Massive Fungal Contamination in Animal Care Facilities Traced to Bedding Supply

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During the course of immunologic studies involving the gastrointestinal colonization of mice with *Candida albicans*, it became apparent that the animals were being exposed to large numbers of *Aspergillus fumigatus* spores which interfered with the *C. albicans* colonization. To determine the source of the *A. fumigatus* exposure and the extent of fungal contamination of the medical school vivarium and four satellite facilities, fungal analyses of feed, bedding, and air were undertaken. Initial samples from the air were collected with 3-h settle plates; air sampling following cleanup was done with an Andersen air sampler. The source of contamination in the mouse rooms was determined to be Beta Chip bedding, which came from the manufacturer highly contaminated. Beta Chip bedding (1 g) obtained from the manufacturer just prior to testing contained 10^4 CFU of *A. fumigatus*, 20 CFU of a zygomycete, and 10 CFU of a *Penicillium* sp. Coarse-grade Beta Chip had approximately one-half those levels of contamination. Pure Cob bedding was highly contaminated also, but with a *Fusarium* sp. and a *Cladosporium* sp. Untreated and heat-treated Sani-Chip as well as all other heat-treated preparations obtained from the manufacturer contained no detectable spores. Rodent chow direct from the manufacturer had no *A. fumigatus*, although it did contain 150 CFU of fungus per g, including 80 CFU of a *Rhodotorula* sp., 60 CFU of *Cryptococcus uniguttulatus*, and 1 CFU of a *Penicillium* sp. Unopened laboratory canine, feline diet, rabbit, and guinea pig chow, however, had 1.5×10^2 to 4.1×10^2 CFU of *A. fumigatus* per g. Air sampling confirmed the presence of numerous *A. fumigatus* spores prior to cleanup and the absence or significant reduction in spore numbers after cleanup with Quatricide PV. These data emphasize the need to examine support materials for microbial contamination prior to their use, especially with research animals being used for microbiologic and immunologic studies. It is recommended that Sani-Chip or heat-treated preparations other than Sani-Chip be used for the housing of rodents.

The gastrointestinal (GI) tracts of inbred adult mice that are intubated with viable *Candida albicans* intragastrically in sufficient numbers, e.g., 2×10^7 cells, retain the fungus for 2 to 3 weeks following intubation (6, 9). The presence of *C. albicans* can be demonstrated by culturing various segments of the GI tract as well as fecal pellets. This animal model has been used in our laboratory to study immune responses to *C. albicans*, and it is clear that the 2 to 3 weeks of association with the fungus is sufficient to prime both the humoral and cellular arms of the murine immune response for enhanced antibody responses and delayed-type hypersensitivity (DH) for secondary responses when subsequently challenged intradermally with viable *C. albicans* (7). We have also used this model to study immune responses in mice in which DH has been down-regulated by intravenous exposure to antigen during the course of immunization (8).

Research on the down-regulatory cells in this model was suspended for 1 year at Tulane University while one of the authors was on leave. When the studies were resumed following the leave, it became clear that mice intubated with *C. albicans* did not become uniformly primed for enhanced immune responses when challenged a second time intradermally with the organism, as had been noted previously (6). Immunologic data acquired during this interval were highly erratic;

some animals developed high levels of DH, while others did not. Prior experience with this model had led us to expect high levels of DH in virtually all immunized mice. Moreover, animals challenged twice intradermally (a second protocol which results in the induction of high levels of DH demonstrable 1 week after the second intradermal challenge) rather than by the intragastric-intradermal routes also displayed somewhat erratic levels of priming. Despite the lack of uniform immunologic responses in the mice, they appeared healthy and their activity was at a normal level.

To attempt to determine the cause of the erratic immunologic data, mice were intubated with large numbers of *C. albicans* and fecal pellets were cultured 4 days later. The intent was to culture samples from the animals at regular intervals thereafter as well to ascertain that the same conditions of the association of the GI tract with *C. albicans* over time prevailed as had been demonstrated previously (6). Fecal cultures were performed on day 4 on four randomly selected mice housed in the same room but selected from cages located in two different hoods. Surprisingly, these cultures, which one would expect to contain at least 10^2 *C. albicans* per two fecal pellets, were negative for *C. albicans* and highly positive for *Aspergillus fumigatus*. Prior to this occasion, *A. fumigatus* had never been isolated from the feces of any mice in our facility, including infant mice as well as adult mice intubated with *C. albicans*. The observation of *A. fumigatus* in the fecal pellets prompted an investigation to determine the source of the *A. fumigatus* and the extent of contamination of the animal care facilities at Tulane University, New Orleans, La.

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MATERIALS AND METHODS

Animals. Male CBA/J mice that were 6 to 8 weeks of age at the time of purchase were acquired from Jackson Laboratories, Bar Harbor, Maine. VAF and COBS mice were purchased from Charles River, Inc., Wilmington, Mass. The CBA/J mice were all housed in one room in the vivarium. Samples from the VAF and COBS mice were cultured immediately upon arrival of the mice in the animal-receiving area of the vivarium facility in the Tulane Medical School. *C. albicans* was cultured, and animals were intubated with the fungus, as described previously (6).

Cultures. Initially, settle plate sampling was performed with petri dishes (15 by 100 mm) containing approximately 20 ml of Sabouraud agar (Difco Laboratories, Detroit, Mich.) and 298 mg of chloramphenicol per liter (Sigma Chemical Co., St. Louis, Mo.). Later, both settle plate sampling and Andersen air sampling were done with dishes of the same size containing malt extract agar consisting of the following ingredients (per liter): 30 g of malt extract, 5 g of peptone, 15 g of agar, and 10 ml of chloramphenicol solution prepared by dissolving 2 g of chloramphenicol in 67 ml of acetone (all from Difco Laboratories). The chloramphenicol was added to the medium after the medium was autoclaved and before it was poured into the plates.

Settle plate sampling was carried out prior to cleanup by placing two open plates of medium on opposite sides of each room for 3 h. Plates were not put in rooms on days during which cages were changed. After cleanup, air sampling was performed with a suction impactor, the Andersen two-stage microbial air sampler (Andersen Samplers Inc., Atlanta, Ga.), operated for 15 min at a rate of 1 ft³ (ca. 3×10^{-2} m³)/min.

Fecal cultures were performed by capturing two fresh fecal pellets with sterile forceps as they were expelled from the animal and placing them into 2 ml of sterile saline for homogenization in a Tissumizer (Tekmar, Co., Cincinnati, Ohio). Two plates were each inoculated with 0.5 ml of the homogenate. Likewise, selected organs and sections of the GI tract were surgically removed from mice and homogenized in 2 ml of sterile saline prior to the plating of 0.5 ml of the homogenate onto each of two plates.

Various types of bedding were obtained from the manufacturer (Northeastern Products, Warrensburg, N.Y.) as either heat treated or untreated. During the heating process, the samples begin at 800 to 850°F (ca. 427 to 454°C) and finish at 200 to 250°F (ca. 93 to 121°C), with the total process requiring 15 to 20 min. For culturing, bedding and feed (Purina Mills Inc., St. Louis, Mo.) were weighed and then emulsified in 10 or 20 ml of sterile saline, and 0.5 ml of each extract was plated onto each of two plates. Appropriate calculations to determine the number of CFU per gram of material were performed. Samples of both opened and previously unopened bedding and feed from the Tulane Medical Center in New Orleans, La., and the Louisiana State University Veterinary School in Baton Rouge were assayed. In addition, bedding and feed were purchased from the manufacturer for culture immediately upon receipt in the vivarium.

A. fumigatus was identified by its typical gross and microscopic appearance on Czapek Dox medium and by its ability to grow at 45°C (1). Aspergilli other than *A. fumigatus*, *Aspergillus flavus*, and *Aspergillus niger* were not identified to the species level and are reported as *Aspergillus* spp. Identification of the yeasts was carried out with the Uni-Yeast Tek rapid identification system (Remel, Lenexa, Kans.) supplemented with an expanded panel of sugar assimilations performed in small culture tubes. Although only random isolates were identified, all non-pigmented and/or encapsulated yeast cells were plated onto birdseed (Staib) agar (1) to rule out the presence of *Cryptococcus neoformans*. Airborne contaminants belonging to the order *Mucorales* were not identified as to genus or species, with the exception of *Syncephalastrum* spp., except to note on the basis of gross and cursory microscopic observations that colonies were either a *Mucor* or *Rhizopus* sp. Molds with hyaline hyphae which did not sporulate under the conditions of incubation employed were reported as nonsporulating hyphomycetes. Nonsporulating molds which were brown or black on culture were reported as dematiaceous molds.

Facilities. Five vivarium facilities were sampled, one at Tulane Medical School and four at satellite locations separated from the Medical School by 1 to 10 mi (ca. 1.6 to 16 km). Each room in the Medical School vivarium, including animal housing, preparation, and storage rooms, as well as comparable rooms in the satellite facilities, was sampled initially with 3-h settle plates. Following cleanup, all rooms were sampled with an Andersen two-stage air sampler. Several research laboratories within the Medical School separated by four to six floors from the vivarium were sampled with settle plates as well.

Cleanup. Following determinations of the source of contamination, all animal facilities were cleaned in the following manner. Animals were removed from the environment, and all surfaces, including walls, ceilings, and cage-holding equipment, were washed with Quatricide PV (Pharmaceutical Research Laboratories, Inc., Naugatuck, Conn.). Quatricide PV contains didodecyl dimethyl ammonium chloride, *n*-alkyl dimethyl benzyl ammonium chloride, and sodium hydroxide. Andersen air sampling was performed several weeks following the cleanup procedure. Animals were housed in the appropriate rooms at the time of sampling.

TABLE 1. Culture analyses of samples from three mice^a

Tissue	CFU of <i>A. fumigatus</i>		
	Mouse 1	Mouse 2	Mouse 3
Spleen	0	0	0
Liver	0	0	0
Lungs	0	3×10^0	3×10^0
Stomach	4.5×10^1	1.2×10^2	1.2×10^1
Intestine	6.9×10^1	1.1×10^2	6.3×10^1
Colon	1.4×10^2	1.0×10^2	5.1×10^1

^a None of the mice had been infected with *C. albicans*, and *A. fumigatus* was the only fungus isolated. The mice were selected at random from cages located in two different hoods in one Medical School vivarium room in which only mice were housed.

RESULTS

Initial observations. CBA/J mice housed in one of two laminar flow hoods in one room in the Medical School vivarium were intubated with 2×10^7 *C. albicans*, and 4 days after the intubation, samples from the mice were cultured with Sabouraud agar with chloramphenicol. One would expect to find approximately 10^2 CFU of *C. albicans* and no other fungal organisms in such cultures. However, no *C. albicans* was found in cultures of the fecal pellets, internal organs, and GI tracts. The fecal cultures were pure cultures of *A. fumigatus*, ranging in numbers from 2.2×10^2 to 4.3×10^2 organisms per two fecal pellets. Subsequently, three mice not infected with *C. albicans* were selected at random from cages in the hood which contained no mice infected with *C. albicans*, and they were sacrificed so that internal organs and sections of the GI tract could be cultured. The culture data are summarized in Table 1. Clearly, *A. fumigatus* spores were present in significant numbers in the GI tracts of these mice. No other fungi, in fact, including *C. albicans*, were detected in the homogenates. It is assumed that the single colonies detected in lung homogenates were spores lodged in the airways and not in the lung tissue per se.

Analysis of bedding and feed. Since *A. fumigatus* was present in relatively large numbers in the GI tracts of all mice tested, it was hypothesized that either the bedding or feed was contaminated with the fungus. The bedding employed for all rodents in Tulane University facilities at that time was Beta Chip, which had not been heat treated by the manufacturer. Initially, clean Beta Chip bedding and Lab Rodent Chow stored in large closed containers in the animal rooms were sampled; later, samples from previously unopened bags stored on site but not within rooms in which animals were housed were removed for testing. Moreover, as there was concern that storage conditions at the supplier's warehouse or in our own facilities might be contributing to the contamination, new bags of bedding of various types were obtained directly from the manufacturer and tested immediately upon arrival in our facility. Various types of feed from the School of Veterinary Medicine at Louisiana State University were obtained for culture as well. That facility is approximately 90 mi (ca. 150 km) from Tulane Medical School.

Culture analyses of the bedding obtained from the manufacturer and of feed taken from previously unopened bags stored in rooms separate from animal housing are summarized in Table 2. As Lab Rodent Chow was not contaminated with *A. fumigatus* and unheated Beta Chip bedding was highly contaminated with it, the source of the *A. fumigatus* in the mice was presumed to be the bedding. Clearly, the heating process employed by the manufacturer in the preparation of bedding

TABLE 2. Culture analyses of bedding obtained directly from the manufacturer in sealed bags and of feed taken from sealed bags kept in storage rooms

Material cultured	No. of organisms (CFU/g)	Fungal species
Bedding		
Beta Chip	1.0×10^4	<i>A. fumigatus</i>
	2.0×10^1	<i>Mucor</i> and <i>Rhizopus</i> spp. ^a
	1.0×10^1	<i>Penicillium</i> sp.
Beta Chip (HT) ^b	0	
Beta Chip (CG) ^c	5.0×10^3	<i>A. fumigatus</i>
	1.0×10^1	<i>Mucor</i> and <i>Rhizopus</i> spp. ^a
Beta Chip (CG) (HT)	0	
Pure Cob	1.2×10^3	<i>Fusarium</i> sp.
	4.0×10^3	<i>Cladosporium</i> sp.
	7.0×10^2	<i>Penicillium</i> sp.
Pure Cob (HT)	0	
Aspen	2.8×10^2	Various zygomycetes
Aspen (HT)	0	
Sani-Chip	0	
Sani-Chip (HT)	0	
Feed		
Lab Rodent Chow (TU) ^d	8.0×10^1	<i>Rhodotorula</i> sp.
	6.0×10^1	<i>C. uniguttulatus</i>
	1.0×10^1	<i>Penicillium</i> sp.
Lab Rodent Chow (LSU) ^e	5.0×10^1	<i>Rhodotorula</i> sp.
	1.0×10^1	<i>Alternaria</i> sp.
	1.0×10^1	<i>Cladosporium</i> sp.
Rabbit chow (TU)	1.5×10^2	<i>A. fumigatus</i>
Rabbit chow (LSU)	8.0×10^1	<i>A. fumigatus</i>
	1.0×10^1	<i>Cladosporium</i> sp.
	1.0×10^1	<i>Syncephalastrum</i> sp.
	4.0×10^1	Dematiaceous molds
	1.0×10^1	Nonsporulating hyphomycete
Laboratory canine chow (TU)	4.1×10^2	<i>A. fumigatus</i>
Feline diet (TU)	1.0×10^1	<i>Rhodotorula</i> sp.
	3.3×10^2	<i>A. fumigatus</i>
	1.0×10^1	Nonpathogenic yeasts
Feline diet (LSU)	0	
Guinea pig chow (TU)	2.7×10^2	<i>A. fumigatus</i>
	6.0×10^1	<i>Penicillium</i> sp.
Guinea pig chow (LSU)	0	
Ferret chow (LSU)	0	

^a These organisms were not differentiated further.

^b HT, heat treated.

^c CG, coarse grade.

^d TU, taken from the Tulane Medical Center vivarium facility.

^e LSU, taken from the Louisiana State University Veterinary School.

which is sold as heat treated resulted in the sterilization of the bedding materials.

Although an exhaustive analysis was not performed, most unopened bags of feed contained several different fungi, and rabbit chow in particular was highly contaminated with *A. fumigatus* in one facility and *A. fumigatus* in conjunction with a number of other fungi in another facility. It is unlikely that the contamination of the feed resulted from exposure to bedding. While unopened bags of feed and bedding are stored in the same room, they are not opened in that room. Further, all feed is stored in the same room, and if the fungus had been acquired by cross-contamination from the bedding, one would have expected the Lab Rodent Chow to have been positive for *A. fumigatus* as well.

Air analyses. Air analyses for fungi were performed in all rooms housing animals as well as those necessary for the sup-

FIG. 1. CFU per room prior to cleanup, based on the total CFU from two settle plates exposed for 3 h in rooms in which various animal species were housed (A), in rooms not directly related to animal-holding areas (B), and in support and storage rooms in the animal areas (C). As appropriate, data for the five vivarium facilities were averaged. The data for rooms housing mice, rats, rabbits, and hamsters are averages of 11, 20, 5, and 3 rooms, respectively. All other data were derived from single rooms.

port activities of the facility in all five animal care facilities maintained by Tulane University. Prior to cleanup, 3-h settle plates, two per room on opposite sides of the room, were used. Following cleanup, Andersen air sampling was performed with the two-stage Andersen air sampler. Facility A is located approximately 1 mi from facility B, facility C is located approximately 10 mi from facilities A and B, and facilities D and E are located three floors apart in the same building, approximately 5 mi (ca. 8 km) from facilities A and B and about 15 mi (ca. 24 km) from facility C.

Data for total CFU and for CFU attributable to *A. fumigatus* were determined with settle plates exposed for 3 h in various rooms of the five animal care facilities. They are collated in Fig. 1. It is clear from these data that the predominant organism in all rooms housing rodents was *A. fumigatus*. Moreover, *A. fumigatus* spores so permeated the air that even by the relatively insensitive measure of settle plates, large numbers of spores were deposited on the plates. Apart from *A. fumigatus*, the most common organism was a *Penicillium* sp., and the next most common was a *Cladosporium* sp. Other randomly distributed fungi identified in low numbers included a *Mucor* sp. and a *Rhizopus* sp., a *Rhodotorula* sp., an *Alternaria* sp., an *Ustilago* sp., a *Syncephalastrum* sp., an *Acremonium* sp., an *Aureobasidium* sp., an *Epicoccum* sp., a *Curvularia* sp., an *Aspergillus* sp., and a *Sporothrix* sp. Several nonpathogenic yeasts, some of which were encapsulated, were also isolated. Representative colonies were tested to rule out *C. neoformans*, and an isolate obtained from the air in one of the rooms housing mice was identified as *Cryptococcus laurentii*.

All rodents are housed in cages with solid floors overlaid with bedding. Rooms in which animals were housed in cages without bedding had few *A. fumigatus* spores detectable with settle plates. The rabbit rooms were almost free of airborne *Aspergillus* sp. spores, despite the fact that the rabbit chow was contaminated with *A. fumigatus* (Table 2). Support rooms, for example, those in which feed and bedding were stored and bottle- and cage-washing rooms, were also highly contaminated with *A. fumigatus* (Fig. 1). Vivarium rooms in non-animal areas, including the surgical suite, were relatively spore free as

determined with settle plates and by Andersen air sampling. As might be expected, the two rooms that animal care workers might be expected to frequent, viz., the restrooms and the lounge, had the greatest number of fungal contaminants outside of the rooms in which animals were housed. In these cases, however, *A. fumigatus* spores were in the minority and those of a *Cladosporium* sp. were in the majority.

As indicated above, many air and feed samples contained nonpigmented yeasts, the majority of which were encapsulated. Representative colonies of all nonpigmented yeasts, encapsulated or unencapsulated, were assayed to rule out the possibility that *C. neoformans*, a pathogenic yeast, was among those yeasts. A few of the encapsulated yeasts were identified to the species level. Thus, *Cryptococcus uniguttulatus* was identified in rodent chow, and *C. laurentii*, as mentioned above, was found in an air sample.

Analyses following cleanup. Following the identification of the source of the airborne *A. fumigatus* spores, all supplies of Beta Chip bedding were incinerated, all animals were removed from all rooms, and all surfaces in all rooms were cleaned with Quatricide PV. Cages were washed in the usual manner in the cage-washing equipment. Approximately 1 month thereafter, each of the rooms was again evaluated, but the air was sampled with the Andersen air sampler operating for 15 min at 1 ft³/min. Since <1 CFU of any fungus per ft³ of air and very few *A. fumigatus* cells were detected on any of the plates, it was clear that the cleanup was effective and that *A. fumigatus* was no longer a problem with regard to contamination of the vivarial environment.

DISCUSSION

We have shown clearly that *A. fumigatus* was a significant air contaminant in several vivarial facilities, in particular in rooms in which rodents were housed; that the source of the *A. fumigatus* in the air was most likely the bedding used, i.e., unheated Beta Chip; and that following a change to Sani-Chip bedding and a cleanup with Quatricide IV, *A. fumigatus* was no longer a significant air contaminant. In addition to being present in large numbers in Beta Chip bedding, *A. fumigatus* spores were present in moderate numbers in unopened bags of rabbit, canine, feline, and guinea pig chow. The chow did not seem to be a major source of contamination of the air, however, since only the rooms in which rodents were housed yielded large numbers of CFU on the settle plates employed. Moreover, rodent chow appeared to be free of *A. fumigatus*, in that no *A. fumigatus* CFU were cultured from it.

This was not designed as a prospective study. It was a study that was prompted by spurious immunological data collected from an animal model in which data had been quite reproducible in the past and by the finding of large numbers of *A. fumigatus* CFU in the fecal pellets of inbred mice housed under seemingly ideal conditions in biocontrol hoods in a university vivarial facility. Since the study was reactive rather than proactive, the air sampling technique, in particular that involving the settle plates, was not ideal for obtaining quantitative data (5). Moreover, we did not count total particles present in the air; therefore, our data are based solely on viable particles. It is reasonable to assume that there would be a number of nonviable fungal particles present in the environment as well. Despite these caveats, the finding in the rodent rooms of relatively large numbers of *A. fumigatus* CFU and relatively small numbers of other fungi that can be expected to be found in the indoor environment with settle plates, in conjunction with the finding of no or low numbers of *A. fumigatus* CFU in rooms housing other animal species, provided solid evidence of a

significant contamination problem with *A. fumigatus*. Moreover, when the putative source of the *A. fumigatus*, i.e., the bedding, was removed and cleanup was effected, the average number of CFU detected in rooms housing animals was <1 CFU/ft³.

Indoor air quality has become an area of increasing concern in recent years. Chemical compounds which may be toxic or allergenic and microorganisms which may produce toxic or allergenic substances are being studied for their roles in decreasing the quality of the air (2, 3, 4, 11, 14–17). Both chemical and biological factors may contribute to the so-called sick building syndrome. For the incident reported here, health problems for workers in the areas involved were not the reason for initiating the study, as is the case in many of the studies reported in the literature. In retrospect, however, symptoms of allergic reactions, including shortness of breath, sneezing, and rhinitis, while working in the animal facilities had become increasingly severe among one principal investigator and two research technicians working with the mice in one of the affected rooms. These symptoms were alleviated to some extent by the wearing of particle masks by workers handling the rodents. At the time the symptoms became severe, the individuals involved attributed the clinical phenomena to the development of hypersensitivities to such animal allergens as urinary prealbumin and epithelial protein (12). An *Aspergillus* sp., while not the most common cause of fungal respiratory allergy (11, 12), is well-known for its potential in this arena (10, 13, 18). Following the cleanup, the respiratory problems disappeared in the investigator and technicians, and they were able to handle the animals without the use of a respiratory filter. It is not surprising that the vivarial staff did not complain of respiratory problems, since all vivarial staff members were required to wear masks when cleaning areas in which animals were housed.

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