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Appl. Environ. Microbiol. **1977, 34(6):715.**

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1977, p. 715-719
Vol. 34, No. 6

Printed in U.S.A.

Production of Thermophilic Actinomycete-Hay Aerosols for Use in Experimental Hypersensitivity Pneumonitis

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Received for publication 18 April 1977

An investigation of the factors that influence the production of dense aerosols of *Micropolyspora faeni* and *Thermoactinomyces vulgaris* from hay cultures revealed that the density, freshness, and moisture content of the hay were important influences. Dry aerosols were produced under optimum conditions from hay cultures of either actinomycete and from sterile hay for inhalation challenges to unimmunized rabbits. Depressions in arterial oxygen tensions and hemolytic complement were monitored after such challenge. This investigation showed that hay cultures are capable of inciting a hypersensitivity-like reaction in the lungs, even in the absence of immunization to the organisms contained within the hay, but the entire hypersensitivity reaction cannot be attributed to *M. faeni* and *T. vulgaris* alone. Hay itself or other microbial or chemical components appear to have some heat-stable component that possibly contributes to the pathogenesis of the disease.

Of all the microorganisms involved as etiological agents of hypersensitivity pneumonitis, the most frequently encountered are the thermophilic actinomycetes *Micropolyspora faeni* and *Thermoactinomyces vulgaris* (8, 20). Although a number of mechanisms of pathogenesis have been proposed (18), little in the way of experimental evidence connecting these specific organisms with the disease is available.

A model recently developed in this laboratory (19) with aerosols of spores of *Aspergillus terreus* or *Aspergillus fumigatus* in rabbits has shown that a spontaneous form of hypersensitivity pneumonitis may be initiated in the absence of immunization to the inciting organism by nonspecific complement activation. The purpose of this investigation is to extend the observations made with the inhaled *Aspergillus* spore model to the thermophilic actinomycetes *M. faeni* and *T. vulgaris* by defining conditions for production of aerosols from these organisms for use in inhalation challenge studies.

MATERIALS AND METHODS

Microorganisms. *M. faeni* and *T. vulgaris* were obtained from the Marshfield Clinic and Research Foundation, Marshfield, Wis. *M. faeni* was grown in half-strength nutrient broth (Difco Laboratories, Detroit, Mich.) at 56°C for 2 weeks. *T. vulgaris* was grown similarly, but in half-strength nutrient broth plus 2.5 g of yeast extract (Difco) per liter.

Hay cultures. Two types of hay were used. Ordinary, year-old dry hay was obtained from a conventional bale. Fresh hay was obtained by immediate laboratory drying (37°C) of freshly cut green forage grasses. After investigating several variables, the following standard conditions were used: 30-g amounts of fresh hay were packed into storage dishes (100 by 80 mm) and sterilized by autoclaving (120°C for 12 min). Each dish was inoculated with 22 ml of broth culture, sealed in parafilm, and incubated for 3 weeks at 56°C.

For controls, sterile hay cultures were prepared by inoculating 30 g of sterile fresh hay with 22 ml of sterile half-strength nutrient broth plus 2.5 g of yeast extract per liter, sealing them in parafilm, and incubating them for 3 weeks at 56°C.

After incubation, the parafilm seal on each culture was broken to permit drying during 1 more week of incubation at 56°C.

Quantitation of spores. One-tenth gram of each hay culture to be quantitated was placed in the bottom of a modified Lucite aerosol chamber. A side opening in the chamber was covered with a plate of nutrient agar. Spores were aerosolized by delivering a stream of air from a pressure pump at 3 lb/in^2 through a Pasteur capillary pipette onto the surface of the culture for 30 min. The number of colonies on each plate was then calculated after incubation.

Laboratory animals. Twenty-four outbred, 3-month-old, female New Zealand white rabbits weighing about 2 to 2.5 kg were obtained from Hilltop Laboratory Animals Inc., Scottdale, Pa. These animals were placed in separate cages and fed under the same conditions. Each animal was allowed at least 2 weeks to adjust to the new environment before the investigations were started.

Serolcgical analysis. Prechallenge serum from each animal was assayed for precipitins to *M. faeni* and *T. vulgaris* by counterimmunoelectrophoresis (9). The antigens used in this procedure were freeze-

pressed extracts of broth cultures of the respective organisms.

Aerosol challenge. The protocol and apparatus used for aerosolizing dry materials for inhalation by rabbits have been described previously (19). Instead of placing plate cultures on the bottom of the exposure chamber, the contents of two of the appropriate cultures were placed in a large plastic bag and pulverized by squeezing the bag several times. The cultures were then emptied and loosely placed in the bottom of the aerosol chamber.

The cultures were aerosolized by delivering a stream of air through a capillary pipette at 3 lb/in^2 onto the surface of the hay from a pressure pump fitted with a filter for 30 min.

The animal exposure experiments were conducted on six groups (A to F) of rabbits, four to each group. Two groups each were exposed to aerosols of either *M. faeni* (A and B), *T. vulgaris* (C and D), or sterile hay (E and F). All four animals from each group were exposed to the same aerosol simultaneously, but the groups were exposed to separately prepared aerosols at widely disparate times.

Blood gas analyses. The detailed procedure and rationale of monitoring pulmonary function as evidenced by depressions in arterial oxygen tension (Pa_{O_2}) has been reported previously (19). All blood gas analyses were performed with a Corning model 161/pH blood gas system (Corning Scientific Instruments, Medfield, Mass.).

Complement assays. Assays of hemolytic complement activity expressed as 50% hemolytic complement (CH_{50}) units per ml were achieved by the method of Mayer (16). Rabbit plasma from each blood sample was diluted 1:10 in Veronal-buffered saline (2) before each determination was made.

RESULTS

Conditions for optimal aerosol production. Few conidia of *M. faeni* are produced on aerial hyphae, and these do not aerosolize well. Since preliminary experiments on creating aerosols of *T. vulgaris* from surfaces of agar media resulted in no more than 1.4×10^3 colonies per culture after 30 min of exposure, additional preliminary experiments were concerned with producing denser microbial aerosols by growing the microorganisms directly on the hay.

The first aspect to be studied was the manner in which hay must be packed to obtain optimal growth. Old hay in amounts varying from 7 to 30 g was packed into glass storage dishes, sterilized, and inoculated with various amounts of *M. faeni* broth culture. After 2 weeks of incubation, the number of colonies produced per gram of hay from the aerosol for each culture was determined. Counts ranged from 1×10^2 colonies per g for 7 g of hay to 2.5×10^3 colonies per g for cultures made with 30 g of tightly packed hay, the maximum amount that could be packed in a storage dish used for culture.

A comparison between "old" and "fresh" hay (see above) was then made. Thirty-gram portions of each type were packed, sterilized, and inoculated with *M. faeni*. After incubation, colony counts from aerosols of each culture were compared. Fresh hay produced counts of about 10^6 colonies per g or about 1,000-fold more than similar quantities of old hay.

To determine the optimum moisture content for production of aerosols of maximum density, eight storage dishes were packed with 30 g of dry, fresh hay, and water was added in amounts varying from 2.5 to 30 ml. Each dish was autoclaved and inoculated with 10 ml of *M. faeni* broth culture. After 1 month of incubation, each culture was weighed, and the difference in weight before and after incubation was determined. This weight loss was attributed to the water content loss of each culture. Colony counts (Fig. 1) from aerosols of these cultures showed that the best aerosols were generated from hay cultures containing 45% water. Counts dropped off precipitously on either side of this value. When the experiment was repeated for *T. vulgaris*, the same optimum moisture content was obtained.

Aerosol challenge studies. Only one animal exhibited a single precipitin line to *T. vulgaris* before aerosolization, and it was assigned to the sterile hay group. Pre- and postchallenge blood gas values from each rabbit of the six test groups were plotted individually and statistically analyzed. These same data were also analyzed collectively by first obtaining the means from each group. From the individual data it was learned that there were statistically significant depressions in some of the rabbits (five of eight for each *M. faeni* and *T. vulgaris* and three of eight for sterile hay) if only 1-, 4-, and 6-h postchallenge values were considered. Two aerosols of each agent were not uniform as seen by differences in the means of each group responding to a given agent.

The mean values for all rabbits exposed to

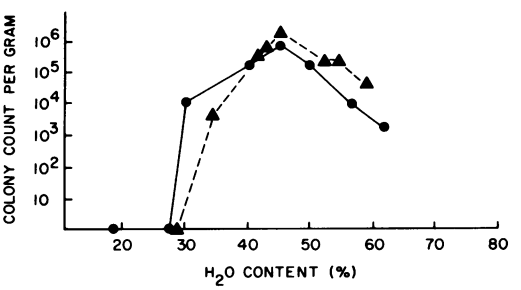

FIG. 1. *Effect of various water concentrations of hay on colony counts of recoverable thermophilic actinomycetes. Symbols: ▲, M. faeni; ●, T. vulgaris.*

each agent are presented in Table 1. Although by inspection it appears that decreases in Pa_{O_2} do occur in each group, a statistical test to compare each group at the 95% level of confidence could not be found. The responses of animals exposed to sterile hay aerosols are markedly uniform until the 24-h reading, at which time depressions were noted. Three animals exposed to sterile hay exhibited severe responses at this time, while two others gave responses at 1 and 4 h, respectively. Two types of patterns were seen in response to the other two agents. The predominant response (Pa_{O_2} depression) to *M. faeni* occured at 4 h postchallenge, whereas that to *T. vulgaris* was most pronounced at 24 h postchallenge.

Data obtained from hemolytic complement assays of these same arterial blood samples were similarly analyzed, except that the data (Table 2) were expressed as the mean percent decrease in CH_{50}. Although no statistical test is available for such data, percent decreases greater than 10% are considered significant (19). As compared with controls, sera from rabbits exposed to the test agent had markedly decreased CH_{50} levels. The *M. faeni* group again exhibited the greatest response at 4 h postchallenge, whereas that for the *T. vulgaris* animals was greatest at 6 h postchallenge.

DISCUSSION

Since cultures of thermophilic actinomycetes on solid artificial media did not aerosolize well enough for inhalation studies, the approach taken in this investigation was to simulate the same kind of aerosols spontaneously produced in nature by growing the microorganisms directly on hay.

Among the factors found to influence the density of aerosols produced from such cultures were: (i) tightly packed hay produced denser spore loads than did loosely packed hay. Hay, presumably, is tightly packed during the baling process after which the growth of these organisms may occur. Although tight packing may serve in nature to insulate the hay so that a spontaneous heat accumulation can occur, the significance of tight packing in this investigation is uncertain since the entire hay sample was incubated at 56°C.

(ii) Fresh hay supported better growth of these organisms than did hay that had been cut and stored. The manner in which the old hay used in this investigation was treated before and after the baling process was unknown, but treatment or storage reduced the capacity of the hay to support the growth of thermophilic actinomycetes. It is known that weather factors prevalent at the time of cutting and curing, the condition of the grass at the time of harvest, the length of storage, and the type of storage conditions greatly influence the content of nitrogenous compounds and vitamins in the stored hay (21). Since growth of thermophilic actinomycetes has been shown to be associated with high nitrogen content (10), some investigators have attempted to improve the nitrogen content of hay for experimental purposes by allowing it to absorb NH_3 fumes (20).

(iii) The amount of moisture in the hay substrate was important and was found to be 45% for maximum aerosol production. This confirms the studies of Festenstein et al. (7), who studied the self-heating of hay and the development of farmer's lung antigens. With a water content below 29%, there was little antigen production. Hays with a water content of 29 to 34% varied widely in their ability to support antigen pro-

TABLE 1. *Mean Pa_{O_2} values after aerosol challenge*

Aerosol	Time postchallenge (h)				
	0	1	4	6	24
M. faeni	88.7 ± 2.7[a]	87.9 ± 4.9	84.3 ± 5.7	85.9 ± 3.9	85.8 ± 3.9
T. vulgaris	92.0 ± 4.2	89.9 ± 5.8	86.1 ± 5.5	87.4 ± 6.3	81.2 ± 8.1
Sterile hay (control)	90.0 ± 2.2	88.0 ± 4.3	88.7 ± 3.6	89.4 ± 3.0	85.1 ± 7.1

[a] Results expressed as the mean of 8 animals ± standard deviation.

TABLE 2. *Mean percent decrease in CH_{50} after aerosol challenge*

Aerosol	Time postchallenge (h)			
	1	4	6	24
M. faeni	9.5 ± 12.3[a]	21.4 ± 14.9	15.8 ± 11.1	14.5 ± 14.2
T. vulgaris	13.2 ± 12.2	15.5 ± 13.5	23.0 ± 14.6	17.4 ± 5.5
Sterile hay (control)	1.6 ± 2.6	5.5 ± 7.6	8.0 ± 7.7	6.6 ± 6.6

[a] Results expressed as the mean percent decrease of 8 animals ± standard deviation.

duction, with the wetter hays usually producing more antigen. Maximum antigen production occurred at a water content of about 47%.

Under optimal conditions, aerosols resulting in a deposition of 10^6 viable fragments per g from 60 g of hay were produced. Gregory and Lacey (11) have calculated that as many as 5×10^7 spores per g of moldy hay may be released when the hay is exposed to the wind. The actual spore load produced by the aerosols could have reached a total of about 6×10^7 (60 g of hay used per aerosol; 10^6 spores per g) within 30 min, but in a volume much smaller than a cubic meter. Lacey and Lacey (14) have calculated that as many as 1.6×10^9 spores per m^3 may be present in highly contaminated, enclosed areas where the disease is most likely initiated. Nevertheless, it is possible that the quality of the moldy hay produced by this procedure was less than that seen in nature. Growth and sporulation of thermophilic actinomycetes in hay in nature is the result of a microbial succession during the molding process (10). This succession was bypassed in this investigation since the hay was first sterilized and inoculated directly with a pure culture.

Growth and sporulation of these organisms in nature is also an aerobic process (12). The cultures prepared for aerosol were incubated in tightly closed and sealed containers (to prevent excessive drying during incubation); hence, it was possible that proper aerobic conditions for growth were not met.

Our laboratories have developed a model of the initiation steps of hypersensitivity pneumonitis (3, 4, 19) wherein unimmunized rabbits are exposed to aerosols of *Aspergillus* spores and their subsequent depressions in arterial oxygen tension and hemolytic complement activity are measured. We have postulated that nonspecific complement activation results in enough pulmonary inflammation to allow antigens to enter the peripheral circulation and immunize the individual. It remained to be determined whether the commonly believed incitants of hypersensitivity pneumonitis, i.e., *M. faeni* and *T. vulgaris*, caused similar responses when administered as aerosol challenges. After such challenge with hay cultures of these organisms, two types of reaction patterns were observed, depending on the organisms used. Rabbits exposed to sterile hay aerosols exhibited both types of reaction patterns, but to a lesser degree. The large depressions at 24 h are of interest when the reaction patterns of these animals are compared with the reaction patterns of unimmunized animals exposed to aerosols of *A. fumigatus* or *A. terreus* (19), since such depressions at 24 h postchallenge were rarely noted in that study.

A question arises as to the immune status of the animals used in this investigation. Precipitins were found in one control animal. It was inadvisable to skin test these animals, as this could have resulted in active sensitization. These animals were undoubtedly exposed to hay at some previous time (bedding, shipping, litter, etc.), and it is possible that they were sensitized to one or more of its components.

Since these animals responded with decreased Pa_{O_2} in the absence of known immunization, the role of complement activation in the absence of specific precipitating antibody was investigated. Decreases in the peripheral hemolytic complement (CH_{50}) levels of sera from animals exposed to *M. faeni* were greatest at 4 h postchallenge, whereas that from the animals exposed to *T. vulgaris* was greatest at 6 h postchallenge. These data extend the in vitro observations of several investigators (5, 6, 15) that *M. faeni* and *T. vulgaris* can activate complement nonspecifically. It was of interest that a few of the rabbits exposed to aerosols of uninoculated hay also had low CH_{50} responses.

An area that needs to be resolved is the exact nature of the agent responsible for the hypersensitivity reaction in the lungs. Fletcher and Rondle (8) failed to extract serologically active material from the spores of *M. faeni*, but Hollingdale (13) was able to extract lipopolysaccharide from the mycelia. This suggests that spore antigens or lipopolysaccharide may not be responsible for the inflammatory reaction in the lungs. If this is the case, mycelia may be a more important component of the aerosol. The lipopolysaccharide in these mycelia may be responsible for complement activation. Lipopolysaccharide has not been isolated from *T. vulgaris* (M. R. Hollingdale, personal communication), but peptidoglycan in the spores or mycelia of this organism could be responsible for complement activation.

It appears from these observations that sterile hay also possesses the ability to activate complement. Edwards (5) compared alternate pathway complement activation by *M. faeni*, *T. vulgaris*, and moldy hay dust. He concluded that these organisms on a weight-for-weight basis do not contribute greatly to the dust sample. His reasoning was that if *M. faeni* and *T. vulgaris* are as active in complement activation in moldy hay as they are in pure culture, they would have to constitute a maximum of 5 and 22% of the dust, respectively, with the rest of the material being inert, and this seemed unlikely. Zaidi et al. (22) investigated the activity of hay dust and *M. faeni* in the pathogenesis of farmer's lung disease in guinea pigs. With a mixture of nonmoldy hay dust and *M. faeni*, the pulmonary

damage was more severe in the early stages than with the dust alone. However, the damage at 30 days differed little from the response to hay dust alone at 7 days. These investigators proposed that *M. faeni* is important only in the early stages of the disease and that hay dust itself is the most important pathogenic factor.

It appears that hay dust may contain material of microbial or plant origin that possesses the ability to activate complement. Lipopolysaccharide from gram-negative epiphytic organisms would be able to nonspecifically activate complement by both the alternate and classical pathways (17). Sterilization of the hay as was done in this investigation would not necessarily eliminate these effects, since lipopolysaccharide is heat stable. Other microorganisms might activate complement by means of heat-stable peptidoglycans (1). Complex polysaccharides of plant origin might also be able to activate complement by the alternate pathway. Edwards (5) has proposed that soluble material may elute from the hay dust and may nonspecifically bind immunoglobulin leading to complement activation.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant OH 00360 from the National Institute of Occupational Safety and Health.

We express our appreciation for the invaluable help of Martin Peterson of the Appalachian Laboratory for Occupational Safety and Health, Morgantown, W.Va., who performed the statistical analysis.

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