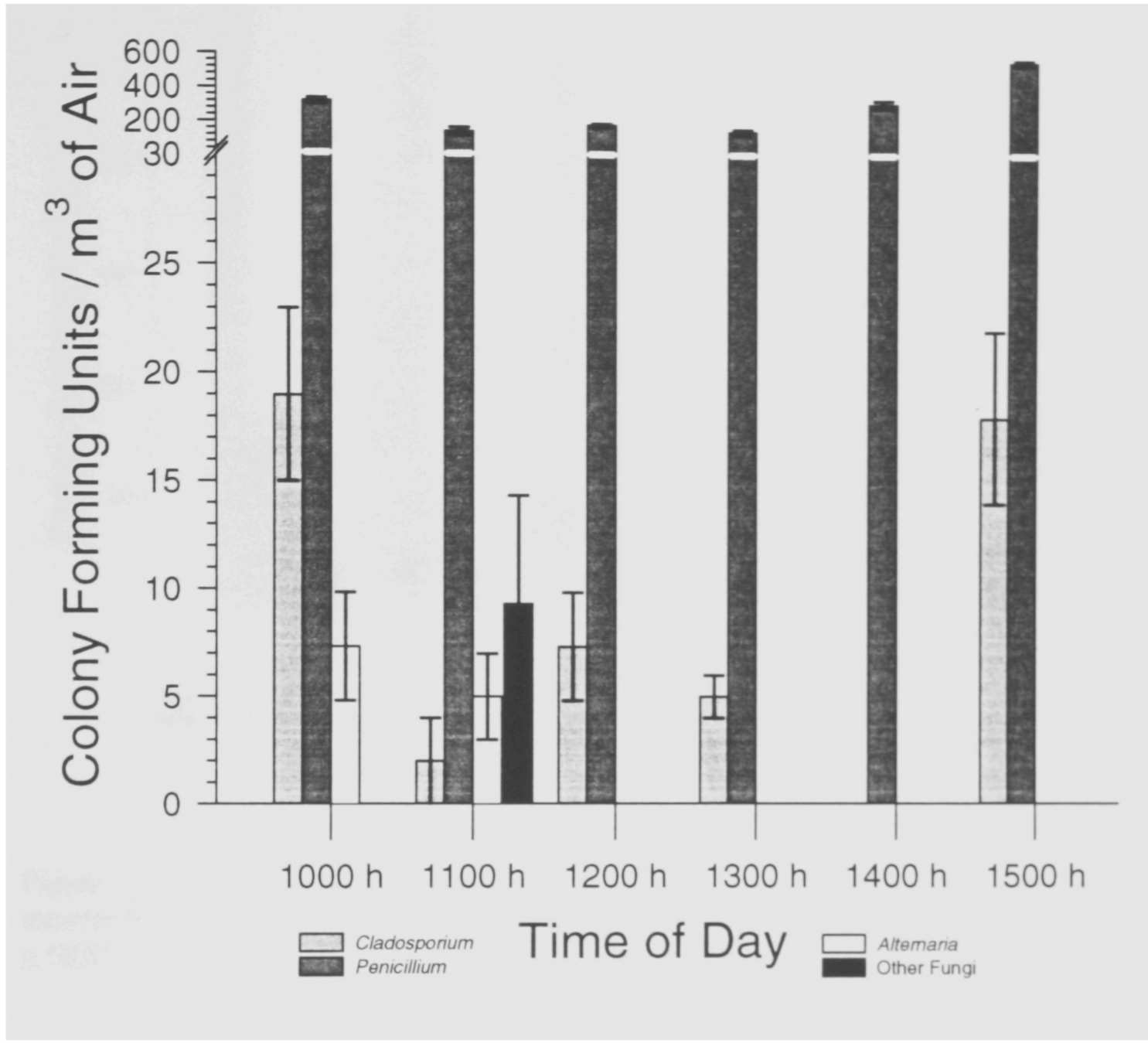

Figure 3.2. Indoor Air Fungal Profiles. At various times, the fungal profiles were measured in indoor air. Each bar represents the mean of three samples. Values are mean ± SEM.

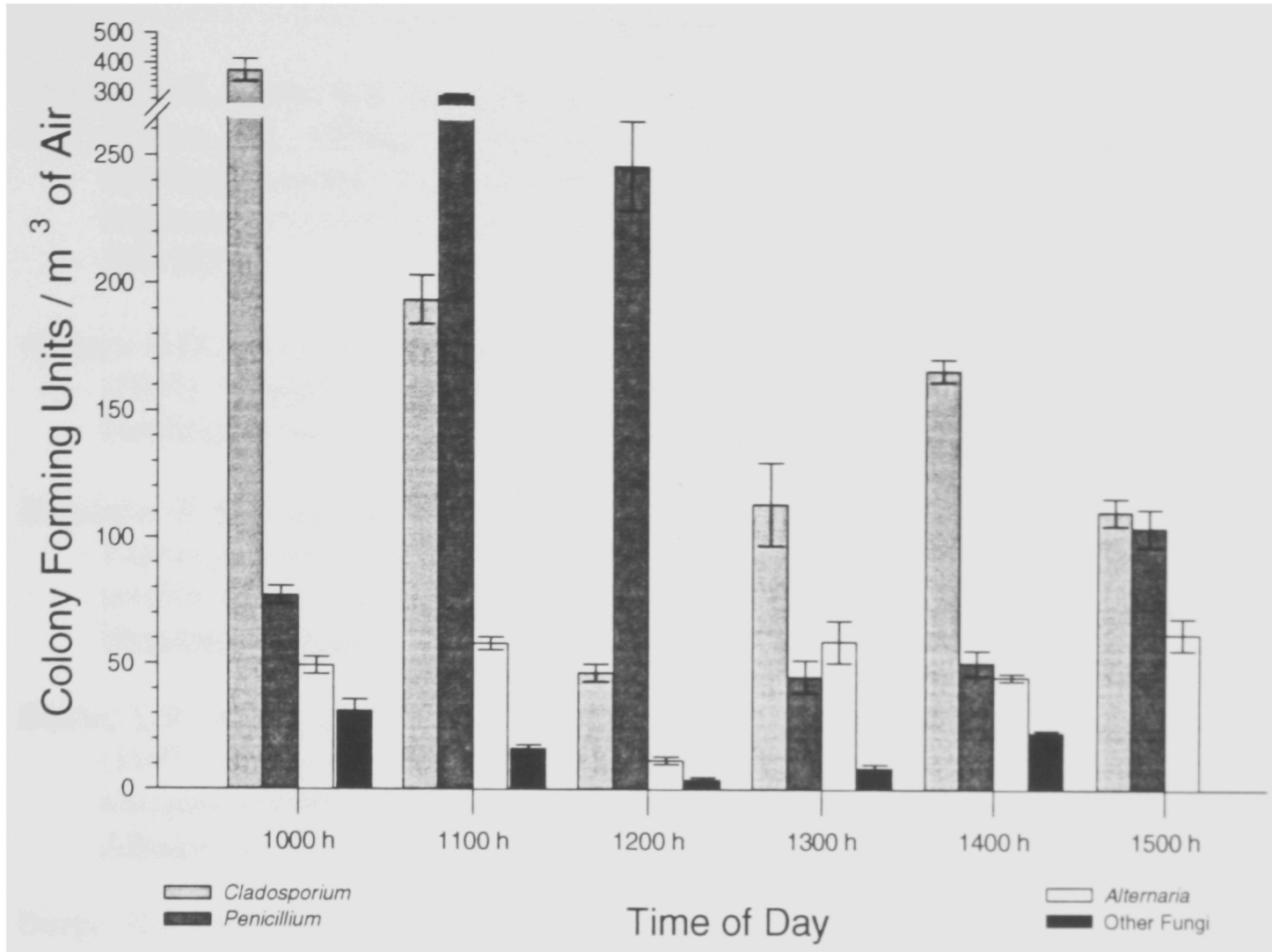

Figure 3.3. Outdoor Air Fungal Profiles. At various times, the fungal profiles were measured in outdoor air. Each bar represents the mean of three samples. Values are mean ± SEM.

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CHAPTER IV

THE PHAGOCYTOSIS OF VIABLE *PENICILLIUM CHRYSOGENUM* CONIDIA BY RAT ALVEOLAR MACROPHAGES INDUCES PRODUCTION OF TNF-α

Introduction

Sick building syndrome (SBS) is a commonly used term for symptoms resulting from indoor air quality (IAQ) problems. Complaints common to SBS include allergic rhinitis, headaches, flu-like symptoms, watering of eyes, and difficulty breathing (Ahearn et al., 1996). SBS has proven difficult to define and no single cause of this malady has been identified (Hodgson, 1992). It has recently come to the attention of the scientific community that fungi and their spores are associated with IAQ problems (Ahearn et al., 1997; Berstein et al., 1993; Boulet et al., 1997; Dales et al., 1991; Dill and Niggemann, 1996; Peat et al., 1993; Roby and Snelle, 1979; Senkpiel et al., 1996). In addition, fungal contamination in indoor environments has been shown to produce allergies in occupants of these buildings (Boulet et al., 1997; Dill and Niggemann, 1996; Peat et al, 1993; Senkpiel et al, 1996).

In a previous twenty-two month study (Cooley et al., 1998), we examined a total of 48 public schools from various parts of the United States, in which there were occupant complaints regarding health concerns and indoor air quality (IAQ). The results of this study demonstrated that *Penicillium* species, especially *P. chrysogenum*, and *Stachybotrys* species propagules were strongly associated with SBS. These studies also support earlier findings that *Penicillium* species has become an important indoor contaminant (Burge et al., 1989).

Inhalation of fungal propagules (spores and conidia) may cause infection and/or inflammation, which is dependent on the nature of the fungal propagules as well as an individual's immune status (Lequours et al., 1986; Parker et al., 1992). Many of these fungal propagules are inhaled each day and are normally cleared from the airways without any symptoms. However, an increasing respiratory burden of fungal propagules such as those seen in SBS may result in respiratory inflammation (Lequours et al., 1986). *P. chrysogenum* conidia are small (1 to 5 μm) and are capable of entering the lower respiratory tract and the alveolar spaces (Brain and Valberg, 1979). It has been shown that bronchial challenges with *Penicillium* species conidia induced immediate- and delayed-type asthma in sensitized subjects (Licorish et al., 1985). Fungal spore-mediated inflammation may be caused by either antibody-dependent or antibody-independent mechanisms. Of the two, less is known about antibody-independent inflammation. Organic dust toxic syndrome (ODTS), an example of an antibody-independent pulmonary inflammation, is associated with exposure to high levels of organic dust, especially organic dusts composed predominately of fungal spores (Shahan et al., 1998). ODTS is characterized by neutrophilic infiltration into the airways with systemic flu-like symptoms occurring 4-8h after exposure (Lequours et al., 1986; Parker et al., 1992). These symptoms are similar to those observed in SBS.

Once a particle enters the alveolar spaces, the pulmonary alveolar macrophages (PAM) are the respiratory system's first line of defense and are known to contribute to inflammation by producing both lipid and protein mediators. We hypothesized that the *P. chrysogenum* conidia contribute to the inflammatory response by inducing the PAMs to produce inflammatory cytokines, such as tumor necrosis factor-α (TNF-α). TNF-α is

an acute phase inflammatory agent that can initiate the coordinate upregulation of cytokines necessary for the maintenance and propagation of the inflammatory process (Elias and Zitnik, 1992). To evaluate this hypothesis, we conducted in vitro experiments using rat alveolar macrophages (RAM) and various concentrations of *P. chrysogenum* conidia and tested for TNF-α production using a sandwich ELISA specific for rat TNF-α.

Materials and Methods

Growth Study

The conidia inocula were prepared from cultures grown 7 days on Sabouraud's dextrose agar (SDA). The conidia were harvested by a gentle washing with sterile ultra-pure phosphate buffer saline (PBS) plus 0.1% Tween 20, washed two times by sedimentation using centrifugation (10,000xg, 10 min), resuspended with PBS, and vortexed extensively. The conidia were then sonicated for 10s to disrupt clumping and filtered through sterile guaze to remove any clumps. After the conidia were singly dispersed, the conidia concentrations were determined using a Neubauer hemacytometer and viability confirmed by plate counts on SDA. The conidia concentration was adjusted to yield $1x10^6$ conidia/ml. The conidia (100µl) were then streaked onto SDA plates (in triplicate) and incubated in the dark at room temperature (RT) at 90% relative humidity (RH). At different times, the conidia were harvested as described above and the viability determined.

Conidia Preparation

The conidia were prepared from cultures grown 5 days on SDA. The conidia were harvested and processed as described above. Groups of conidia were rendered non-viable by exposure to ultra-violet (UV) irradiation for four hours or incubation in absolute methanol for five minutes. All of the conidia were washed two times by sedimentation using centrifugation (10,000 x g, 10 min), resuspended with PBS, and vortexed extensively. The conidia were then sonicated for 10s to disrupt clumping and filtered through sterile guaze to remove any clumps. After the conidia were singly dispersed, the conidia concentrations were determined using a Neubauer hemacytometer and viability confirmed by plate counts on SDA.

In Vitro RAM Viability

To examine the RAM viability, Promega's CytoTox 96® Assay was used to indirectly measure the release of lactate dehydrogenase (LDH), a cytosolic enzyme present in viable mammalian cells.

After the incubation media was removed, 100µl of Eagle medium without supplements were added to each well. To lyse the RAMs, the plates were incubated at -80°C for approximately 30 min, followed by thawing at 37°C for 15 min and this process was repeated. The plates were then centrifuged (250 x g, 4 min) to pellet cellular material. To conduct the enzymatic assay, 50µl from each well was transferred to a fresh, flat bottom microplate and the assay was conducted as per Promega's instructions. Briefly, 50µl of the substrate mixture was added to each well. The plates were covered

with foil to protect the substrate from light and incubated at room temperature (RT) for 30 min. Fifty microliters of the stop solution was added to each well and the absorbance was read on a microtiter plate spectrophotometer (DynaTech MR 4000) at 490nm.

The absorbance readings from the control wells containing known numbers of RAM ($1x10^5$, $1x10^4$, and $1x10^3$) were plotted and a standard curve was generated from a linear regression using a Sigma Plot computer program. The numbers of viable RAMs from the experiments were interpolated from this standard curve.

In Vitro Phagocytosis Assays

After each rat was euthanized by an overdose of halothane, the trachea was exposed, and a 22-gauge angiocatheter was inserted into the trachea and tied in place with suture. The lungs were lavaged by slowly instilling 10ml of sterile endotoxin-free Hanks balance salt solution (HBSS) that had been warmed to 37°C and then slowly removing the HBSS. This was repeated a total of five times. The total number of viable rat alveolar macrophages (RAM) in the bronchioalveolar lavage (BAL) was determined using a Trypan Blue Exclusion (TBE) assay and counting on a hemacytometer. The BAL was centrifuged (1000 x g, 10 min) to sediment the cells. Erythrocytes were removed by immersing the cell pellet in a hypertonic saline solution (1.6% NaCl) and centrifugation (1000 x g, 10 min) to sediment the cells. The BAL cells were suspended in Eagle medium without calcium (Ca^{++}), magnesium (Mg^{++}), and L-glutamine and supplemented with 5% (v/v) sterile endotoxin-free heat-inactivated fetal bovine serum (FBS) to yield $1x10^6$ RAM per ml. The TBE assay was repeated. One hundred microliters containing approximately $1x10^5$ viable RAM were added to each well of microtiter plates and

incubated for 2 hours at 37°C, 5% CO_2, and 95% RH to allow adherence. The wells were washed extensively with Eagle medium to remove non-adherent cells and 190µl of sterile Eagle medium supplemented with Ca^{++}, Mg^{++}, and L-glutamine and 10% FBS was added to each well. After one hour of incubation, control wells (minimum of 3 wells per plate) were examined for RAM viability using the cytotoxicity assay. Viable and non-viable *P. chrysogenum* conidia (suspended in 10µl PBS) were added to the RAM wells (RAM to spore ratios were 1:1, and 1:10), and, in some experiments, lipopolysaccharides (LPS) (suspended in 10µl PBS) were added to the RAM wells (0.01ng/ml, 0.1ng/ml, and 1.0ng/ml). The plates were incubated at 37°C, 5% CO_2, and 95% RH for up to 8 hours. At various times (1h, 2h, 4h, 6h, or 8h), the supernatant was removed. In some of the experiments, RAMs, including control wells, were assayed for viability using the TBE or the cytotoxicity assay. For the experiments utilizing the cytotoxicity assay to measure cell viability, control wells containing $1x10^5$, $1x10^4$ and $1x10^3$ RAMs were plated to produce a standard curve to enumerate the number of viable RAMs in the experiment. The number of viable RAMs were interpolated from the standard curve, divided by their original count and multiplied by 100 to obtain percent viability. The supernatant was passed through a 0.22µm filter and tested for TNF-α. The phagocytosis assays were repeated three times.

In Vitro TNF-α Analysis

The supernatants from the phagocytosis experiments were assayed for TNF-α using a sandwich ELISA specific for rat TNF-α. Briefly, in these assays, flat-bottom 96-

well microtiter plates (Nunc Maxisorb) were coated overnight at 4°C with anti-rat TNF-α capture antibody (Pharmagene). Each plate was washed with PBS+0.1% Tween 20 (PBS-T), and blocked with PBS+3.0% bovine serum albumin (BSA). After four washes with PBS-T, eight two-fold dilutions in duplicate of the appropriate control or standard and the test BAL was added to the respective wells. The plates were incubated for 2 hours at RT, washed four times with PBS-T, and anti-rat TNF-α biotinylated detecting antibody (mAb) (Pharmagene) added and incubated for 1 hour at RT. The plates were washed four times with PBS-T, avidin-horseradish peroxidase (HRP) was added, and the plates were incubated for 1 hour at RT. Plates were washed an additional 4 times with PBS- and 2, 2'-Azino-bis (3-ethylbenzthiozoline-6-sulfonic acid) plus 30% hydrogen peroxide. After 20-30 min, the HRP enzyme reaction was stopped by adding 2mM sodium azide. Absorbance was read on a microtiter plate spectrophotometer (DynaTech MR 4000) at 410nm. The optical densities of the unknown samples were interpolated from a standard curve using a Sigma Plot computer program.

In Vivo Acute Exposures

Female BALB/c mice (5 to 6 weeks of age) and female C57Bl mice (5 to 6 weeks of age) were used in these experiments. The BALB/c mice were lightly anesthetized with oxygen and halothane and 50µl of fluid (25µl/nostril) were instilled in both nostrils. The control mice received 50µl of PBS intranasally (IN) and no fungal conidia. One group of mice received $1x10^5$ viable conidia (25% viability). The final group of mice received

$1x10^5$ conidia either render non-viable by UV-irradiation or methanol incubation. The C57Bl mice were inoculated using the same procedure as the BALB/c mice.

After inoculation of the conidia into the nostrils of lightly anesthetized mice, the animals were held in the upright position for 2 min for complete inhalation of the conidia and resumption of normal breathing. Twenty-four hours after the inoculation, the mice were euthanized by an overdose of halothane, and a 24 gauge angiocatheter was inserted into the trachea and tied in place with suture. The lungs were lavaged by slowly instilling 1ml of sterile endotoxin-free HBSS that had been warmed to 37°C and slowly removing the HBSS. This was repeated a total of 4 times. The BAL cells were pelleted by centrifugation (1000 x g, 10 min). The BAL cells were suspended in PBS and the alveolar macrophages were enumerated using the TBE assay and counting on a Neubauer hemacytometer. The BAL fluid was adjusted to yield $2x10^5$ macrophages per ml and 200µl was centrifuged (Cytospin) to pellet the BAL cells onto cytospin slides ($4x10^4$ macrophages per slide). The slides were fixed by immersing the slide into absolute methanol for one minute, stained with Wright-Giemsa stains, and a cell differentiation count (CDC) performed by counting 1000 BAL cells to determine the type of cells in the airway.

Statistics

Data were analyzed by a computer program (Sigma Stat) employing the Mann-Whitney rank sums test (U test), the Kruskal-Wallis one-way ANOVA (H test), Spearman's product moment correlation, and Dunn's multiple comparisons and partial correlations (Freund and Simen, 1992).

Results

Growth Study

We conducted growth studies to determine the ideal time to harvest conidia where we could obtain the highest number of viable conidia after the conidia were singly dispersed. Conidia harvested on the fifth day produced the highest number of singly dispersed conidia with the highest average viability (24.5% ±7.3%) (Figure 4.1).

In Vitro RAM Viability

In vitro experiments using RAM obtained from a lung lavage and attached to microtiter plates were conducted. One group of *P. chrysogenum* conidia was rendered non-viable. Viable (25% viability) and 100% non-viable conidia were incubated with the RAM (conidia to RAM ratios of 1 to 1 and 1 to 10) or with LPS (0.01ng/ml, 0.1ng/ml, and 1.0ng/ml) at 37°C, 5% CO_2, and 95% relative humidity (RH) for 8 hours. At various time periods, the RAMs were assayed for viability using the cytotoxicity assay (Figure 4.2). The results of a microscopic examination of the assay suggested that viable *P. chrysogenum* conidia were causing the RAM to clump while non-viable conidia did not. However, the viability of the RAM were not significantly decreased.

TNF-α Assays

To determine if the conidia was capable of inducing TNF-α, in vitro experiments using RAM obtained from a lung lavage and attached to microtiter plates

were conducted. The RAMS were incubated with LPS (0.01ng/ml, 0.1ng/ml, and 1.0ng/ml) at 37°C, 5% CO_2, and 95% relative humidity (RH) for 8 hours. One group of *P. chrysogenum* conidia was rendered non-viable. Viable (25% viability) and 100% non-viable conidia were incubated with the RAM (conidia to RAM ratios of 1 to 1 and 1 to 10) at 37°C, 5% CO_2, and 95% relative humidity (RH) for 8 hours. The media was removed, filtered through a 0.22μm centrifuge filter tube and examined for TNF-α using a sandwich ELISA specific for rat TNF-α. The results demonstrated that LPS induced a significant ($P<0.05$) release of TNF-α (Figure 4.3). The viable conidia also exhibited an increase in TNF-α, with the 1:1 ratio of viable *P. chrysogenum* conidia inducing a TNF-α response that was significantly greater than what the 1.0ng/ml dose of LPS induced (Figure 4.4). The non-viable conidia did not induce a significant increase in TNF-α.

Acute In Vivo Exposures

Female BALB/c mice and C57Bl mice were instilled intranasally (IN) with $1x10^6$ *P. chrysogenum* (viable and non-viable) conidia suspended in 50μl of phosphate buffer saline (PBS) (25μl per nare). Twenty-four hours after the acute IN inoculation, the mice were euthanized and a lung lavage (BAL) was conducted. The airway cells were pelleted, suspended in PBS and pelleted onto a Cyto-spin slide. The BAL cells were stained and cell differentiation counts (CDC) were performed. The results of this experiment suggested that viable conidia that were IN inoculated into the BALB/c mouse model could induce significant increases ($p < 0.002$) in airway neutrophils

when compared to the controls (PBS only) and 100% non-viable conidia (Figure 4.5). No eosinophils were recovered from any of the samples. There were no significant differences between the conidia render non-viable by either UV-irradiation or methanol. However, in the C57Bl mouse model, there were no differences in the CDC in any of the groups with the exception of the group receiving viable conidia (Figure 4.6). The animals in that group had a significant increase ($P < 0.01$) in airway eosinophils, but there was not a significant increase in airway neutrophils.

Discussion

The growth studies suggest that the viability of conidia could be a factor. Not all spores produced by a fungal colony are viable (Burge et al., 1977). Sporulation is triggered by a decrease in nutrients. As a colony ages, conidia are produced, using the stored essential nutrients. Some of these nutrients that are essential for conidia viability, may be in short supply, and eventually become depleted. When this happens, the conidia will become non-viable. Viable conidia do not look or stain different from non-viable conidia. Counting the conidia on a hemacytometer and then using serial dilutions and plating the conidia on a medium, the viability, in terms of percentage, can be determined.

The in vitro results indicated that low doses of the conidia were phagocytized. However, as the concentration of viable spores increased, the PAMs began to clump, but the RAM viability was not significantly decreased. All of the conidia that were rendered non-viable by various methods demonstrated no clumping or cell death. The TNF-α ELISA results demonstrated that the viable conidia induced the production of TNF-α that

was significantly higher than the LPS supernatants. The non-viable conidia did not induce any significant increases in TNF-α.

The in vivo acute studies suggest that viable *P. chrysogenum* conidia induce inflammatory reactions (airway neutrophilia) in the BALB/c mouse model. The C57Bl mouse model did not demonstrate airway neutrophilia. However, this mouse model did have a significant increase in airway eosinophils. When antigens are instilled IN, the BALB/c mouse model tends to be a low IgE responder, whereas the C57Bl mouse model tends to be a high IgE responder (Holt et al., 1987). An increase in airway eosinophils is an indication of an allergic response. This suggests that long-term exposure to low doses of viable *P. chrysogenum* conidia may be capable of inducing allergic symptoms (increases in serum IgE along with peripheral and airway eosinophilia) in the C57Bl mouse model and warrant a more detailed examination.

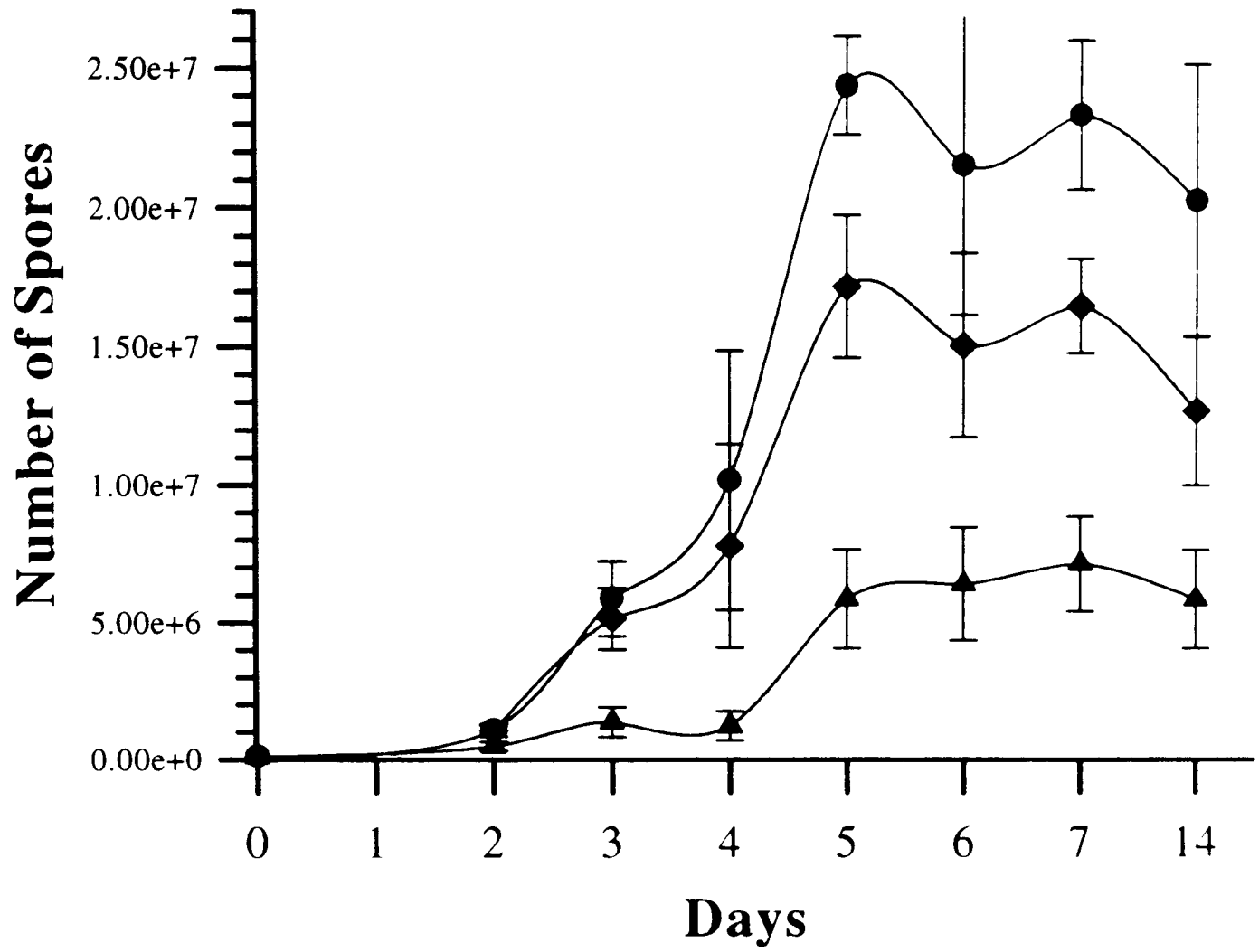

Figure 4.1. *Penicillium chrysogenum* Growth Study. *P. chrysogenum* conidia were inoculated onto SDA, pH 5.6, and incubated at 22°C. At various time periods, the conidia were harvested by a gentle wash using PBS. The conidia were sonicated for 10s to disrupt clumping and filtered through sterile gauze to remove debris and clumps. After the conidia were monodispersed, the conidia concentrations were determined using a Neubauer hemacytometer. The viability of the monodispersed conidia was confirmed by serially dilutions onto SDA and incubated at 22°C. The circle (●) represents the total conidia count. The diamond (◆) represents the non-viable conidia. The triangle (▲) represents the viable conidia. The error bars represent the standard deviation (SD).

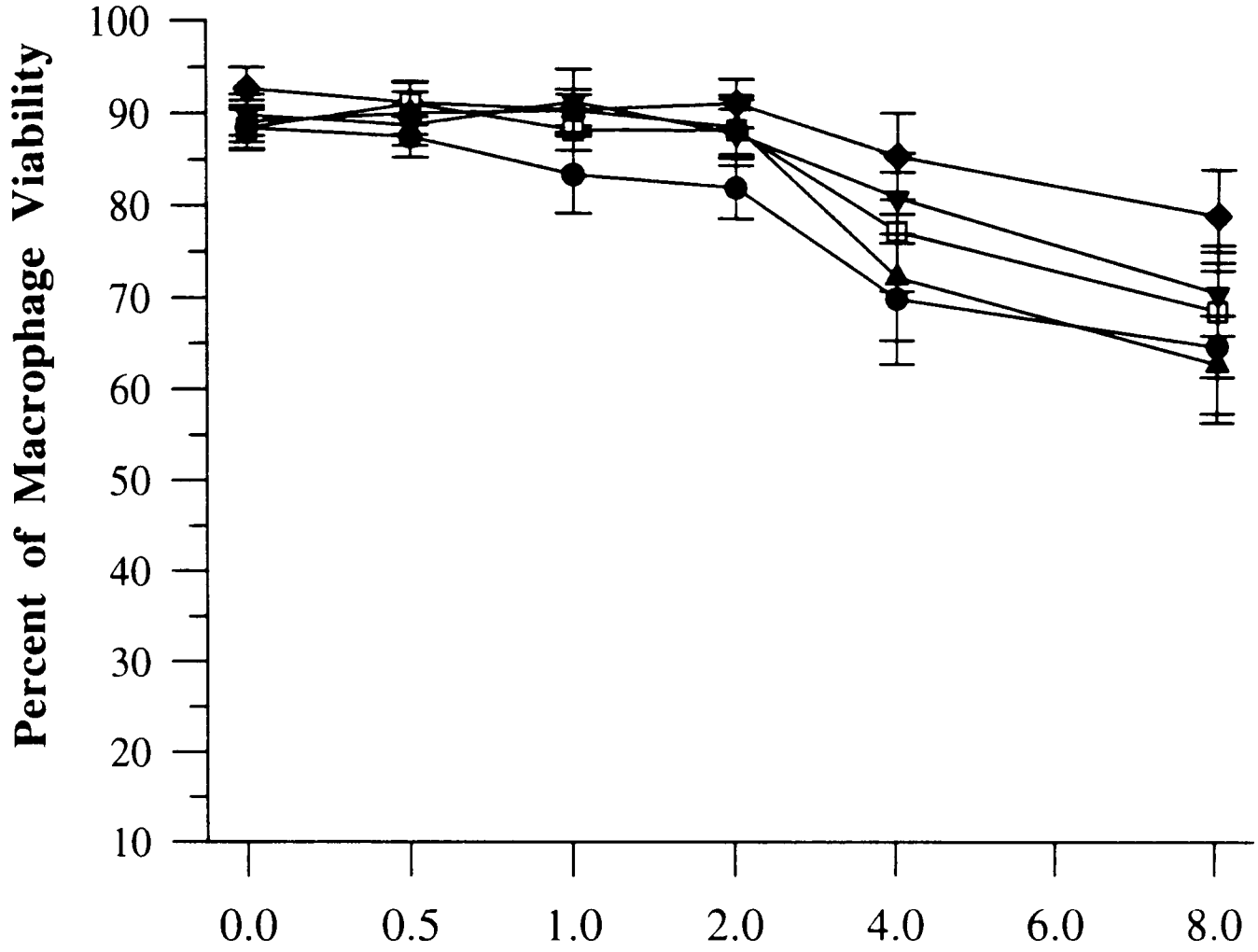

Figure 4.2. Viability of Rat Alveolar Macrophage after In Vitro Inoculations with Viable and Non-viable *P. chrysogenum* Conidia. Rat alveolar macrophages (RAM) were obtained from a lung lavage and plated to microtiter plates. Viable and non-viable *P. chrysogenum* conidia were incubated with the RAM (conidia to RAM ratios of 1:1 and 1:10) at 37°C, 5% CO_2, and 95% RH for up to 8 hours. At various time periods, the RAMs were assayed for viability using the cytotoxicity assay. The diamond (◆) represents the Controls which received PBS only. The inversed triangle (▼) represents animals that received 1:1 non-viable conidia. The square ( ) represents animals that received 1:10 non-viable conidia. The triangle (▲) represents animals that received 1:1 viable conidia. The circle (●) represents animals that received 1:10 viable conidia. The error bars represent the standard deviation (SD).

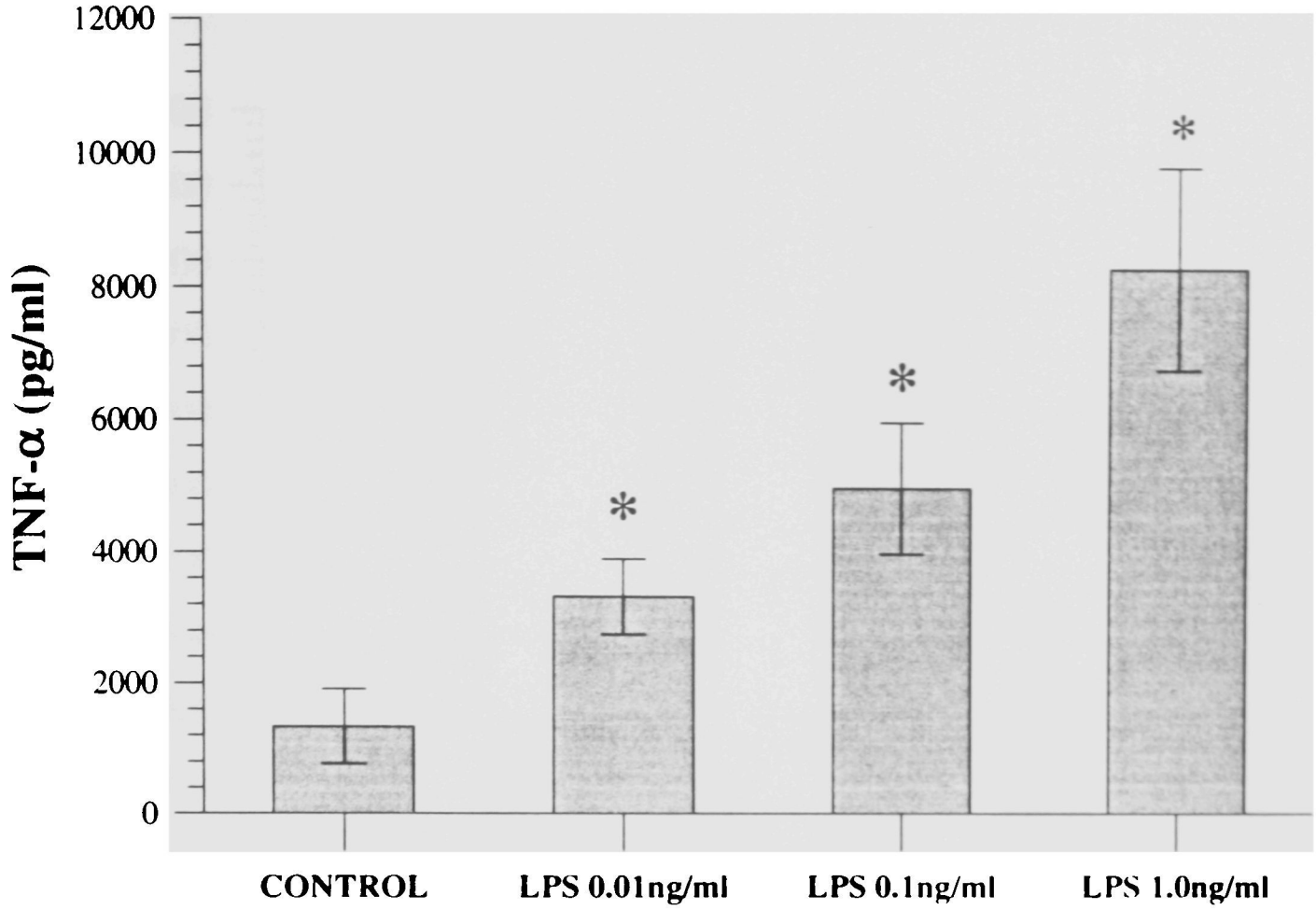

Figure 4.3. In Vitro Analysis of TNF-α after Incubation of Rat Alveolar Macrophages and LPS. Rat alveolar macrophages (RAM) were obtained from a lung lavage and plated to microtiter plates. Various amounts of LPS was incubated with the RAM (0.01 ng/ml, 0.1 ng/ml, 1.0 ng/ml) at 37°C, 5% CO_2, and 95% RH for 8 hours. The CONTROLS received media only. The media was removed from each well, filtered, and assayed for TNF-α using a sandwich ELISA assay specific for rat TNF-α. The single asterisk (*) represents $P<0.05$ as compared to controls. The error bars represent standard deviation.

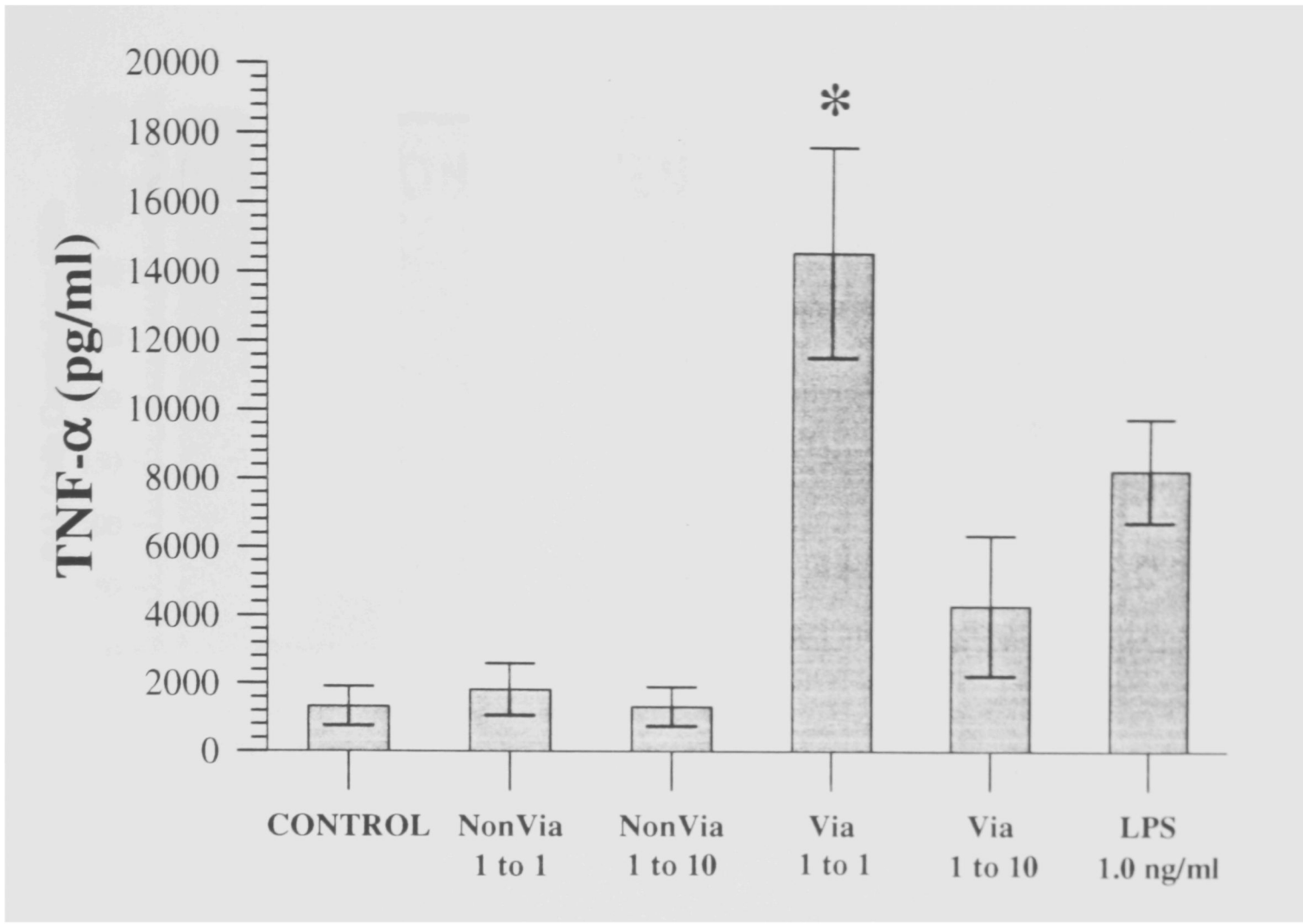

Figure 4.4. In Vitro Analysis of TNF-α after Incubation of Rat Alveolar Macrophages with Viable and Non-viable *P. chrysogenum* conidia. Rat alveolar macrophages (RAM) were obtained from a lung lavage and plated to microtiter plates. Viable and non-viable *P. chrysogenum* conidia were incubated with the RAM (conidia to RAM ratios of 1:1 and 1:10) at 37°C, 5% CO_2, and 95% RH for up to 8 hours. The CONTROLS received media only. The media was removed from each well, filtered, and assayed for TNF-α using a sandwich ELISA assay specific for rat TNF-α. The LPS (1.0 ng/ml) was taken from Figure 4.3 to demonstrate a comparison. The single asterisk (*) represents $P<0.05$ as compared to controls. The error bars represent standard deviation.

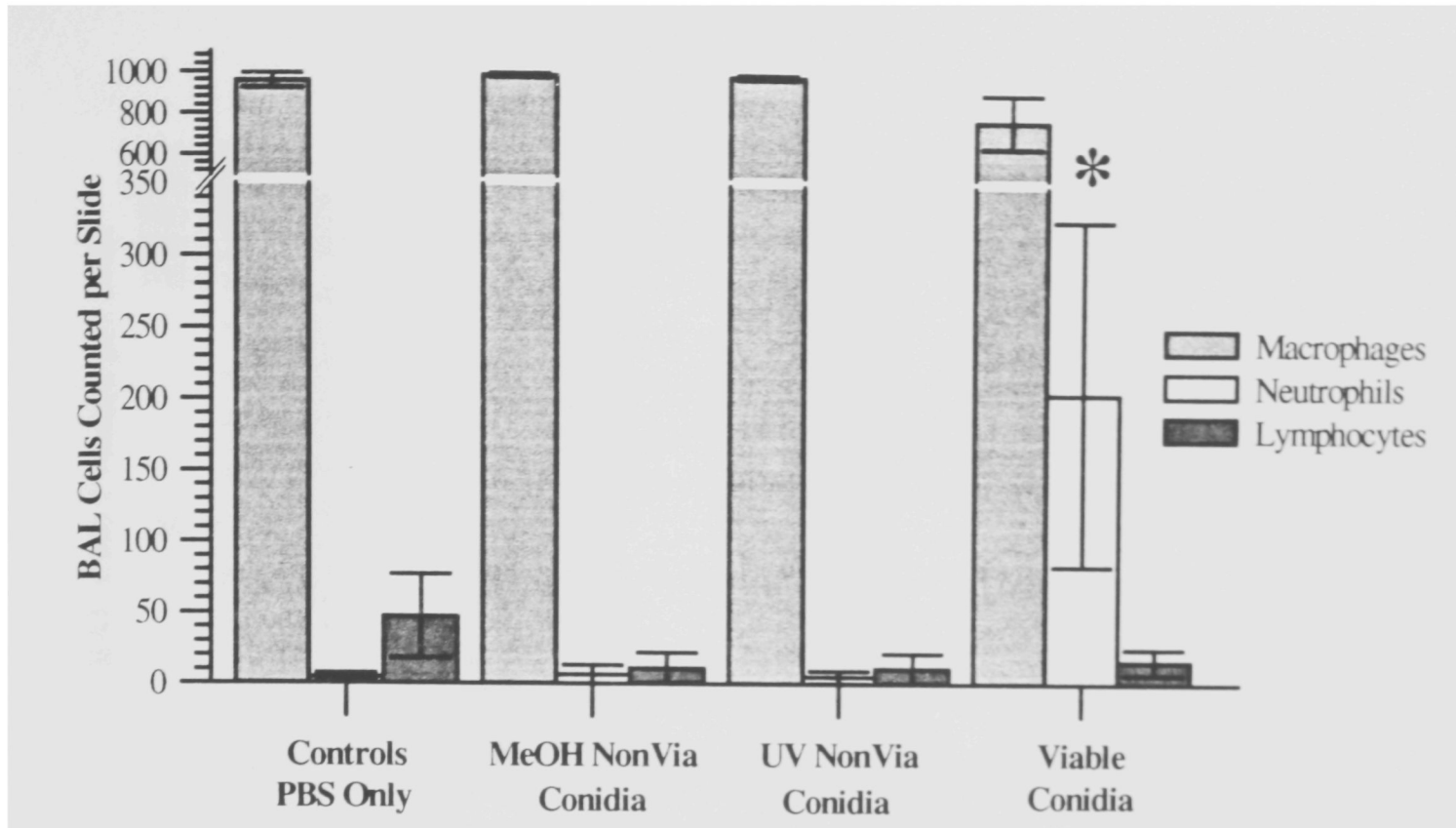

Figure 4.5. In Vivo Analysis of BAL Cells obtained from BALB/c Mice after an Acute Intranasal Inoculation of Viable and Non-viable *P. chrysogenum* conidia. BALB/c female mice were inoculated IN with $1x10^5$ *P. chrysogenum* (viable and non-viable) conidia suspended in 50µl of PBS. Twenty-four hours after the acute IN inoculation, the mice were euthanized and a lung lavage was conducted. The airway (BAL) cells were pelleted onto slides, stained, and a CDC was performed. The Controls received PBS only. A total of 1000 BAL cells were counted per slide per mouse. The error bars represent the standard error of means. The asterisk (*) represents $P < 0.002$.

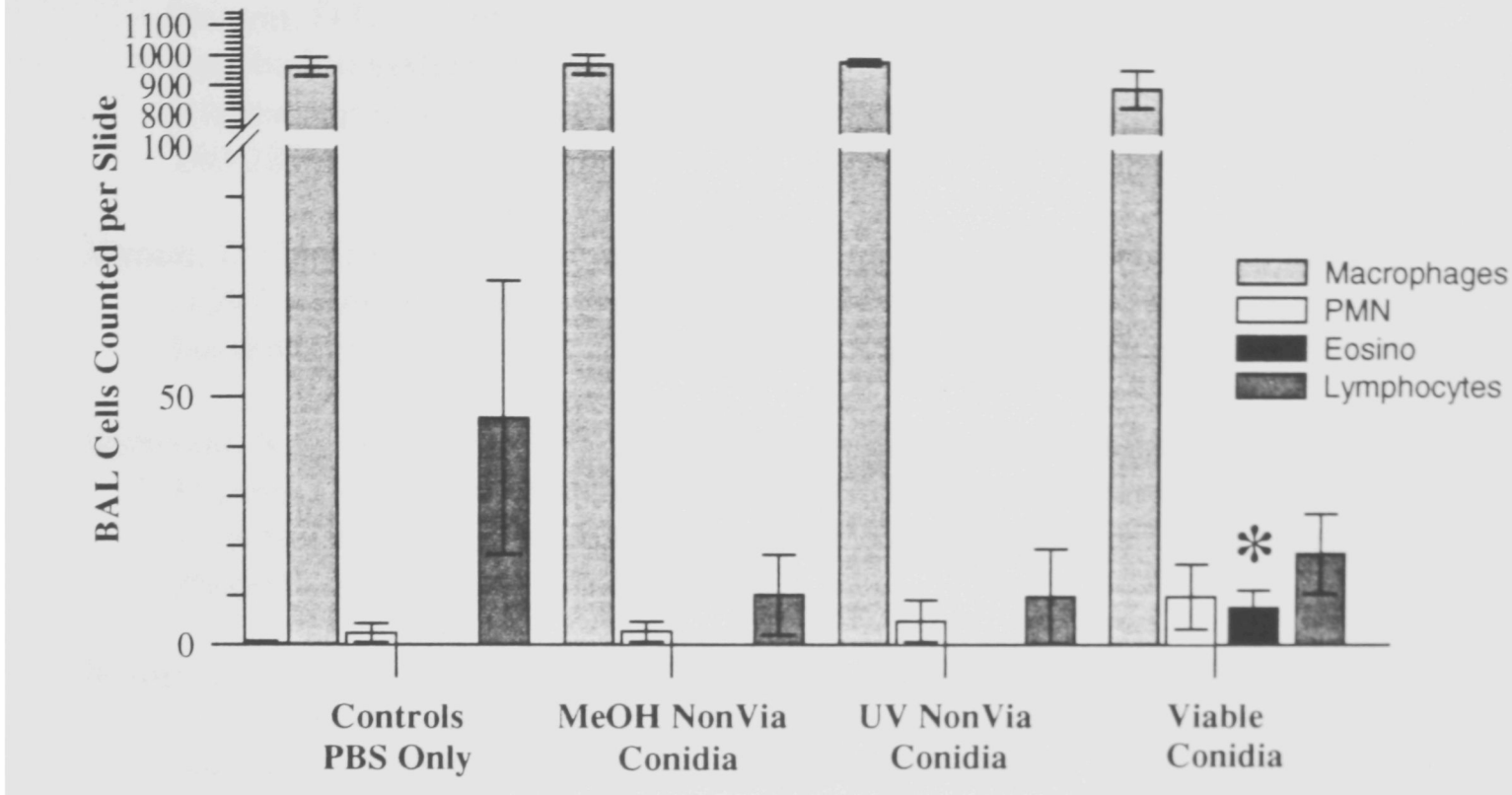

Figure 4.6. In Vivo Analysis of BAL Cells Obtained from C57Bl/6 Mice after an Acute Intranasal Inoculation of Viable and Non-viable *P. chrysogenum* conidia. C57Bl female mice were inoculated IN with $1x10^5$ *P. chrysogenum* (viable and non-viable) conidia suspended in 50µl of PBS. Twenty-four hours after the acute IN inoculation, the mice were euthanized and a lung lavage was conducted. The airway (BAL) cells were pelleted onto slides, stained, and a CDC was performed. The Controls received PBS only. A total of 1000 BAL cells were counted per slide per mouse. The error bars represent the standard error of means. The asterisk (*) represents $P < 0.01$.

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CHAPTER V

CELLULAR AND HUMORAL RESPONSES IN AN ANIMAL MODEL INHALING *PENICILLIUM CHRYSOGENUM* CONIDIA

Introduction

Numerous studies have attempted to elucidate the cause of sick building syndrome (SBS) (Finnigan et al., 1984; Feder, 1985). While no one cause for the symptoms induced by indoor air quality (IAQ) problems is likely to exist, the presence of fungi in sick buildings is becoming consistently associated with this problem (Mishra et al., 1992; Miller, 1992).

In a previous study, we presented evidence that demonstrated that *Penicillium* species, especially *P. chrysogenum*, and *Stachybotrys* species are strongly associated with SBS (Cooley et al., 1998). These studies also supported earlier findings that *Penicillium* species have become an important indoor contaminant (Burge et al., 1989). It has been shown that bronchial challenges with *Penicillium* species conidia induced immediate and delayed-type asthma in sensitized subjects (Verhoeff et al., 1995). These allergic reactions appear to result from the inhalation of fungal products, but the mechanism(s) responsible for these phenomena remain unclear. In another previous study, we demonstrated that viable *P. chrysogenum* conidia induced a TNF-α response in RAMs in vitro while non-viable conidia did not. These findings also demonstrated that an acute dose of viable conidia instilled intranasally (IN) in a C57Bl mouse model induced an inflammatory response that consisted of eosinophils and neutrophils. This study was designed to examine: (1) the deposition, clearance, and retention of *P. chrysogenum*

conidia introduced IN in a murine model and (2) the consequences of the instillation of *P. chrysogenum* conidia in the mammalian lung by using a mouse model.

Materials and Methods

Conidia

P. chrysogenum was grown on Sabouraud's Dextrose Agar (SDA), pH 5.6, for 5 days. The conidia were harvested by gentle washing with sterile endotoxin-free phosphate buffered saline (PBS). Some conidia were rendered non-viable (NV) by exposure to absolute methanol for five minutes. Conidia were counted on a hemocytometer and adjusted to yield $2x10^7$, $2x10^6$, $2x10^5$, and $2x10^3$ singly dispersed conidia per ml with average diameter of 3.5 μm. Serial dilutions were plated on SDA 5.6 pH plates to determine the percent conidia viability.

Animals

Female C57B1/6 mice (5 to 6 weeks of age) and female BALB/c mice (5 to 6 weeks of age) were maintained in a HEPA filtered room in HEPA filtered metal cages. The mice were lightly anesthetized, placed in an upright position, and inoculated IN with 50μl (25μl per nare) of conidia in PBS. The mice were held in this upright position for 2 minutes to allow for complete instillation of the dose. Controls received only PBS. For the three week study, animals were inoculated IN twice per week for three weeks.

Deposition, Clearance, and Retention Experiments

At various time-periods after acute IN inoculations, the C57Bl/6 mice were euthanized, and the lungs and tracheas were aseptically removed, placed in 10ml PBS, and homogenized. Serial dilutions were plated on SDA 5.6 pH plates to determine the percent conidia viability. There were 3 mice in each group and the experiment was repeated.

Bronchioalveolar Lavages and Blood Samples

Bronchioalveolar lavages (BAL) were performed. The BAL cells were sedimented by centrifugation, stained, and a cell differential count (CDC) performed. The BAL supernatant was filtered (0.22 μm syringe filter) and assayed for cytokines. Blood smear slides were stained and CDC performed. Serum was assayed for total IgE.

Cytokine and Immunoglobulin Analysis

The BAL were assayed for tumor necrosis factor-α (TNF-α), interleukin-4 (IL-4), and interleukin-5 (IL-5), utilizing sandwich ELISAs (previously described) specific for mouse cytokines (Phramagene) (Watson et al., 1993), and total serum IgE using sandwich ELISAs (previously described) specific for murine immunoglobulins (Phramagene) (Coffman and Carty, 1986).

Statistics

Data were analyzed by a computer program (Sigma Stat) employing the Mann-Whitney rank sums test (U test), the Kruskal-Wallis one way ANOVA (H test),

Spearman's product moment correlation, and Dunn's multiple comparisons and partial correlations (Freund and Simen, 1992).

Results

Deposition Experiments

In C57B1/6 mice receiving $1x10^6$ conidia (25% viability) IN, conidia were recovered from the lungs 15 min and 3h, 6h, 9h, 12h, 24h, and 36h after inoculation. Eighteen percent ($4.7x10^4$) of the viable conidia were deposited in the lungs with 4% ($1.0x10^4$) being retained, most likely in the alveolar spaces. Viable conidia were recovered up to 36h post-inoculation (Figure 5.1). The clearance efficiency of the deposited viable conidia was 0% for the first 6h. The clearance efficiency at 9h was 60%, at 12h, 79%, at 24h, 99%, and at 36h, 99.5%.

TNF-α Production

C57Bl/6 mice exposed by inhalation to *P. chrysogenum* conidia demonstrated an increase in TNF-α. There were at least 3 mice in each group and time-period. Mice were inoculated IN with varying concentrations of *P. chrysogenum* conidia and at different time-periods, the lungs were lavaged to ascertain TNF-α production (Figure 5.2). The mice received $1x10^6$ conidia (26% viability) resulting in a dose of $2.6x10^5$ CFU, $1x10^5$ conidia (26% viability) resulting in a $2.6x10^4$ CFU dose, or $1x10^6$ NV conidia. The results demonstrated a dose and time-dependent increase in TNF-α production in the viable conidia preparations. TNF-α production induced by 10^6 viable conidia was

significantly different ($P<0.05$) from the controls. There was an increase, although not significant ($P=0.09$), in the number of neutrophils in the BAL cells recovered from the mice that received $1x10^6$ viable conidia.

Three-Week Study

The C57Bl/6 and the BALB/c mice were inoculated IN with $1.0x10^4$ conidia (viability 25%) (n=6), $1.0x10^2$ conidia (viability 25%) (n=6), $1.0x10^4$ NV conidia (n=6), and PBS (controls, n=10) twice per week for 3 weeks. The C57Bl/6 mice receiving 10^4 viable conidia demonstrated a significant increase ($P<0.05$) in total serum IgE along with peripheral eosinophilia, while mice receiving 10^2 viable and 10^4 NV conidia did not (Figure 5.3). In addition, the BAL from the C57Bl/6 mice receiving 10^4 viable conidia had significant increases ($P<0.05$) in IL-4 (Figure 5.3). However, with the exception of an increase in airway neutrophils in the group receiving 10^4 viable conidia, none of the BALB/c mice groups showed any differences. In all mice, the BAL cell, with the exception of the BALB/c mice noted above, and IL-5 levels did not differ significantly from the controls.

Discussion

There are three methods by which rodents may be inoculated for aerosol deposition. These are (1) natural "physiologic" inhalation, (2) intratracheal instillation, and (3) intranasal instillation (Brain and Valberg, 1979). Each method has its advantages and disadvantages. Natural inhalation requires expensive and complex equipment and

chambers along with the technical expertise to generate a constant aerosol. With intratracheal instillation, the same (measured) amount of material can be introduced into each animal. However, this requires deep anesthetization, and repeated anesthetization required for a chronic study could induce comas in the animals. Intranasal instillations require only light anesthetization, but the material must pass through the extensive nasal turbinate network. In addition, the primary objection to intratracheal and intranasal instillation is the high probability that patterns of particle distribution are unlike those resulting from natural inhalation (Brain et al., 1976). However, examination of the immune response to an aerosol deposited into the lungs of an animal depends on the ability of the investigator to introduce known amounts of the material into the lungs. In an earlier study, we demonstrated that occupants in a room in which *P. chrysogenum* was growing were constantly exposed to viable *P. chrysogenum* conidia, with little or no variations in the fungal ecology of the room (McGrath et al., 1999). Since a chronic study using a known amount of conidia introduced into lungs was the objective of our laboratory, we chose to employ intranasal inoculation. Because rodents are obligate nose breathers, the deposition, clearance, and retention of *P. chrysogenum* conidia in a mouse model were examined.

The retention of a particle is determined by the deposition and clearance of the particle. The output of particles previously deposited in the lungs is called clearance and refers to the processes that physically expel the particles from the lungs. This mechanism includes absorption, sneeze, cough, mucociliary transport, and alveolar clearance mechanisms involving pulmonary macrophages (Brain and Valberg, 1979). It should be kept in mind that clearance is often of greater significance than deposition. Therefore,

clearance efficiency may be the determining factor for total integrated exposure, and, consequently, the probability of a pathologic or physiologic response, especially when the particles are viable conidia.

In the C57Bl mouse model, our data demonstrated that approximately 80% of the viable conidia were either trapped in the nasal turbinates, swallowed, or expelled. The remaining 20% of the viable conidia were deposited distal to the larynx. The clearance efficiency of the viable conidia remained close to 0% for the first 6h, but the PBS carrier liquid was probably absorbed. By 12h, the clearance efficiency was approximately 80%. This suggests that the remaining viable conidia ($1x10^4$) had been deposited and retained in the alveolar spaces, where the primary clearance mechanism involves the pulmonary macrophages. At 36h, approximately 0.5% of the *P. chrysogenum* conidia remained viable. This is an important observation in that many researchers and clinicians have been under the impression that the spores (conidia) are unlikely to release any antigen unless they germinate, a process that requires several hours, because the mucociliary tract and the alveolar macrophages will remove most of the fungal propugales prior to germination (Platt-Mills et al., 1998).

Our data demonstrate that the IN inoculation of viable *P. chrysogenum* conidia into the lungs of C57B1/6 mice results in inflammatory and allergic-like reactions. Our data shows that acute doses of viable *P. chrysogenum* conidia induced significant ($P<0.05$) increases in TNF-α, while NV conidia induced no such inflammatory reaction. TNF-α is a cytokine produced by cells and is involved in the inflammatory cascade (Watson et al., 1993). Our data also demonstrated that a 3 week exposure to 10^4 viable *P chrysogenum* conidia induced the production of significant ($P<0.01$) increases in total

serum IgE and peripheral eosinophilia in the C57Bl/6 mouse model. The total IgE increases in the mouse model are supported by the significant increases ($P<0.05$) in the BAL IL-4 from the same conidia group. Since IL-4 is required for IgE production (Coffman and Carty, 1986), the production of IL-4 in the lungs suggests that the viable conidia were inducing the IgE increase. Since the BAL cells and IL-5 levels from all of the mice did not differ from the controls, this indicates that perhaps the exposure period (3 weeks) was not long enough, especially since we chose not to prime the mice by intraperineal (IP) injection. It is likely that priming animals using IP injections for aerosol sensitization may involve pathways that are not used during a natural sensitization. However, IN priming will probably require longer exposure periods, along with increasing the variability of allergic response in the animals.

Our data suggest that the viability of the *P. chrysogenum* conidia may be an important factor in the animal's response. The slow clearance of the viable conidia suggest that there is ample time for the conidia to produce a substance or compound that may contribute to the observed allergic response. These data also support that genetic susceptibility (atopic), at least in rodents, may be a factor. It has been demonstrated that C57Bl/6 mouse models are genetically predisposed (high IgE responder) to IN inoculations of allergens while BALB/c mouse models (low IgE responder) are not (Holt et al., 1987). One of the most important precipitating factors in asthma is the inhalation of aeroallergen. It is thought that long-term, low level exposure to indoor aeroallergen (e.g., dust mites, molds, pets) may play a role in the generation of asthma and contribute to the chronic and recurring symptomatology (Duff and Platts-Mills, 1992). This study presents more information that the inhalation of fungal conidia (specifically viable

Penicillium species) may play a role in allergic diseases. This could be by causation or exacerbation.

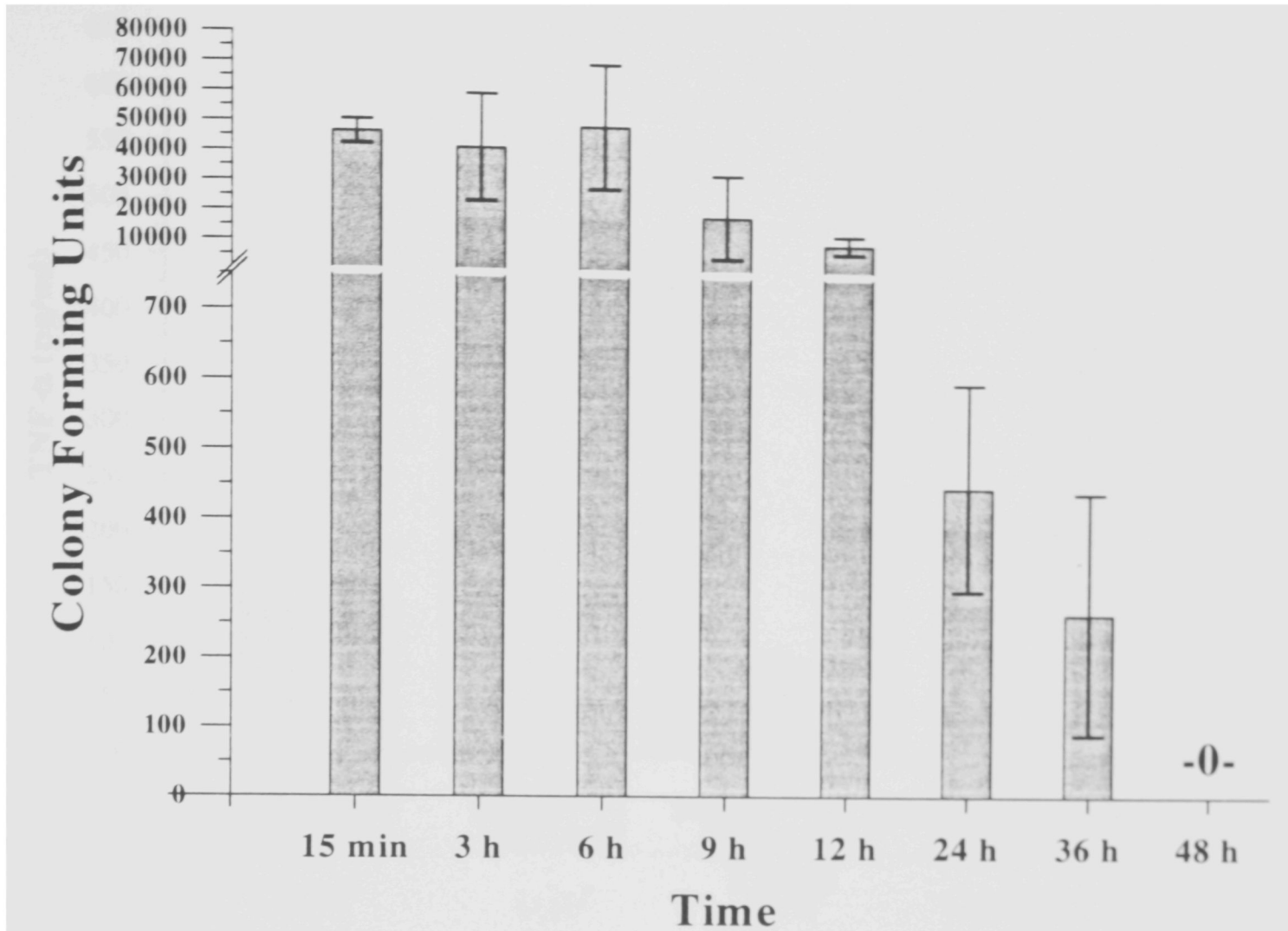

Figure 5.1. Deposition, Clearance, and Retention of *P. chrysogenum* conidia in C57Bl/6 Mice. Female C57Bl/6 mice were inoculated IN with $1x10^6$ *P. chrysogenum* conidia (viability 26%) resulting in a dose of $2.6x10^5$ CFU. At various time-periods after the acute inoculation, the mice were euthanized, the lungs and tracheas aseptically removed, homogenized and serial dilutions plated on SDA plates to determine percent conidia viability. Each time-period had a minimum of six mice. The -0- indicates no viable conidia were recovered. The error bars represent the standard error of means (SEM).

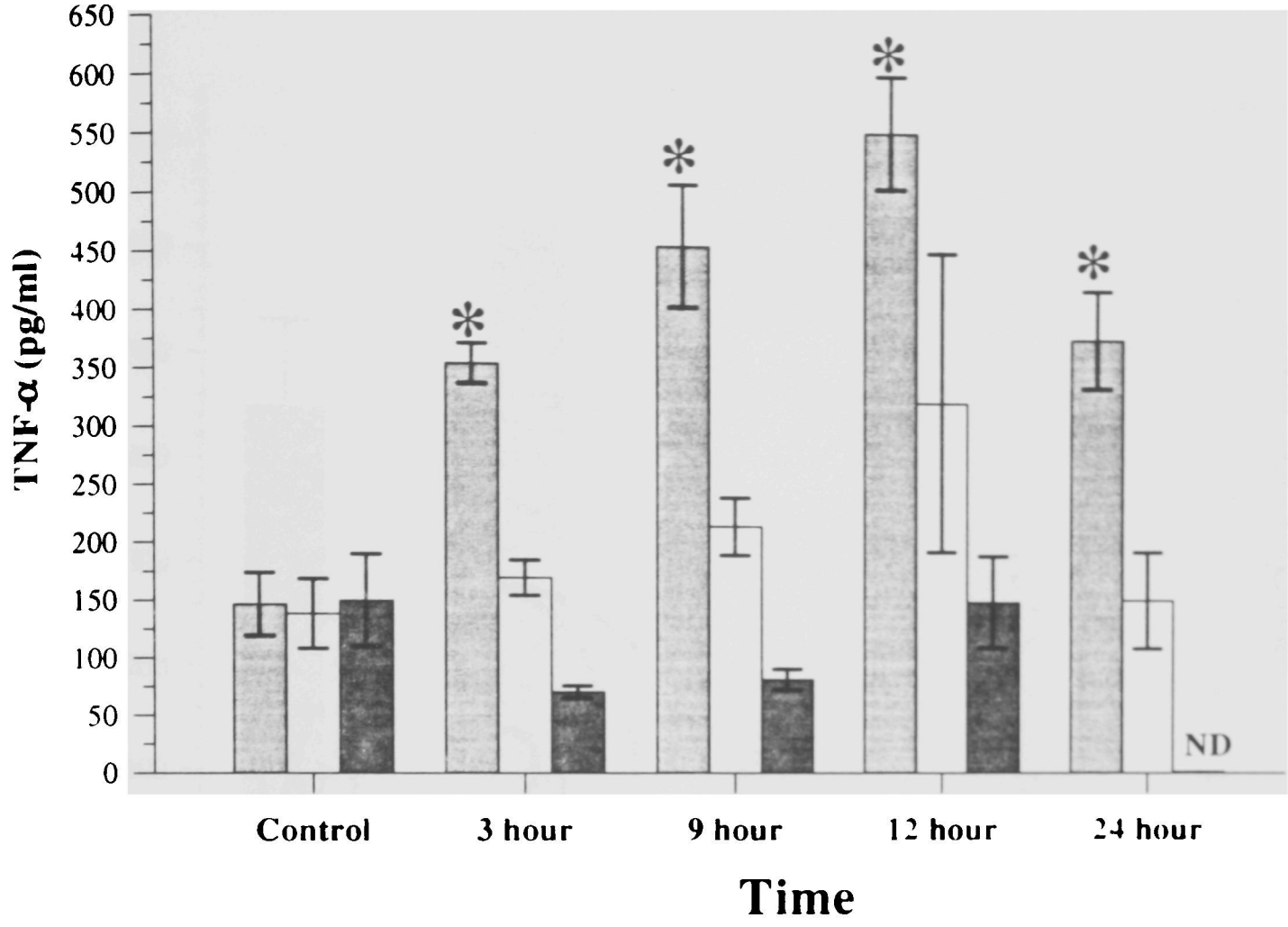

Figure 5.2. In Vivo Analysis of TNF-α after Intranasal Inoculation of Viable *P. chrysogenum* conidia. Female C57Bl/6 mice inoculated IN with varying concentrations of *P. chrysogenum* conidia and at different time-periods, the lungs were lavaged and assayed for TNF-α utilizing a sandwich ELISA specific for murine TNF-α. The light grey bars represent mice that were inoculated IN with $1x10^6$ viable *P. chrysogenum* conidia; the white bars represent mice that were inoculated IN with $1x10^5$ viable *P. chrysogenum* conidia; and the dark grey bars represent mice that were inoculated IN with $1x10^6$ NV *P. chrysogenum* conidia. There were at least three mice in each group and time-period. The single asterisk (*) represent $P<0.05$. The ND represents Not Done. The error bars represent the SEM.

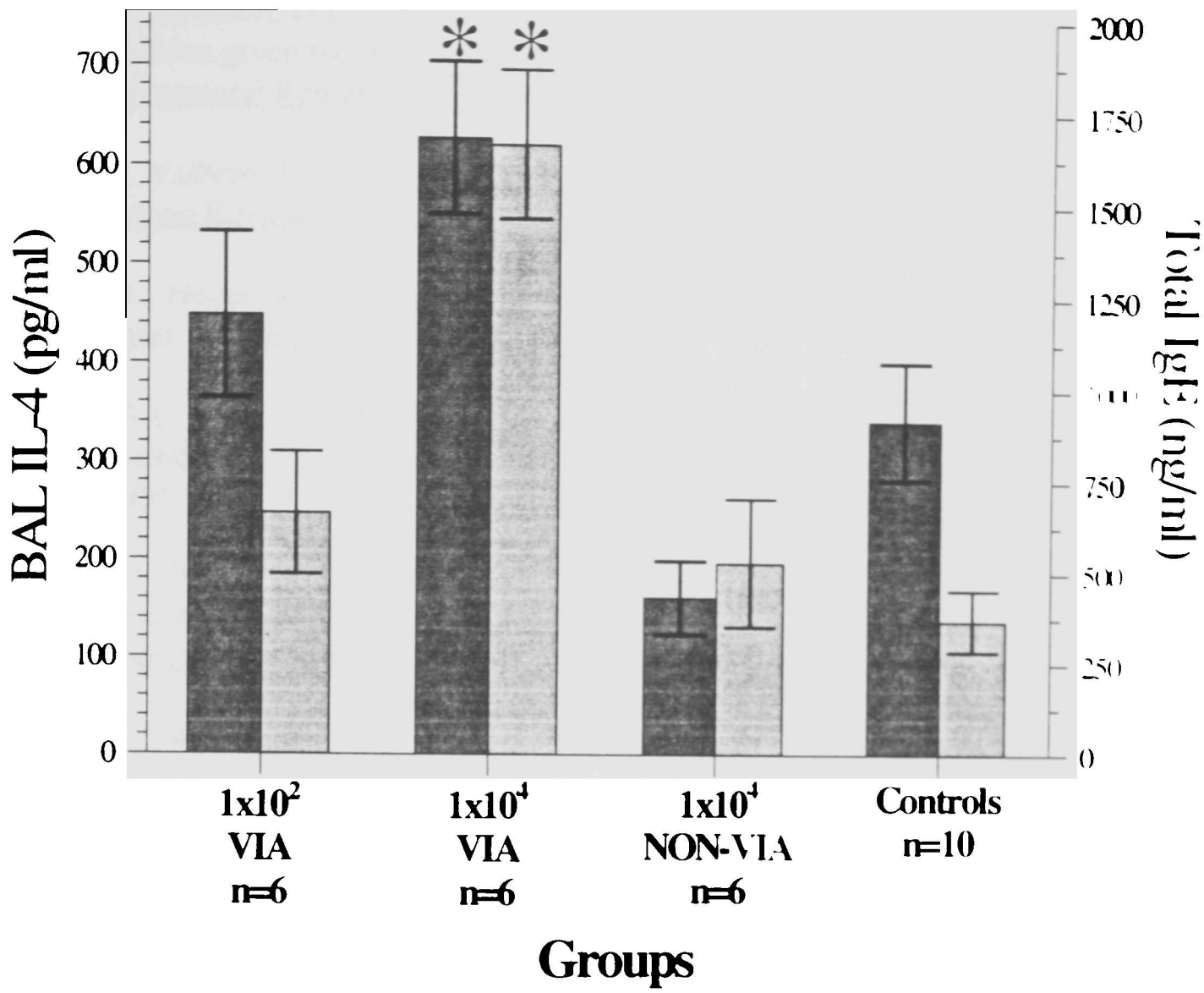

Figure 5.3. Analysis of BAL IL-4 and Serum IgE after Three Weeks of Intranasal Inoculations with Viable and Non-viable *P. chrysogenum* conidia. Female C57Bl/6 mice were inoculated IN twice per week for three weeks with varying concentrations of *P. chrysogenum* conidia. Twenty-four hours after the final inoculation, the mice were euthanized, blood processed for serum, and the lungs lavaged. The BAL was assayed for IL-4 (represented by the dark grey bar) utilizing a sandwich ELISA specific for murine IL-4. The serum was assayed for total IgE (represented by the light grey bar) using a sandwich ELISA specific for murine IgE. The single asterisk (*) represents $P<0.05$ The error bars represent the SEM.

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CHAPTER VI

AN ANIMAL MODEL FOR ALLERGIC PENICILLIOSIS INDUCED BY THE INTRANASAL INSTILLATION OF VIABLE *PENICILLIUM CHRYSOGENUM* CONIDIA

Introduction

Sick building syndrome (SBS), a term for symptoms resulting from poor indoor air quality (IAQ), has proven difficult to define and no single cause of this disease has been identified (Hodgson, 1992). Complaints include rhinitis, difficulty in breathing, headaches, flu-like symptoms, and watering of the eyes, which suggests both upper and lower respiratory tract diseases (Spangler and Sexton, 1983; Mishra et al., 1992).

In a previous study, we demonstrated that *Penicillium* species (especially *P. chrysogenum*) and *Stachybotrys* species are strongly associated with TBS (Cooley et al., 1998). These studies also supported earlier findings that *Penicillium* species have become an important indoor contaminant (Burge et al., 1989). It has been shown that bronchial challenges with conidia of *Penicillium* species induced immediate- and delayed-type asthma in sensitized subjects (Licorish et al., 1985). While these allergic reactions appear to result from the inhalation of fungal products, the mechanism(s) responsible for these phenomena remains unclear.

In allergic diseases, T-cell activation and IL-4 production follow the presentation of allergens by antigen presenting cells (APC) to T-cells (Lack et al., 1995). Further T-B-cell cooperation leads to the production of IgE. Interaction of specific IgE antibodies on the surface of effector cells (mast cells and basophils) with allergen triggers the early phase of immediate-type hypersensitivity responses. These events initiate the development of allergic inflammation. These events are further characterized by

infiltration of eosinophils and release of eosinophil components (eosinophil cationic protein, eosinophil peroxidase and the eosinophil major protein), followed by all the signs and symptoms of an inflammatory process. An important linkage between sensitization and inflammation is represented by T cells that secrete mediators not only involved in IgE synthesis (IL-4), but also responsible for eosinophil recruitment (IL-5), activation and survival (GM-CSF) (Azzawi et al., 1990; Walker et al., 1991; Bentley et al., 1993; Watson et al., 1993).

Little is known about the role of fungal propagules in the pathogenesis of allergic diseases and as trigger-factors for clinical signs of allergic disease. This study was designed to examine the consequences of the introduction of *P. chrysogenum* conidia into the mammalian lung. Because of the importance of *P. chrysogenum* conidia in SBS (Cooley et al., 1998), we have developed an animal model to determine the consequences of nasal instillation of these conidia. In an earlier study, we measured the deposition, clearance, and retention of *P. chrysogenum* conidia that were instilled IN in a mouse model (Cooley et al., 1999). In that report, we showed that the acute instillation of viable *P. chrysogenum* conidia induced inflammatory reactions in a dose dependent manner, while the instillation of non-viable conidia did not. In this study, the question that we wanted to address was what were the consequences of long-term instillation of *P. chrysogenum* conidia. This study demonstrated that long-term exposure to low doses of viable *P. chrysogenum* conidia leads to significant increases in serum IgE accompanied with peripheral eosinophilia, airway eosinophilia and an increase in airway neutrophils, along with significant increases in IL-4 and IL-5. These results are significant because

they point to the presence of an inflammatory response due to the introduction of viable *P. chrysogenum* conidia into the mammalian lung.

Materials and Methods

Conidia

The *P. chrysogenum* culture employed in this study was isolated from a building that was experiencing TBS (Cooley et al., 1998). The *P. chrysogenum* was grown on Sabouraud's dextrose agar (SDA), pH 5.6, 22°C, and 95% relative humidity for 5 days. The conidia were harvested by gentle washing with sterile endotoxin-free phosphate buffered saline (PBS). Some groups of conidia were harvested using absolute methanol. After five minutes in the methanol, all conidia were non-viable (NV). All of the conidia were then washed two times by sedimentation (centrifugation at 10,000 x g, 15 min) and resuspended with PBS and vortexed extensively. The conidia were sonicated for 10 s to disrupt clumping, filtered through sterile 20µm cell filters (Falcon) to remove any remaining clumps or debris, and counted on a hemacytometer. This procedure yielded an average of 25% (±5%) viable singly dispersed conidia, averaging 3.5µm in diameter (range 1µm to 5µm). The singly dispersed conidia (viable and NV) were adjusted in PBS to yield $2x10^5$ or $2x10^3$ conidia per ml. Serial dilutions were plated on $SDA_{s\ 6pH}$ plates to determine the percent viability of the conidia.

Animals

Female C57Bl/6 mice (Harlan), 5 to 6 weeks old, were maintained in a high efficiency particulate arrestor (HEPA) filtered room and in HEPA filtered suspended stainless steel cages, without bedding. Once per week for 6 weeks, mice were lightly anesthetized (isoflurane and oxygen mixture), placed in an upright position, and inoculated IN with 50µl (25µl per nare) of PBS containing $1x10^4$ NV conidia, $1x10^4$ conidia (viability 25% ± 5%), or $1x10^2$ conidia (viability 25% ± 5%). The mice were held in this upright position for 2 minutes to allow for complete instillation of the dose. Controls were inoculated IN with 50µl (25µl per nare) of PBS. Twenty-four hours after the six-week inoculation, the mice were anesthestized (halothane and oxygen mixture) and euthanized by cardiac puncture.

All animals were under the care of trained technicians and a full-time veterinarian in the Laboratory Animal Resource Center. Animals were fed autoclaved feed and water ad libidum. All animals were treated in accordance to the policies established by the TTUHSC institutional animal care and use committee.

Blood and Lung Lavage

Samples for hematological examination were drawn by retro-orbital bleeding (Van Herck et al., 1997). The venous blood smear slides were stained with Wright-Giemsa stain and a cell differentiation and count (CDC) was performed. Total blood was also removed by cardiac puncture, processed to obtain serum, and stored at -80°C

For the lung lavage, the trachea was exposed and a 22 gauge angiocatheter was inserted and tied in place with suture. The lungs were lavaged (4 times) by slowly

instilling 1 ml of sterile endotoxin-free Hanks balance salt solution (HBSS) (37°C) followed by gentle aspiration. The lungs were aseptically removed and placed in 10ml of 10% neutral formalin without inflation. The total number of macrophages (viable and NV) in the bronchioalveolar lavage (BAL) was determined using the Trypan Blue Exclusion assay counted on a hemacytometer. The BAL was centrifuged (1000 x g. 10 min) to sediment the cells. The BAL cells were suspended in PBS to yield $2x10^5$ macrophages per ml. Samples (200µl) of the BAL cells were pelleted onto microscope slides utilizing a cytospin centrifuge (Shannon). The cytospin slides were fixed in absolute methanol, stained with Wright-Giemsa stain, and a CDC was performed. The BAL supernatants were passed through 0.22µm cellulose acetate filters and stored at -80°C.

Cytokine and IgE Analysis

The BAL supernatants were assayed for IL-4, IL-5, and GM-CSF. The amount of cytokines released was determined using a sandwich ELISA specific for murine cytokines (Watson et al., 1993). Serum was assayed for total IgE using a sandwich ELISA specific for murine IgE (Coffman and Carty, 1986).

Briefly, in these assays, flat-bottom 96-well microtiter plates (Nunc Maxisorb) were coated overnight at 4°C with the appropriate capture antibody. Each plate was washed with PBS+0.1% Tween 20 (PBS-T), and blocked with PBS+3.0% bovine serum albumin. After four washes with PBS-T, eight two-fold dilutions in duplicate of the appropriate control or standard and the test BAL or serum were added to the respective

wells. The plates were then incubated for 2 hours at room temperature (RT), washed four times with PBS-T, and the appropriate biotinylated detecting antibody (mAb) added and incubated for 1 hour at RT. The plates were washed four times with PBS-T, avidin-horseradish peroxidase (HRP) was added, and the plates were incubated for 1 hour at RT. Plates were washed an additional 4 times with PBS- and 2, 2'-Azino-bis (3-ethylbenzthiozoline-6-sulfonic acid) plus 30% hydrogen peroxide. After 20-30 min, the HRP enzyme reaction was stopped by adding 2mM sodium azide. Absorbance was read on a microtiter plate spectrophotometer (DynaTech MR 4000) at 410 nm. The optical densities of the unknown samples were interpolated from a standard curve using a Sigma Plot computer program.

Alveolar Macrophages and Electromicroscopy

To evaluate conidia-phagocyte interactions, pulmonary alveolar macrophages were obtained from the lungs of euthanized C57Bl/6 mice that had received IN inoculations of *P. chrysogenum* conidia 3 hours, 6 hours, and 24 hours earlier. Lungs were lavaged with HBSS as described above. Cells were recovered by centrifugation at 1000-x g for 10 min. For transmission electromicroscopy, $1x10^5$ alveolar macrophages from mice that had received IN inoculations of viable *P. chrysogenum* conidia were fixed in glutaraldehyde (3%) in 0.1M sodium cacodylate buffer (pH 7.3) for 1 hour at room temperature. The samples were then post-fixed in osmium tetroxide (1%) and prepared for ultrastructural analysis by standard methods (Hutson, 1978).

For the histopathological examinations, the lungs were aseptically removed and fixed in buffered 10% formalin. Five-micrometer sections were prepared and stained

with hematoxylin-eosin stain and examined blind. The sections were evaluated for the presence of lung eosinophilia, foreign material, fibrosis, alveolar macrophages, and inflammatory cells.

Statistics

Statistical analysis was performed using Sigma Stat, a statistical software package designed by Jandel (SPSS), to analyze the data for analysis of variance (ANOVA) with α=0.05 and β=0.80 using the group of mice that received PBS only as the control.

Results

Serum IgE Levels, BAL IL-4, and Peripheral Eosinophils

Animals were inoculated IN with varying numbers of *P. chrysogenum* conidia (both viable and NV) once a week for a six-week period and examined for total serum IgE and BAL IL-4 production. Mice inoculated with 10^2 and 10^4 viable *P. chrysogenum* conidia had a significant increase in the total serum IgE (P=0.002 and P=0.017, respectively) when compared to controls, while the mice that received 10^4 NV conidia group did not (Figure 6.1). The IN instillation of 10^2 and 10^4 viable *P. chrysogenum* conidia induced significant increases in IL-4 (P=0.002 and P=0.017, respectively), which strongly correlated with the total serum IgE increases (Figure 6.1). IN instillation of 10^2 or 10^4 viable *P. chrysogenum* conidia also induced a significant peripheral eosinophilia (P≤0.001) in these animals (Figure 6.2).

BAL Cellular Responses

The BAL from the animals were also examined. Figure 6.3 shows that IN instillation of 10^4 viable *P. chrysogenum* conidia induced a significant eosinophilia ($P \leq 0.01$) in the lungs of mice. An increase, although not significant ($P=0.059$), in BAL neutrophils was also observed. Figure 6.3 also shows that 10^4 viable *P. chrysogenum* conidia, as compared to the controls, induced a significant increase in IL-5 ($P=0.004$). Although the IN instillation of 10^4 NV conidia induced a significant neutrophilia ($P \leq 0.01$) in these animals (Figure 6.4), no eosinophils were found in their BAL (Figure 6.3). IN instillation of 10^2 viable conidia did not induce either neutrophilia or eosinophilia in the BAL. While the control mice had a background level of neutrophils, no eosinophils were found in the BAL. The BAL were also assayed for GM-CSF, but there was not a significant ($P=0.09$) in the production of this cytokine in the mice that were instilled IN with 10^4 viable and NV *P. chrysogenum* conidia.

Histopathology and Electromicroscopy

A histological examination of the lung tissue revealed a few peribronchial lymphoid aggregates with plasma cells and some minor infiltration of eosinophils into the lung tissue of mice that were IN instilled with 10^4 viable *P. chrysogenum* conidia while the other groups (10^2 viable conidia, 10^4 NV conidia, and the controls) did not reveal any eosinophils. However, the histopathological evaluation of the lung tissue samples showed no evidence of fibrosis or tissue remodeling.

Figure 6.5 is a series of electron micrographs of alveolar macrophages taken from the BAL of mice 3 h, 6 h, and 24 h after IN instillation of 10^4 viable *P. chrysogenum* conidia. These micrographs show *P. chrysogenum* conidia have been phagocytosed and

are within phagosomes and in various stages of destruction. Figure 6.5D is a micrograph of a control *P. chrysogenum* conidia from a preparation that had not been injected into mice, but that had been prepared for morphological examination as described above.

Discussion

This study examining the IN instillation of *P. chrysogenum* conidia into C57B1/6 mice has produced interesting findings. The data demonstrate that the IN inoculation of *P. chrysogenum* conidia into the lungs of C57B1/6 mice does result in inflammatory reactions. The data also demonstrate that sensitization to the viable *P. chrysogenum* conidia via mucosal exposure was efficient and did not require the potentially immunomodulatory intraperitoneal or subcutaneous priming (with adjuvant) used in the ovalbumin models to evoke airway inflammation. Long term exposure to 10^2 and 10^4 viable *P. chrysogenum* conidia caused significant (P=0.002 and P=0.017, respectively) increase in total serum IgE. Significant increases in total IgE as compared to controls are an indicator of an allergic response (Bentley et al., 1993). In addition, the same animals demonstrated significant (P<0.01) peripheral and airway eosinophilia following long-term exposure to 10^4 viable conidia. Significant increases in eosinophils, especially airway eosinophils, as compared to controls, are an indication of an allergic response (Kay, 1992; Azzawi et al., 1990; Watson et al., 1993; Herz et al., 1996). These cellular and IgE increases are supported by the significant increases in IL-4 and IL-5 in the BAL of mice inoculated IN with 10^4 viable conidia. These cytokines are required for IgE synthesis and eosinophil recruitment, respectively. The production of these cytokines was measured 24 hours after IN inoculation. Although the BAL IL-5 produced by the

mice that received 10^4 viable conidia was significantly different from the controls, we do not know if the production of this cytokine is increasing or decreasing at 24 hours. This is the same for BAL GM-CSF, a cytokine needed for activation and survival of eosinophils. The BAL GM-CSF from mice that had received 10^4 viable conidia had increased at 24 hours, although not significantly (P=0.09) when compared to controls. Nevertheless, the significant production of these cytokines (IL-4 and IL-5) in the lungs of mice inoculated with 10^4 viable conidia, coupled with the airway eosinophilia, strongly suggests that the instilled conidia are inducing this reaction. Since the IN instillation of NV conidia did not induce the same responses, it is likely that the viable conidia are producing a compound responsible for these reactions.

We have previously shown the deposition, clearance, and retention of *P chrysogenum* conidia in the lungs of C57B1/6 mice (Cooley et al., 1999). The results from this earlier study demonstrated that approximately 18% of the viable conidia that were inoculated IN were deposited into the lungs, and after 9 hours, 4% of the viable conidia were recoverable from the lungs. In addition, viable conidia were recoverable up to 36 hours after the initial inoculation. These results are significant in that the data demonstrated that viable conidia could remain in the lungs long enough to be able to produce substances. Those data also suggested that the remaining viable conidia were deposited and retained in the alveolar spaces where the primary clearance mechanism involves the pulmonary macrophages. The micrographs clearly demonstrate the phagocytosis of *Penicillium* conidia and probable degradation.

We have previously demonstrated that viable conidia could remain in the lungs long enough to produce substances that could induce allergic reactions (Cooley et al.,

1999). Our results suggest that viability and the production of an unknown substance and not some cell wall constituents (e.g., (1-->3)-β-glucans) are responsible for the chronic airway inflammation observed in fungal-induced allergies. This is because cell wall constituents such as (1-->3)-β-glucans are found in NV as well as viable conidia. However, long term exposure to 10^4 viable conidia induced increases (P=0.09) in airway neutrophils, while 10^4 NV conidia induced a significant increase (P<0.01) in airway neutrophils. Whether this was due to some cellular component of the conidia or was only an irritant effect is not known.

Although a histological examination of the lung tissue revealed minor infiltration of eosinophils in the group that received 10^4 viable conidia, no lung tissue damage or remodeling was observed. This might be due to the short duration of the study. Earlier studies (IN inoculations of *P. chrysogenum* conidia once per week for 3 weeks) did not demonstrate any significant changes in airway IL-5 or GM-CSF, or any airway eosinophils (Cooley et al., 1999). This suggests that chronic airway inflammation in our model may require a longer exposure period to induce identifiable airway damage and remodeling.

The present study presents additional information that the inhalation of fungal propagules (specifically *Penicillium* species) and their viability may play a role in eosinophilic bronchitis. This relationship could be causal or merely exacerbation. Our earlier work showed that SBS and the dominance of *Penicillium* species are strongly associated (Cooley et al., 1998). Other studies have suggested that there are associations between damp housing, chronic childhood respiratory symptoms, and sensitization to house dust mites and mold allergens, including *Penicillium* species (Strachan, 1993,

Verhoeff et al., 1995). If further studies continue to show this same trend, then it should be clear that *Penicillium* species conidia should be removed (to whatever degree possible) from indoor air of houses and public buildings. This removal could theoretically result in a drastic reduction in at least the exacerbation of fungal-induced SBS in developed countries.

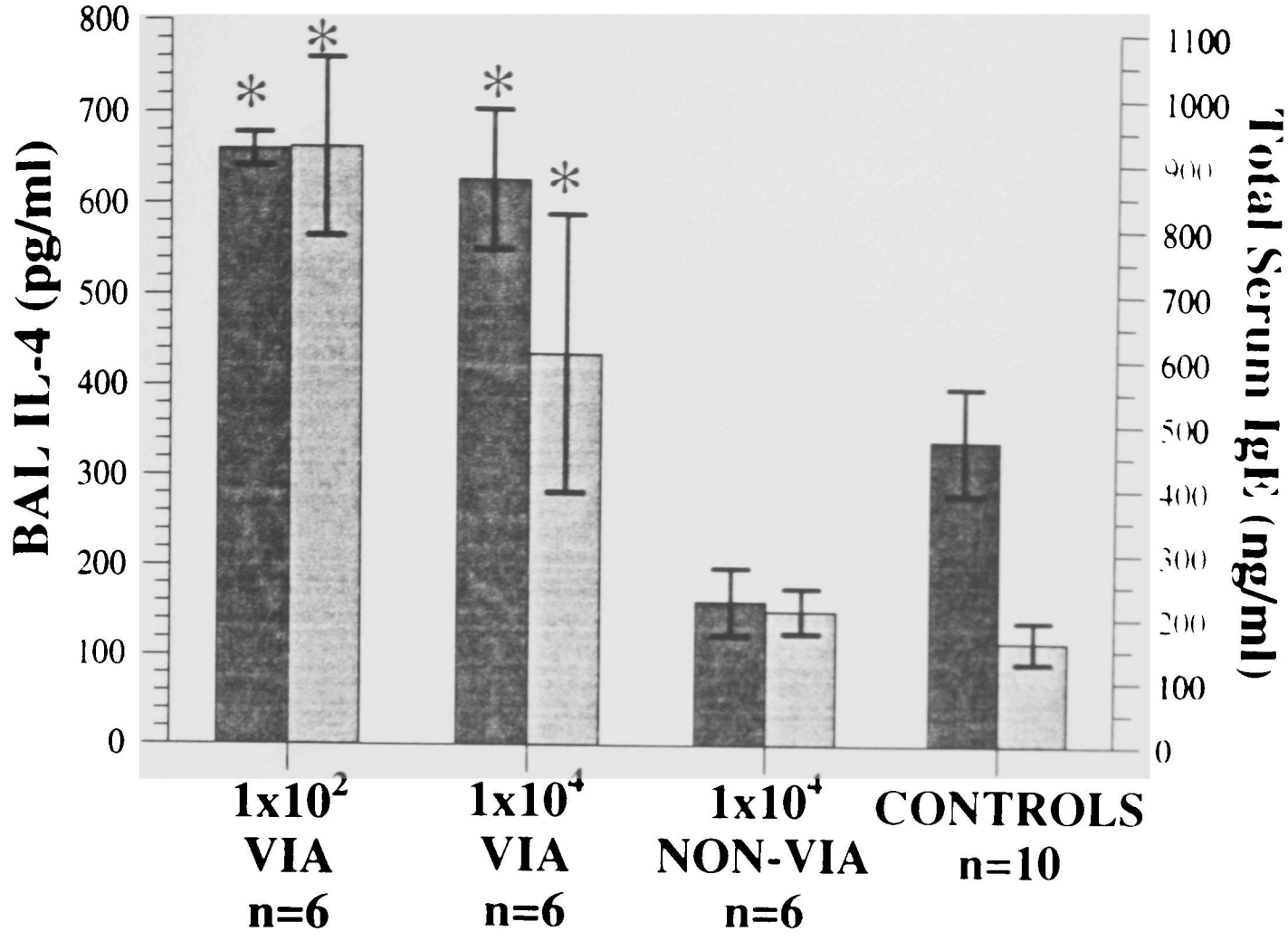


Figure 6.1. Analysis of BAL IL-4 and Serum IgE after Six Weeks of Intranasal Inoculations with Viable and Non-viable *P. chrysogenum* conidia. Female C57Bl/6 mice were inoculated IN with viable (VIA) and non-viable (NON-VIA) *P. chrysogenum* conidia once a week for 6 weeks. Twenty-four hours after the final IN inoculation, the mice were euthanized and their serum was assayed for total IgE (light grey bars) using a sandwich ELISA specific for murine IgE. The BAL was assayed for IL-4 (dark grey bars) using a sandwich ELISA specific for murine IL-4. A single asterisk (*) represents statistical significance compared to controls (CONTROLS) ($P \leq 0.02$). The error bars represent standard error of means (SEM).

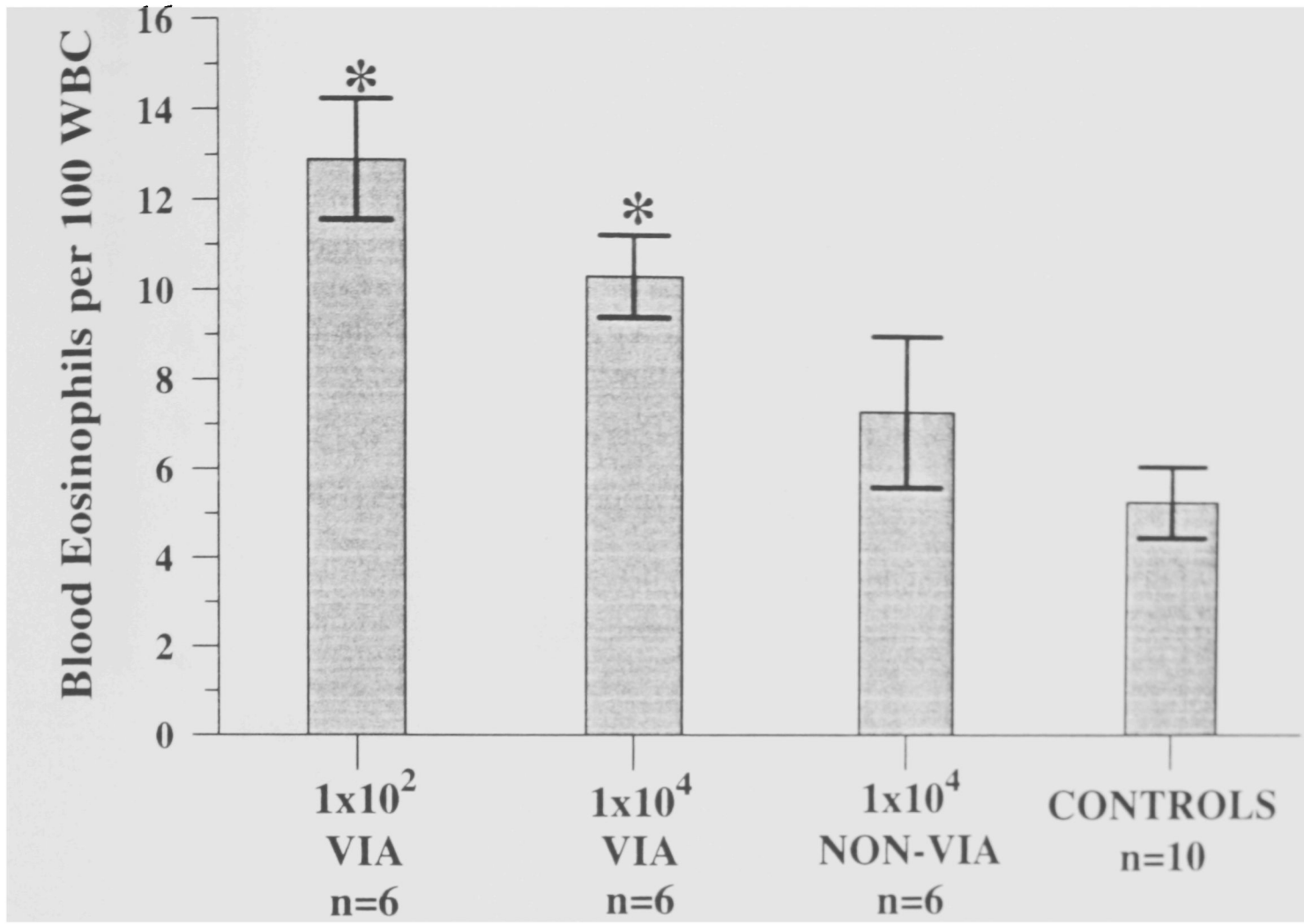


Figure 6.2. Blood (Peripheral) Eosinophils after Six Weeks of Intranasal Inoculations with Viable and Non-viable *P. chrysogenum* conidia. Female C57Bl/6 mice were inoculated IN with viable (VIA) and non-viable (NON-VIA) *P. chrysogenum* conidia once a week for 6 weeks. Twenty-four hours after the final IN inoculation, and before euthanization, peripheral blood smear slides were made, stained, and the eosinophils counted per 100 white blood cells. A single asterisk (*) represents statistical significance as compared to controls (CONTROLS) ($P \leq 0.002$). The error bars represent SEM.

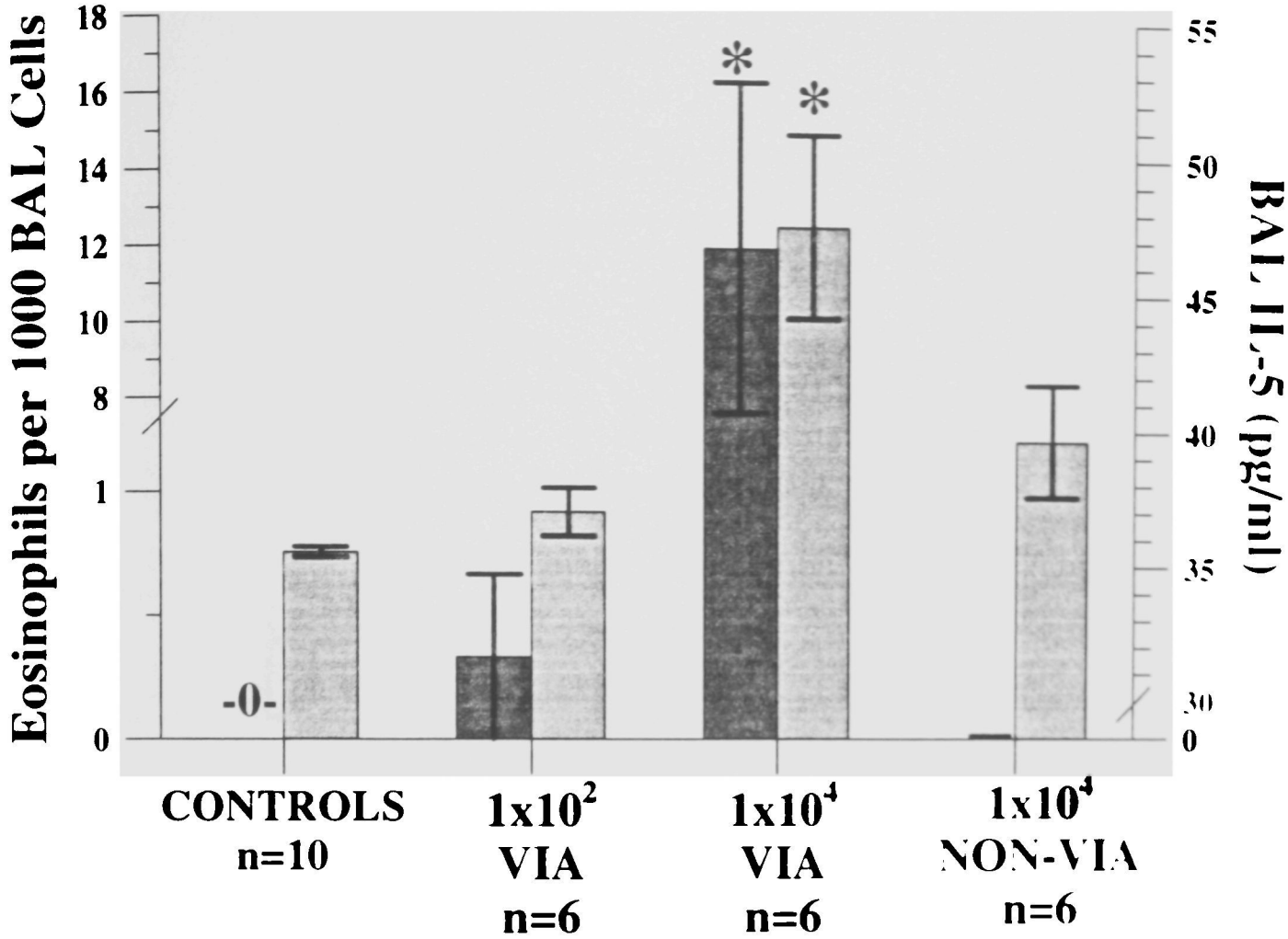


Figure 6.3. BAL (Airway) Eosinophils and BAL IL-5 after Six Weeks of Intranasal Inoculations with Viable and Non-viable *P. chrysogenum* conidia. Female C57BL/6 mice were inoculated IN with viable (VIA) and non-viable (NON-VIA) *P. chrysogenum* conidia once a week for 6 weeks. Twenty-four hours after the final IN inoculation, the mice were euthanized, the lungs were lavaged, and 1000 BAL cells per animal were counted. The dark gray bars represent the number of eosinophils. The BAL was assayed for IL-5 (light grey bars) using a sandwich ELISA specific for murine IL-5. A single asterisk (*) represents statistical significance as compared to controls (CONTROLS) ($P<0.01$). The error bars represent SEM.

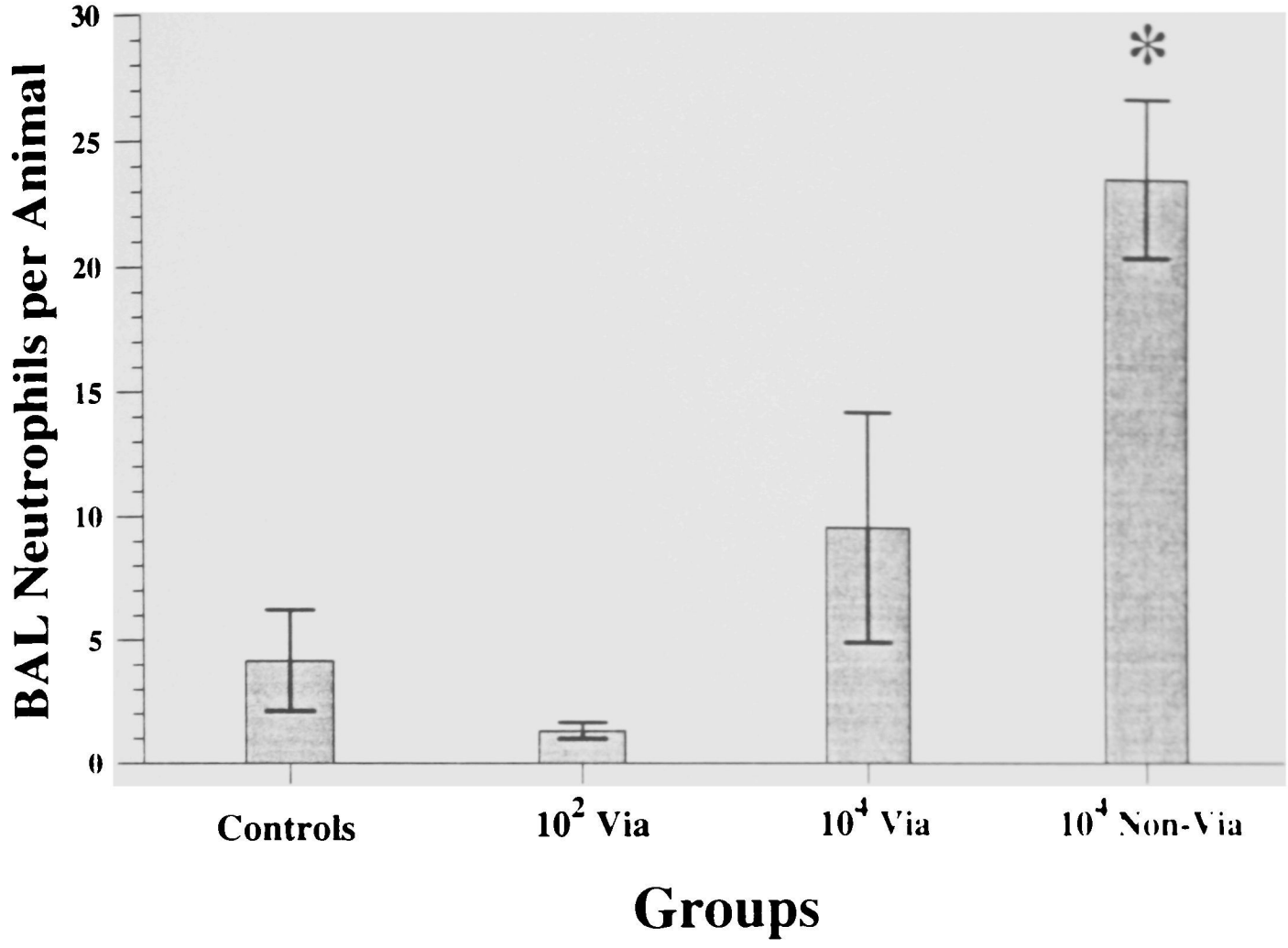
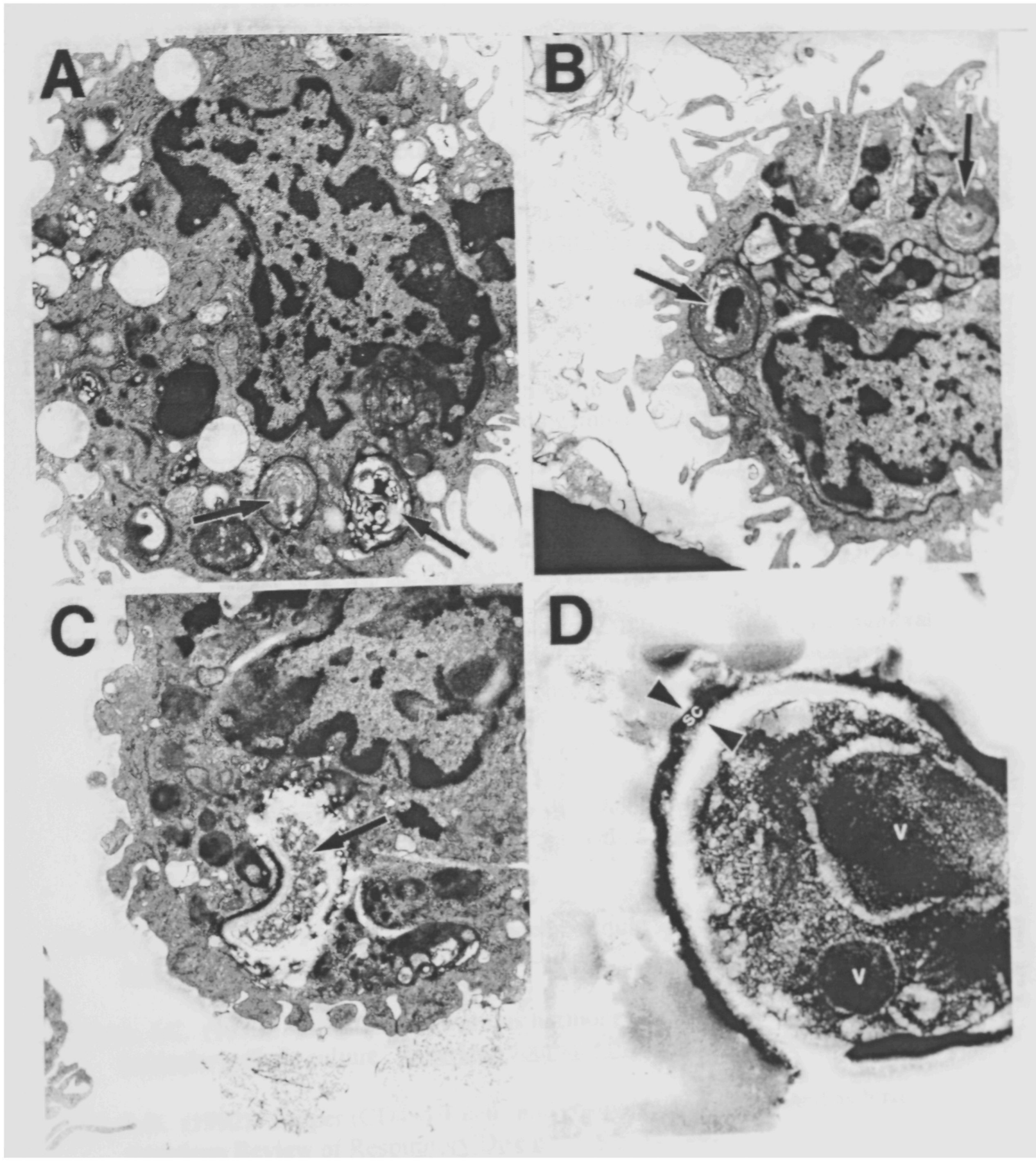


Figure 6.4. BAL (Airway) Neutrophils after Six Weeks of Intranasal Inoculations with Viable and Non-viable *P. chrysogenum* conidia. Female C57Bl/6 mice were inoculated IN with viable (VIA) and non-viable (NON-VIA) *P. chrysogenum* conidia once a week for 6 weeks. Twenty-four hours after the final IN inoculation, the mice were euthanized, lavaged and their BAL cells were pelleted onto slides, stained, and the neutrophils counted per 1000 BAL cells. A single asterisk (*) represents statistical significance as compared to controls (CONTROLS) ($P \leq 0.001$). The error bars represent SEM.

Figure 6.5. Micrographs of BAL Alveolar Macrophages after Acute Intranasal Inoculations with Viable and Non-viable *P. chrysogenum* conidia and a *P. chrysogenum* conidium. Figures 5A-C illustrate the ultrastructure of alveolar macrophages taken from the BAL obtained from C57Bl/6 female mice at 3 h (A), 6 h (B), and 24 h (C) after instillation of viable conidia (all at 10,000 x). Phagocytosed conidia (arrows) at various stages of digestion were commonly observed within phagosomes of the macrophage at all times studied. Temporal correlation of conidia destruction was not apparent as many macrophages even after 3 h contained conidia in various stages of breakdown. Residual bodies were present in cells at all times, typical of alveolar macrophages. Figure 5D illustrates the ultrastructure of *P. chrysogenum* conidia (43,750 x) before instillation [spore coat (sc) between the arrow heads and spore vacuoles (v)]. This morphology is comparable to the minimally damaged instilled conidia captured in Panel C. The conidia in Panels A and B are apparently in later stages of destruction.


A
B
C
D
SC
V
V

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CHAPTER VII

DISCUSSION

When Charles Blackley proved that grass pollen grains were the cause of seasonal hay fever, he took the first steps to understanding why some plants and animals in our environment are important causes of sensitization and symptoms (Blackley, 1959). Dr. Blackley was well aware of the size of pollen grains; he knew that they could fly up to least a thousand feet. He also demonstrated that the injection of aqueous extracts of pollen into humans would give rise to wheal and flare responses. Dr. Blackley also described his own asthmatic response after the inhalation of *Penicillium* mold.

As outdoor air quality has improved over the last 20 to 30 years, an awareness of the potential effects of indoor air pollution on health has emerged. People in the United States spend more than 80% of their time indoors at work, at school, and at home. Concerns about indoor air, which at first brought skepticism, are gaining respectability as various attributes of the indoor environment and its effect on health and well being are being investigated. The complaints or symptoms that occupants in buildings with an IAQ problem experience are commonly called sick-building syndrome. This collection of ailments, which is defined by a set of persistent symptoms occurring in at least 20% of those exposed, is typically is of unknown etiology, but is reduced sometime after an affected individual leaves the building. However, the issue has remained controversial because many of the health problems associated with indoor air pollution have nonspecific symptomology and a wide range of potential toxicants and sources. Although scientific uncertainties remain, the cost of SBS has become increasingly

apparent. SBS was initially considered to be a comfort problem that was perhaps less important than the money saved by emphasizing energy efficiency. More recently, the high costs of SBS in terms of employee productivity and morale have been recognized (Samet and Spengler, 1991).

It has recently come to the attention of the scientific community that fungi and their spores (conidia) are associated with indoor air quality problems or SBS (Ahearn et al., 1997; Bernstein et al., 1993; Boulet et al., 1997; Dales et al., 1991; Dill and Niggemann, 1996; Peat et al., 1993; Senkpiel et al., 1996). Fungal propagules can cause an immunopathology with an exaggerated or inappropriate immune response, called hypersensitivity reaction or common allergy. Fungal spores are a known cause of allergic diseases (Gravesen, 1979) and have been identified as one of the major indoor allergens (Pope, 1993). Even though fungal contamination in indoor environments has been shown to produce allergies in occupants of these buildings (Lehrer et al., 1983; Miller, 1992; Licorish et al., 1985; Verhoeff et al., 1995), there were few studies showing which fungi and their spores are associated with SBS (Roby and Sheller, 1979). Our studies were made possible due to our association with an IAQ company. The uniqueness of this study was that sites were made available because school officials contacted the IAQ company. This allowed us access to all samples, data, questionnaires, and occupant generated complaints from buildings that were experiencing IAQ problems.

A useful definition of causation in diseases that are generally not in infectious origin is known as contributory cause. It requires a demonstration that the presumed cause precedes the effect, and that altering the cause alters the effect. It requires neither that all those who are free of the contributory cause will be free of the effect nor that all

those who possess the contributory cause will develop the effect. However, the presence of the contributory cause must increase the probability of the occurrence of the effect and its absence must reduce the probability of the effect (Morton, 1990).

Even using the concept of contributory cause, due to the multitude of factors and confounders involved in SBS, causality has proven difficult to demonstrate. When contributory cause cannot be definitively established, a series of supportive criteria for contributory cause was developed. These included the appropriateness of temporal relationships, the strength of associations, the consistency of observations, the biologic plausibility, and a dose-response relationship (Morton, 1990). These criteria may help support the argument that a factor is a contributory cause and reduce the likelihood that the observed association is due to chance or bias. However, they do not prove the existence of a contributory cause. A major problem, however, is that it is often difficult to control independent variables because of the diversity of the study population, its motility, or a lack of personal (total) exposure. In addition, it is difficult to segregate the individual pollutant from copollutants and other confounders. Thus, at best, associations can be drawn only between the exposure and the effect. A causal relationship is rarely discernible even with strong statistical significance.

The first phase of our research was devoted to developing a database consisting of the quantification and identification of microorganisms isolated from air and swab samples along with temperature and humidity measurements, and complaint forms received from the IAQ company. The purpose of an examination of this database was to determine if any correlation existed between IAQ complaints, outdoor versus indoor air samples of microorganisms (bacterial and fungal) and/or these microorganism's products.

occupant complaints (symptoms), relationships of time and location of these complaints, and increases in respiratory infections.

The strength of our study was that 48 public schools that were experiencing SBS were examined. Our studies of these sites demonstrated that *Penicillium* (*P. chrysogenum*) and *Stachybotrys* species were associated with indoor areas that had SBS complaints. In 25 of these buildings, we consistently isolated *Penicillium* species from the interior of complaint areas. What strengthens our postulate that these fungi may be a contributory cause is that our study demonstrated that after remediation of the buildings, which removed interior fungal growth, there was a significant reduction in IAQ occupant complaints. These findings suggest that *Penicillium* (*P. chrysogenum*) species are strongly associated with SBS. However, all that we had demonstrated was a temporal relationship, a consistency of observations, and a strong association.

These data demonstrate that the *Penicillium* species, especially *P. chrysogenum*, can adapt to an environment in which man is most comfortable, and are apparently capable of successfully competing with most conidial fungi. Its conidia are small (average 3.5µm in diameter) and are capable of entering the lower respiratory tract. However, for a particle to enter the respiratory tract, it must be aerosolized.

Aerodynamic particle size (the diameter of a sphere that describes the aerodynamic behavior of a particle) is the primary factor that controls how particles behave in an aerosol. For a spherical particle, such as *Aspergillus* and *Penicillium* species spores or conidia, aerodynamic diameter is the same as the actual diameter. *Penicillium* conidia and *Aspergillus* spores have aerodynamic diameters of 3 to 4µm and can stay airborne for days. For most elliptical particles, the smallest diameter more

closely represents aerodynamic particle size. *Cladosporium* spores are pyriform-shaped with the smallest diameter of 4µm and a length of 8µm. Because aerodynamically large particles, such as *Stachybotrys* species spores (7 to 9µm in diameter), fall faster than small ones, these spores are not usually isolated from air samples.

The concentration of any aerosol can be modeled by equations that include terms describing the source strength (i.e., the number of particles available for dispersion), the rate of dispersion from each source, the space into which the particles are dispersed, and the decay rate of the aerosol (i.e., the rate of particle loss). However, most of these terms that must be included in model equations are unknown for biologic aerosols. Sources are usually discontinuous, such as individual colonies growing on a wall surface or areas of contamination within a HVAC system. Dispersion mechanisms can be intrinsic, as is the case when living fungi actively discharge spores into the air. More commonly, dispersion results from mechanical disturbances that are sporadic, such as floor cleaning or the vibrations and movement of air in a HVAC system.

We observed that the average concentration of viable *Penicillium* conidia in complaint areas in "tight" or "sick" buildings was 200 CFU/m^3 of air. However, this was the "average" concentration and many building sites had 5- to 10-fold higher concentrations. In non-complaint areas or outside, the average concentration of viable *Penicillium* propagules rarely exceeded 10 CFU/m^3 of air, with many measurements showing no viable *Penicillium* propagules. An important point that our study demonstrated is that in indoor areas with visible interior *Penicillium* growth, the exposure to *Penicillium* propagules was significantly higher and constant when compared to the outdoor air, which contained varying concentrations of different fungi. This suggests that

the number of viable *Penicillium* propagules sampled from interior complaint areas are not discontinuous, varying with time and dispersion mechanisms, but may be an indication of the loading or amount of *Penicillium* colonies contaminating the interior. However, in some non-complaint areas, the *Penicillium* propagules were higher than outdoors. This could be explained that the further from the source, the fewer the viable propagules sampled form the air. Another valid explanation is occupant variability; that is, not all of the occupants are equally sensitive to molds. These findings again support a temporal relationship, a consistency of observations, a strong association, and even an indication of biologic plausibility (the conidia are aerosolized and small enough to enter the respiratory tract). However, for fungi to be allergenic, the exposure and the dose are important variables (Pope et al., 1993), although the sensitivity of the population may vary.

The relationship between exposure (the amount of an agent in an aerosol) and dose can be represented as the aerosol level (Factor 1) times the fraction penetrating into the airways (Factor 2) times the fraction deposited at the effective site (Factor 3) equals exposure. To accurately measure exposure, each of the three factors must be taken into consideration. We commonly use measured ambient levels of aerosols or fungal spores as a surrogate for exposure, considering for each particle type that factors 2 and 3 are constant. However, these factors are not constants, but variability within well-defined groups, such as elementary school-age children or non-smoking adults, is small enough that the variability can be ignored (Burge, 1995). Likewise, we use exposure (measured ambient levels) as a surrogate for dose. This may be appropriate for exposure to relatively simples particles such dust mite fecal materials or cat dander. However, we

believe that the release of antigen from fungal spores (conidia) is complex and strongly dependent on the kind of spore involved. We have demonstrated that *P. chrysogenum* conidia must be viable to induce an inflammatory and allergenic reaction. Another important factor may be how long the spore has been separated from the parent organism and is subjected to the harsh outdoor environment (radical changes in temperature and humidity and UV light), which could rapidly cause loss of viability, or the relative mild indoor environment (constant temperature and humidity and no UV). This complex scenario may, in part, explain the extreme disparities evident in the literature on fungus-related allergy (Lopez and Salvaggio, 1985).

Using the observed data, we postulated that in buildings experiencing SBS that have higher indoor concentrations of *Penicillium* species than the outdoor, then *Penicillium* species (*P. chrysogenum*) may be a contributing factor. Using this approach, our hypothesis was that *Penicillium* species product(s) are a contributing factor in the allergic reactions suffered by many of the occupants of buildings experiencing SBS. To establish biological plausibility and a dose-response relationship, the overall strategy was to determine if *P. chrysogenum* products do induce adverse biological responses and to establish quantitative relationships between the exposure *of P. chrysogenum* conidia and the incidence of adverse responses.

However, another variable was introduced when our growth studies suggested that fungal conidia viability was an important factor. When *P. chrysogenum* was grown under laboratory conditions, we observed that only approximately 25% of the conidia obtained were viable. To address the issue of viability, we rendered all of the conidia non-viable by a variety of methods, including heating at 60°C for 30 minutes, ultraviolet

(UV) irradiation for 4 hours, and incubation in absolute methanol for five minutes. Since it was obvious that heating the conidia would denature potential products, this method was not used in any experiments. In addition, the possibility existed that incubation in methanol could leach potential antigens from the conidia. However, since conidia incubated in absolute methanol for five minutes did not demonstrate any differences such as an increase in inflammatory cells or TNF-α as compared to the UV-irradiated conidia, we decided to use methanol as the method of rendering conidia non-viable.

The first step in our strategy was to establish that *P. chrysogenum* conidia are capable of inducing adverse biological responses. The methods that we used were in vitro assays and short-term (acute) animal studies in a mouse model. Since one of the first lines of defense of the immune system to inhaled particles are the pulmonary alveolar macrophages (PAM), we chose to conduct in vitro assays using these cells. The question that we examined was "what are the effects of *P. chrysogenum* conidia upon PAMs?" Our results demonstrated that *P. chrysogenum* conidia (products) must be viable to be capable of inducing increases of TNF-α in PAMs. TNF-α may represent an essential cytokine in the pathogenesis of lung allergic inflammatory responses. Once released, TNF-α can activate epithelial, endothelial, and inflammatory cells, and promote granulocyte adherence and migration (Bittleman and Casale, 1994). It has been shown that BAL TNF-α levels are increased in symptomatic, but not asymptomatic, asthmatics (Broide et al., 1992).

For our next step, a mouse model was chosen for the acute in vivo animal bioassays. Extrapolation is the process of relating empirical study findings to real world

scenarios. Studies in animals are the most dependent on this process. To demonstrate biological plausibility, a dose response that was similar to the concentration observed in the study was determined. Under normal activity, a human adult who is exposed to this concentration (200 CFU/m^3 of air) for 40 hours would have a weekly cumulative dose in the lungs of approximately $3x10^3$ viable *Penicillium* conidia. If the home is contaminated, then an adult's weekly cumulative dose could more than double, approaching $1x10^4$ viable *Penicillium* conidia. Since our early studies suggested that only approximately 25% of the conidia obtained were viable, to obtain a yield of $3x10^3$ viable *Penicillium* conidia, we would have to intranasally inoculate the mice with approximately $1x10^4$ total *Penicillium* conidia. If the target site is the lung (tissue distal to the trachea) then, teleologically, one could argue that each species evolved with similar functional demands (i.e., O_2 - CO_2 exchange) and environmental stresses on the pulmonary system. In the average human adult, most particles (>90%) larger than 10µm in aerodynamic diameter are deposited in the nose or oral pharynx, and cannot penetrate to tissues distal to the larynx (Brain and Valberg, 1979; Heyder, 1982). Particles that penetrate beyond the upper airways are available to be deposited in the bronchial region and the deeper-lying airways. The alveolar region has significant deposition efficiencies for particles smaller than 5µm. In general, approximately 90% of inhaled particles with aerodynamic diameters greater than 3µm are deposited on the mucus overlying ciliated epithelium, while 90% of inhaled particles with aerodynamic diameters 3µm or less are deposited in the non-ciliated regions of the lower respiratory tract distal to the terminal bronchioles (Brain and Valberg, 1979; Heyder et al., 1975). *Penicillium* conidia are

spherical in shape and have aerodynamic diameters ranging from 1μm to 5μm with an average of 3.5μm. This suggests that less than 50% of the *Penicillium* conidia ($1x10^3$) inhaled would reach the alveoli spaces.

However, rodents are obligate nose breathers. They have extensive nasal turbinates with significantly more nasal surface area on a per body weight basis than do humans. This makes it difficult to extrapolate nasal inoculations to humans. When using intranasal instillations, the inoculum must pass through these extensive nasal turbinate networks. The primary objection to intranasal instillation is the high probability that patterns of particle distribution are unlike those resulting from natural inhalation (Brain et al., 1976). However, since our strategy was to examine chronic doses, we had to use a method that would allow for repeated anesthetization with a drug that the animal model would tolerate. Intratracheal instillation would require deep anesthetization, which, over a period, the animal would develop tolerance and would eventually overdose. Intranasal instillation only requires the animal to be lightly anesthetized, thus avoiding the chance of overdose. To examine the immune response to the *Penicillium* conidia deposited into the lungs of an animal, we determined the number of viable conidia being deposited, cleared and retained in the lungs of our animal models.

In the C57Bl mouse model, we demonstrated that approximately 4% ($1x10^4$) of the viable inoculum ($2.5x10^5$) were deposited in the alveoli spaces. Extrapolating these findings to a dose similar to what humans might receive would mean the mouse model would have to be inoculated IN with $1x10^5$ *Penicillium* conidia (25% viability).

The results of the deposition, clearance, and retention assays were very important in that we demonstrated that *P. chrysogenum* conidia remained viable for at least 36

hours. These data suggest that the viable conidia remain in the alveolar spaces long enough to germinate and products of this attempted germination could induce adverse effects.

In the next step, we sought to determine the acute effects of *P. chrysogenum* conidia (viable and non-viable) in the BALB/c and the C57Bl mouse model. The results of these experiments demonstrated that viable conidia were capable of inducing a significant increase in inflammatory cells (eosinophils and/or neutrophils) in mammalian lungs while non-viable conidia did not. These findings also confirmed that there was no observable difference between rendering the conidia non-viable by methanol and UV irradiation.

The next step in our strategy was to establish quantitative relationships between exposure of *P. chrysogenum* conidia (products) and the incidence of adverse responses. Again, we used the mouse model and posed two questions. The first question was "if an acute dose of *P. chrysogenum* conidia induced inflammation in the lungs, what would be the effects of long term exposure in the lungs to inhaled spores?" The second question was, "could the *P. chrysogenum* conidia products induce an allergic reaction in the animal model?" To generate a low dose, we reduced the amount of the inoculum to $1x10^4$ and $1x10^2$ conidia (25% viability).

When the animal models were inoculated IN for three weeks, the C57Bl mouse model demonstrated significant increases in total IgE and BAL IL-4 along with peripheral eosinophilia. However, the BALB/c mice did not demonstrate an increase in IgE or BAL IL-4. Although the BALB/c mouse model inoculated IN with viable conidia displayed airway neutrophilia, these animals did not appear to be a good model for

determining if the conidia were capable of inducing allergic reactions. However, these data suggest that atopic individuals may be at a higher risk to develop allergic reactions to fungal products, especially viable *P. chrysogenum* conidia.

In our final series of experiments, the C57Bl mouse model was inoculated intranasally (IN) weekly for 6 weeks with 10^4 viable (25% viability) *P. chrysogenum* conidia. These data suggest that long-term inhalation of viable *P. chrysogenum* propagules induces inflammatory responses, such as increases in serum IgE, IL-4, and IL-5, along with peripheral and airway eosinophilia and airway neutrophilia, which are mediators of allergic reactions. The results also suggest that viable *P. chrysogenum* conidia may be producing a substance that is necessary to induce these responses.

This study examining the IN instillation of *P. chrysogenum* conidia into C57Bl/6 mice has produced interesting findings regarding the etiology of asthma. Asthma is a common and chronic inflammatory condition of the airways whose cause is not completely understood. The prevalence of asthma is currently undergoing an unprecedented upswing in many developed countries, increasing from fewer than 7 million cases in 1980 to nearly 15 million in 1997 (Smith et al., 1997). The general, but not unanimous, view among epidemiologists is that the increase in deaths, hospitalizations, and physician visits for asthma reflects a true increase in the prevalence of the disease (Boushey and Fahy, 1995). The reasons for this are unclear, but most speculation centers on the changes in building insulation driven by the rising cost of energy, a reduction in the turnover of indoor air, and the increased exposure to indoor pollutants (Boushey and Fahy, 1995). This increase in asthma prevalence closely parallels the appearance of SBS. The recognition of the fact that asthma is an

inflammatory disease has focused attention on the mechanism(s) that produce this inflammation (Lack et al., 1995). Understanding this disease begins with a histopathological examination of asthmatic airway and lung tissue that reveals the presence of a chronic inflammatory response. Recent studies have suggested predominance of a particular subtype of lymphocyte in asthmatic airways. These studies have examined lymphocytes recovered from bronchial lavage fluid obtained from asthmatic and healthy subjects, as well as analyzing the pattern of cytokine production by in situ hybridization. The findings demonstrate predominance of the TH2 subset in asthmatic subjects (Robinson et al., 1992). This subset of T-helper lymphocytes is characterized by its production of a cluster of interleukins such as IL-4, IL-5, and GM-CSF. IL-4 is important in directing plasma cells to make IgE. IL-5 promotes in vitro survival and activation of eosinophils and induces eosinophilia in vivo. GM-CSF also contributes to eosinophil maturation and differentiation. In another study, it was shown that there was an increase in the number of TH2 cells expressing these interleukins in the BAL fluid obtained 24 hours after an antigen challenge in asthmatics, and the number of these cells correlated to the increase in the interleukins and the number of eosinophils in the BAL (Robinson et al., 1993).

Our studies demonstrated that the IN inoculation of *P. chrysogenum* conidia into the lungs of C57B1/6 mice not only results in allergic reactions, but may also result in asthma-like reactions. The data also demonstrate that sensitization to the viable *P. chrysogenum* conidia via mucosal exposure was efficient and did not require the potentially immunomodulatory intraperitoneal or subcutaneous priming (with adjuvant) used in the ovalbumin models to evoke airway inflammation. Our findings demonstrate

that long-term exposure to viable *P. chrysogenum* conidia caused a significant increase in total serum IgE, which is an indicator of an allergic response (Bentley et al., 1993). In addition, the same animals demonstrated significant peripheral and airway eosinophilia following long-term exposure to 10^4 viable conidia. Significant increases in airway eosinophils are an indication of an allergic response and one of the most important indicators of asthma-like reactions in mammals (Kay, 1992; Robinson et al., 1992; Azzawi et al., 1990; Watson et al., 1993; Herz et al., 1996). Our data also demonstrated that these cellular and IgE increases are supported by the significant increases in IL-4 and IL-5 in the BAL of mice inoculated IN with 10^4 viable conidia. These cytokines are required for IgE synthesis and eosinophil recruitment, respectively. The BAL GM-CSF from mice that had received 10^4 viable conidia had increased at 24 hours, although not significantly. Nevertheless, the significant production of these cytokines (IL-4 and IL-5) in the lungs of mice inoculated with 10^4 viable conidia, coupled with the airway eosinophilia, strongly suggests that the instilled viable conidia are inducing a response similar to that observed in an asthmatic reaction. These findings are supported by an epidemiological study that demonstrated that exposure to *Penicillium* species was a risk factor for childhood asthma (Garrett et al., 1998). However, causation is still elusive and these findings can only demonstrate associations.

Our work showed that SBS and the dominance of *Penicillium* species are strongly associated. Our results suggest that viability of *P. chrysogenum* conidia and the production of an unknown substance are responsible for the chronic airway inflammation observed in fungal-induced allergies and asthma. Our studies present additional information that the inhalation of fungal propagules (specifically *P. chrysogenum*) and

their viability may play a role in SBS and eosinophilic bronchitis. Our studies also suggest that atopic individuals may be at more risk when exposed to buildings with interior *Penicillium* species growth. This relationship could be causal or merely exacerbation. Other studies have suggested that there are associations between damp housing, chronic childhood respiratory symptoms, and sensitization to house dust mites and mold spores (allergens), including *Penicillium* species (Garrett et al., 1998; Martin et al., 1987; Verhoeff et al., 1995). This study suggests that measures to decrease exposure, especially in the public schools, to interior growth of fungi, such as *Penicillium* species, should be an important part of the management of SBS. This removal could theoretically result in a drastic reduction in at least the exacerbation of fungal-induced SBS in developed countries.

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