

Experimental production of hypersensitivity pneumonitis with bagasse and thermophilic actinomycete antigen

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Albino New Zealand rabbits and Sprague-Dawley rats were repeatedly exposed to either: (1) ultrasonically aerosolized actinomycete-laden bagasse extract or (2) Micropolyspora faeni antigen via the intratracheal, intravenous, or intramuscular route. Response was evaluated in terms of pulmonary morphologic changes, development of serum precipitins, skin reactivity, and peripheral lymphocyte ^3H -TdR incorporation. Some "normal" unaerosolized rats housed in cages with bagasse flooring demonstrated antibagasse precipitins by Ouchterlony gel diffusion and/or varying degrees of peribronchial and perivascular lymphoid hyperplasia. Peripheral lymphocytes from aerosolized rats failed to incorporate significant ^3H -TdR in the presence of bagasse extract but bagasse was shown to inhibit phytohemagglutinin-induced lymphocyte transformation. Rabbits immunized repeatedly intratracheally with actinomycete antigen in saline solution developed serum precipitins, delayed skin reactivity, actinomycete induced peripheral lymphocyte ^3H -TdR incorporation plus intense alveolar and interstitial pneumonitis characterized by prominent alveolar macrophage proliferation. Rabbits immunized via the intramuscular route in Freund's adjuvant demonstrated identical immunologic findings but no extensive pulmonary lesions. Our results indicate that repeated respiratory tract exposure to thermophilic actinomycete antigen can induce pulmonary mononuclear cell infiltrates, serum precipitins, and antigen-induced peripheral lymphocyte DNA synthesis. The histopathologic lesions and delayed skin reactions to M. faeni in our intratracheally immunized rabbits suggest that cell-mediated (type IV) hypersensitivity is present in this experimental animal model.

Bagassosis is a hypersensitivity pneumonitis resulting from prolonged inhalation of dried sugar cane fiber dust (bagasse) by certain industrial workers.¹ Affected patients, and to a lesser extent, unaffected sugar cane industry workers demonstrate circulating precipitating antibody against extracts of crude moldy bagasse and certain thermophilic actinomycetes isolated from bagasse.² However, the pathogenetic mechanisms involved in production of bagassosis and related hypersensitivity pneumonitides are not fully understood, the immunologic response of experimental animals to inhaled antigens having largely been limited to the anaphylactic response.

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Experimental animal models should aid in further understanding of the pathogenetic mechanisms involved in hypersensitivity pneumonitis. In this study we attempt to gain information on the immune response of rodents to ultrasonically aerosolized and intratracheally inoculated bagasse and thermophilic actinomyceete antigen.

MATERIALS AND METHODS

Experimental animals

Rats. Random-bred female Sprague-Dawley rats, 200 to 300 grams, housed in cages with fresh bagasse straw flooring* but in an area separate from preparation and insufflation of concentrated bagasse antigen, were used for aerosol immunization and as appropriate saline-nebulized and nonnebulized controls. Control Charles River CDF strain germ-free rats were housed in the LSU germ-free center in standard Trexler-type flexible film isolaters without bagasse flooring, fed autoclaved Purina diet 5010-C, and cultured biweekly to ensure sterility.

Rabbits. Twenty-five white New Zealand rabbits were immunized intramuscularly, intravenously, or intratracheally with purified, ground, lyophilized *M. faeni* in saline solution.

Antigen

Bagasse-insufflated rats. Crude bagasse, previously baled and stored in an open field at high environmental temperature and humidity for several months, was used. Lipid was removed by several washes with ether, paper filtration, and drying. Defatted bagasse was extracted in deionized distilled water 1:10 w/v with periodic adjustment of pH to 7.3 by addition of 0.1N NaOH over a 24 hour period. The supernatant was passed through double-thickness filter paper, Seitz filtered, lyophilized, and reconstituted daily in 10 per cent solution for ultrasonic nebulization. Bagasse antigen for lymphocyte transformation studies was prepared as above followed by dialysis against distilled water, sterile Millipore filtration, and dilution to 3 to 300 μ g per milliliter in medium 199.

M. Faeni-inoculated rabbits. The thermophilic actinomyceete *Micropolyspora faeni* was grown in our laboratory for 5 days in nutrient broth. Growth were centrifuged, washed 3 times with distilled water, disrupted with glass beads (0.25 to 0.30 mm.) in a model MSK Braun Cell Homogenizer at 4,000 cycles per minute for 4 minutes, and lyophilized for immunization. *M. faeni* antigen for skin test, precipitin, and in vitro lymphocyte transformation studies consisted of 0.018M NaCl extract prepared as previously described.³

Immunization and operational procedure

Bagasse-insufflated rats. Rats to be nebulized were placed in a large metal box† containing an inlet, exhaust outlet, and appropriate tubing and exhaust connections as previously described.⁴ An ultrasonic nebulizer producing a particle size of approximately 1 μ (Ultramist III, Macrosonics Corporation, Rahway, N. J.) was used for insufflation of bagasse extract into the box at a rate of 15 to 20 ml. per hour daily. Control germ-free rats were insufflated with saline without removal from their aseptic plastic tent environments.

Twenty rats were exposed to bagasse extract by daily one-hour insufflation. One animal was put to death and bled every second day excluding weekends during the initial 2 week period, and 2 were put to death weekly thereafter over a total period of 9 weeks. Thirty rats were divided into the following groups and killed and bled at regular intervals for 8 weeks: 12 unaerosolized rats housed in a room separate from the unconcentrated antigen preparation and insufflation area; 6 rats housed in a separate area and ultrasonically nebulized daily for one hour with 15 to 20 ml. of sterile physiologic saline solution; 6 germ-free rats maintained in a control aseptic environment and ultrasonically nebulized for one

*Material routinely used for animal cage flooring in this area.

†Graciously provided by Dr. Jordan Fink, Medical College of Wisconsin.

hour daily for 3 weeks with 15 to 20 ml. of sterile physiologic saline; 10 nonnebulized germ-free rats maintained in a controlled germ-free environment. All rats except germ-free animals were housed in cages with commercial bagasse straw bedding. Saline-aerosolized and unaerosolized germ-free animals were used for assessment of the development of serum precipitins in animals with no environmental organic dust inhalant antigen exposure.

At the times the animals were put to death, the following procedures were performed:

1. Sera were frozen at -20° C. and analyzed for precipitins by Ouchterlony double diffusion against lyophilized, reconstituted crude bagasse antigen. Antigen concentrations, agar, and template size were as previously described.^{1,3} Gamma globulins were precipitated from aliquots of rat serum according to the method of Kendall.⁵ Precipitates were dialyzed against distilled water, lyophilized, and redissolved in Veronal buffer in $\frac{1}{40}$ the original serum volume. Double-diffusion studies were performed with the use of concentrated and unconcentrated samples.

2. Skin tests were attempted with crude bagasse extract with 1.0, 10.0, and 100 μ g concentrations in 0.05 ml. volume, but toxic reactions were noted in "normal" rats at all concentrations and this material was deemed unsuitable for these purposes.

3. Lymphocytes were harvested from peripheral nodes and tritiated thymidine (^{3} H-TdR) incorporation in the presence of bagasse antigen determined as previously described.⁶ All cultures were maintained in triplicate and incubated in a 5 per cent CO_2 atmosphere at 37° C. for 4 days. Triplicate phytohemagglutinin (PHA) controls (0.1 ml. of $\frac{1}{60}$ solution Difco PHA) and unstimulated controls were performed in all cases. Only results with good control PHA transformation were recorded.

4. At the time the animals were put to death, randomly selected portions of each lung of control and experimental animals were removed and fixed in 10 per cent formalin for microscopic examination. The tissue was embedded in paraffin and sections were stained with hematoxylin.

Actinomycete immunized rabbits. Thirteen were inoculated intramuscularly or intravenously with crude homogenized, lyophilized *M. faeni* antigen in total doses ranging from 10 to 300 mg. Seven animals received 10 to 100 mg. intravenously in saline solution (10 mg. in a single injection or 20 mg. every 4 days over a total 12 to 16 day interval); 6 received 10 to 60 mg. intramuscularly in Freund's adjuvant in a single injection or in two 30.0 mg. inoculations at 4 day intervals. Twelve were immunized by local intratracheal injection of *M. faeni* with the use of a 21 gauge needle and sterile technique under light pentobarbital anesthesia. In a single dose 1 to 10 mg. was administered. The larger doses (80 to 300 mg.) were given in 20 to 50 mg. doses every 4 days over a 12 to 20 day interval.

Fourteen days after immunization animals were skin tested with 0.1 ml. of dialyzed *M. faeni* cellular extract prepared according to the method of Biguet and co-workers.⁷ After recording 15 minute, 3 hour, and 24 hour skin reactivity, selected animals in each group were bled for serum precipitin determination and killed at 48 to 72 hours; this was followed by peripheral lymph node cell harvesting for antigen-induced lymphocyte ^{3} H-TdR incorporation studies. Dialyzed 0.018M NaCl extracted, lyophilized *M. faeni* antigen was also used for lymphocyte ^{3} H-TdR incorporation studies. Lyophilized antigen was reconstituted directly in tissue culture medium in 10, 100, and 500 μ g per milliliter concentrations. For precipitin analysis this lyophilized antigen was reconstituted in 0.9 per cent saline solution at a 20.0 mg. per milliliter concentration.^{1,3}

RESULTS

Morphological studies

Rats. Ultrasonic nebulization of crude bagasse antigen in rats was associated with the pathologic changes demonstrated in Fig. 1. This photomicrograph illustrates the entire spectrum of pathology noted. Perivascular lymphocytic infiltration of small pulmonary vessels was the earliest change noted (Fig. 2). Following this, increased numbers of lymphocytes were found in the pulmonary interstitial areas with eventual thickening of alveolar septae (Fig. 2). After

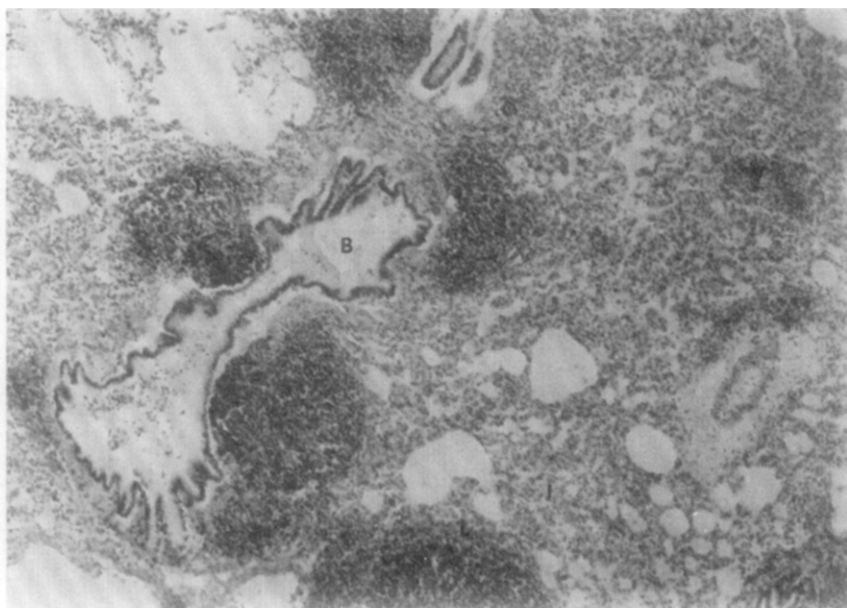


FIG. 1. Rat lung exposed to bagasse aerosol for 4 weeks. The bronchiole (**B**) contains a cellular exudate and is surrounded by hyperplastic lymphoid tissue (**L**). Pulmonary parenchyma is atelectatic with increased cellularity of the interstitium (**I**). Perivascular accumulations of lymphocytes are indicated by (**V**). (Original magnification X40.)

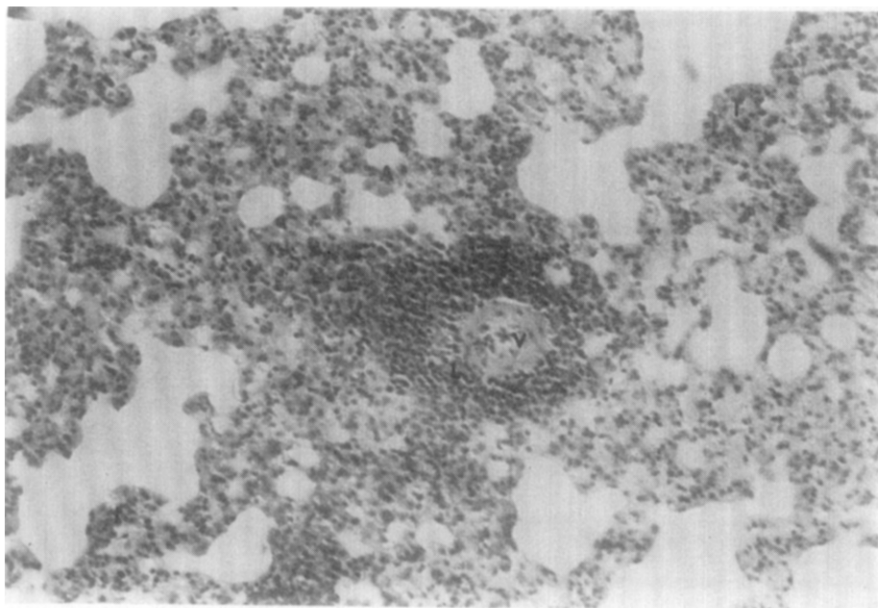


FIG. 2. Rat lung exposed to bagasse aerosol for one week. A small pulmonary vessel (**V**) is surrounded by lymphocytes (**L**). Alveolar walls (**A**) are edematous and there is early infiltration of the interstitium (**I**) by lymphocytes. (Original magnification X100.)

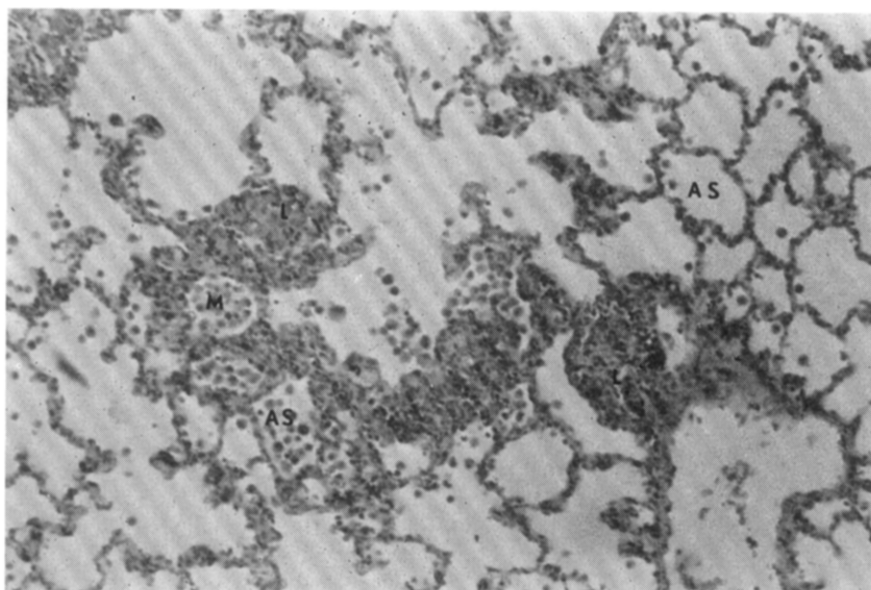


FIG. 3. Rabbit lung inoculated intratracheally with 10.0 mg. of *Micropolyspora faeni* antigen 14 days prior to death. Note increase in macrophages (M) within alveolar spaces (AS) and lymphocytic infiltration (L) of interstitial and perivascular areas. (Original magnification X100.)

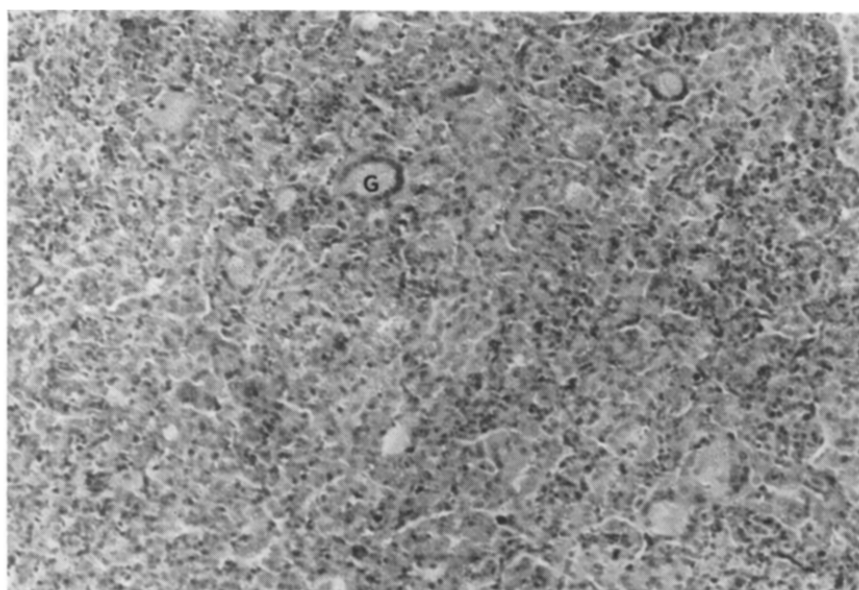


FIG. 4. Rabbit lung repeatedly inoculated intratracheally with 80.0 mg. (20.0 mg. every fourth day) of *Micropolyspora faeni* antigen 17 days prior to death. Extensive parenchymal changes are illustrated with loss of normal lung architecture. Lymphocytes are less prominent and there are numerous mononuclear and giant cells (G). (Original magnification X100.)

TABLE I. Antibagasse double-diffusion precipitin reactions in bagasse-aerosolized, control unaerosolized, and control germ-free rats

Antigen	No.* of positive precipitin reactions†			
	Bagasse-aerosolized	Nonaerosolized control	Control germ-free	
			Saline-aerosolized	Nonaerosolized
Crude bagasse‡	16/18	4/12	0/6	0/10

*Positive reactions/number of samples tested.

†10× Concentrated rat gamma globulins.

‡Same batch of bagasse used for immunization (20 mg. per milliliter antigen concentration).

prolonged exposure the number of small lymphocytes decreased with an associated increase in larger mononuclear cells in the perivascular and interstitial areas. When nebulization was continued for 4 or more weeks, bronchial inflammatory changes and peribronchial lymphoid hyperplasia were sometimes present as seen in Fig. 1. Control germ-free animals nebulized in an identical fashion with saline had none of these pathologic changes. Control saline-aerosolized and unaerosolized rats housed in cages with bagasse flooring had minor changes of perivascular accumulation of lymphocytes, but the intense lymphoid hyperplasia and interstitial pneumonitis demonstrated in Fig. 1 was not present and there was no difficulty in distinguishing pulmonary lesions in bagasse-aerosolized and control rats when slides were examined in blind fashion.

Rabbits. More extensive and somewhat different pathologic changes were noted in rabbits given intratracheal injections of 10 to 60 mg. of purified bagasse antigen. Fig. 3 shows the early changes of increased numbers of alveolar macrophages and perivascular accumulation of lymphocytes and monocytes. In Fig. 4 there is loss of normal alveolar architecture with sheets of large mononuclear cells and marked multinucleated giant cell formation. Intratracheal injection of saline produced no changes in the lungs of control rabbits. Rabbits immunized intramuscularly with *M. faeni* in adjuvant demonstrated only scattered areas of mononuclear cell pulmonary infiltrates as is commonly noted after parenteral injection of Freund's adjuvant. Animals given *M. faeni* intravenously in saline solution also revealed only minimal increases in alveolar macrophages without lymphoid hyperplasia or interstitial changes.

Precipitins, skin tests, and lymphocyte transformation

Rats. Occasional unconcentrated sera of control and bagasse-aerosolized rats housed in cages with bagasse flooring demonstrated clear precipitin arcs against bagasse or thermophilic actinomyces antigen by gel double diffusion. However, in most cases, these arcs diminished markedly or were no longer visible after washing and staining. Using 10× unconcentrated ammonium sulfate precipitated rat gamma globulin, well-defined stainable precipitin arcs were demonstrated in virtually all sera of bagasse-aerosolized rats and in many unaerosolized control rats but in none of the CDF germ-free rats (Table I).

TABLE II. ^3H -TdR uptake (counts per minute) of bagasse-aerosolized and control aerosolized or nonaerosolized rat peripheral lymph node cells following stimulation with bagasse extract

Animal No.	Duration of aerosolized bagasse exposure (days)	Bagasse antigen concentration* ($\mu\text{g}/\text{ml.}$)	^3H -TdR uptake by cells/counts/min. (mean) †	x Greater
Aerosolized				
1	7	50	9,015	2.03
2	10	50	2,017	0.37
3	12	25	9,980	1.91
4	14	50	23,038	2.69
5	14	75	10,294	0.96
6	16	75	5,186	1.02
7	17	25	20,880	3.31
8	19	25	13,630	1.33
		100	11,710	2.85
9	21	25	14,933	2.10
10	42	25	2,496	1.62
11	42	25	3,334	0.65
Controls				
1	6 (saline)	25	1,354	1.23
2	21 (saline)	50	4,719	1.79
3	14 (saline)	25	3,414	0.91
4	21 (saline)	25	3,793	0.79
5	19 (saline)	25	2,482	0.89
6	24 (saline)	25	1,944	0.94
7	No exposure	50	1,598	0.72
8	No exposure	50	2,683	0.93

*Several concentrations between 3 and 300 μg per milliliter were employed in each animal without dose-response effect. Only results with 25 to 100 μg concentrations are included.

† Mean of triplicate determinations.

Results of in vitro bagasse-induced lymphocyte ^3H -TdR incorporation are listed in Table II. Only three of 11 bagasse-aerosolized rats exhibited lymphocyte ^3H -TdR incorporation rates greater than 2.5 times that of the unstimulated controls. The actual degree of ^3H -TdR incorporation could have been greater, since unstimulated control counts were generally higher in this group than in saline-aerosolized animals. Two of 3 control animals immunized with bagasse in Freund's adjuvant exhibited ^3H -TdR incorporation rates greater than 2 times those of unstimulated controls. Although these rates were clearly higher than those observed in our saline-aerosolized controls, they were of questionable significance as an indication of antigen-induced lymphocyte transformation and suggested that bagasse might be unsuitable material for lymphocyte transformation studies. Studies to determine a possible inhibitory effect of bagasse extract on PHA- and antigen-induced lymphocyte transformation were then initiated in the guinea pig. This species was used for this purpose because of comparatively high PHA-induced lymphocyte ^3H -TdR incorporation rates when compared with the rat⁸ and possibly with the rabbit.⁹ Results are listed in Table III. In all animals studied a clear-cut depression of PHA-induced lymphocyte ^3H -TdR incorporation was noted when bagasse extract was added to PHA in tissue culture media. A small volume of bagasse (0.1 ml.) was added and bagasse did not stimulate unsensitized normal rat lymphocytes (Table II). Thus, this depressive effect cannot be accounted for on a dilution basis or by reagent competition.

TABLE III. ^3H -TdR incorporation by nonsensitized guinea pig lymphocytes after stimulation with PHA or PHA plus crude bagasse extract

Animal No.	Antigen (bagasse and PHA concentration)	Mean*	x Greater	% Decrease with bagasse
1	Unstimulated control	10,990	—	—
	PHA 0.1 ml.	201,549	18.33	—
	PHA 0.1 ml. + bagasse 300 $\mu\text{g}/\text{ml}$.	92,500	8.43	54.2
2	Unstimulated control	1,124	—	—
	PHA 0.1 ml.	73,116	65.21	—
	PHA 0.1 ml. + bagasse 300 $\mu\text{g}/\text{ml}$.	54,447	48.43	25.6
3	Unstimulated culture	4,018	—	—
	PHA 0.1 ml.	231,982	57.74	—
	PHA + bagasse 300 $\mu\text{g}/\text{ml}$.	118,081	23.38	59.6
4	Unstimulated culture	4,651	—	—
	PHA 0.1 ml.	69,348	14.91	—
	PHA + bagasse 200 $\mu\text{g}/\text{ml}$.	28,703	6.17	58.6
5	Unstimulated control	1,464	—	—
	PHA 0.1 ml.	105,662	72.13	—
	PHA 0.1 ml. + bagasse 100 $\mu\text{g}/\text{ml}$.	58,945	40.24	44.3
6	Unstimulated culture	2,074	—	—
	PHA 0.1 ml.	25,961	12.51	—
	PHA + bagasse 75 $\mu\text{g}/\text{ml}$.	9,722	4.68	73.27
7	Unstimulated culture	1,628	—	—
	PHA 0.1 ml.	26,115	15.80	—
	PHA + bagasse 25 $\mu\text{g}/\text{ml}$.	15,956	9.80	38.00

*Mean of 5 determinations.

TABLE IV. Precipitin reactions and skin reactivity in 25 rabbits immunized by various routes with pure culture thermophilic actinomycete antigen, plus 7 controls

Antigen* dose (mg.)	Route of immunization†	No. of positive serum precipitin reactions‡	Skin test§ 500 $\mu\text{g}/\text{ml}$. (50 μg)		
			15 min.	3 hr.	24 hr.
10	I.M.	3/3	0	11	13
60	I.M.	3/3	0	10	11
10	I.V.	0/3	0	0	0
30	I.V.	0/2	0	0	0
100	I.V.	2/2	0	0	0
1	I.T.	0/2	0	5	0
10	I.T.	0/6	0	5	7
80	I.T.	2/2	0	8	5
300	I.T.	2/2	0	9	11
Unimmunized controls	—	0/7	0	0	0

*Washed, ground, and lyophilized *Micropolyspora faeni*, CBS A-94.

†I.M. = intramuscular; I.V. = intravenous; I.T. = intratracheal.

‡No. of positive reactions/No. of animals in group.

§Mean diameter of erythema and edema (15 minutes and 3 hours) or erythema and induration (24 hours) in all animals of each group.

TABLE V. ^3H -TdR uptake (counts per minute) by peripheral lymph node cells of rabbits immunized with ground lyophilized thermophilic actinomycete antigen, following stimulation with *M. faeni* extract

A. Rabbits immunized					
Animal No.	Immunization route*	Dosage (mg.)	^3H -TdR uptake (mean)† by cells (counts/min.)		x Greater
			Antigen-stimulated‡	Control	
153	I.V.	10	247	584	0.42
151	I.V.	10	3,140	2,573	1.22
150	I.V.	10	7,587	2,962	2.06
155	I.V.	30	1,225	355	3.45
156	I.V.	30	765	973	0.97
252	I.V.	100	16,466	962	17.11
158	I.V.	100	3,116	499	6.24
240	I.T.	10	2,398	2,406	0.99
241	I.T.	10	21,455	3,539	6.06
243	I.T.	80	3,305	519	6.36
245	I.T.	300	3,948	324	12.18
246	I.T.	300	3,044	633	4.80
271	I.M.	10	4,935	574	8.58
272	I.M.	10	4,940	729	6.77
274	I.M.	60	1,020	116	8.79
275	I.M.	60	4,379	142	29.83
278	I.M.	60	1,210	199	6.08
B. Nonimmunized controls					
Animal No.	Unstimulated control	PHA	x Greater	Antigen-stimulated‡	x Greater
166	3,614	41,297	11.43	8,374	2.31
167	3,136	40,607	12.94	4,166	1.32
168	3,614	41,297	11.43	8,374	2.31
262	3,263	39,987	12.25	4,021	1.23

*I.V. = intravenous; I.T. = intratracheal; I.M. = intramuscular.

†Mean of triplicate determinations.

‡100 μg per milliliter concentrations.

Rabbits. Results of intradermal tests, serum precipitin, and lymphocyte transformation studies in 25 rabbits immunized by various routes with purified thermophilic actinomycete antigen are listed in Tables IV and V. Antigen dosage plus route and method of immunization were important in the development of serum precipitins and skin reactivity. Animals immunized intramuscularly with actinomycete antigen incorporated in Freund's adjuvant uniformly developed serum precipitins plus 3 hour and 24 hour delayed skin reactivity. The intratracheal and intravenous routes of immunization resulted in development of serum precipitins only at higher immunizing doses. Skin reactivity generally did not develop in these groups except at the highest 300 mg. intratracheal dose level. Biopsy of selected 24 hour skin test sites revealed intense accumulations of mononuclear cells, some in perivascular distribution resembling classic delayed hypersensitivity reactions.

Of the 17 animals used for lymphocyte transformation studies, those immunized intramuscularly with Freund's adjuvant demonstrated good peripheral lymph node cell ^3H -TdR incorporation upon in vitro exposure to actinomycete antigen. Lymphocyte ^3H -TdR incorporation in animals immunized intravenously

was poor except at the high dose level, but the intratracheal route of immunization resulted in good ^3H -TdR incorporation in all but one animal at the three dose levels employed. All of the animals exhibiting significant actinomycete-induced peripheral lymph node ^3H -TdR incorporation revealed serum precipitins, suggesting that sufficient antigen gained access to immunologically competent peripheral lymphoid cells in these cases.

DISCUSSION

This study was undertaken to compare two aspects of the immune response of rodents to bagasse and thermophilic actinomycete antigen: (1) the development of pulmonary lesions and serum-precipitating antibody in the response to ultrasonically aerosolized bagasse in the rat; (2) the humoral and cell-mediated immune response to thermophilic actinomycete antigen administered by various routes in the rabbit.

Our results indicate that prolonged daily insufflation of rats with ultrasonically aerosolized bagasse extract leads to development of an intense interstitial pneumonitis characterized by prominent lymphocytic infiltration. This pneumonitis largely represented a quantitative difference from the focal lymphocytic infiltrates present in normal rat lung¹⁰ and was not identical to the predominantly alveolar macrophage-plasma cell infiltrates noted in human hypersensitivity pneumonitis. The interstitial pneumonitis produced in our rabbits immunized intratracheally with actinomycete antigen was also characterized by lymphocytic infiltration and edema, but alveolar macrophage proliferation, as noted in human hypersensitivity pneumonitis, was a prominent feature of these infiltrates.

These findings are consistent with earlier studies in which similar pulmonary infiltrates were produced in the rabbit and guinea pig after intratracheal injection or insufflation of ground bagasse powder.^{11, 12} Recent studies have also demonstrated pulmonary lesions similar to those noted in human hypersensitivity pneumonitis after ultrasonic nebulization of rats with crude pigeon excreta antigen⁴ and of rabbits with purified ovalbumin.¹³

Our bagasse-aerosolized rats also demonstrated serum precipitins against bagasse extract by Ouchterlony double diffusion. However, with the use of concentrated ammonium sulfate-precipitated serum gamma globulin, some "normal" unaerosolized rats housed in cages with bagasse flooring demonstrated antibagasse precipitins, likely reflecting prolonged environmental exposure to small amounts of antigen.

Bagasse-aerosolized rats demonstrated no peripheral lymph node cell ^3H -TdR incorporation in the presence of bagasse extract. These extracts are generally not suitable for skin testing purposes and in the current study were shown to interfere with lymphocyte function as determined by inhibition of PHA-induced lymphocyte ^3H -TdR incorporation. Two possible explanations of this inhibitory phenomenon are that aflatoxins, carcinogenic mycotoxins which are known to inhibit concanavalin A-induced lymphocyte ^3H -TdR incorporation,¹⁴ might have been present in our "moldy" bagasse samples or that crude bagasse bound PHA and prevented lymphocyte stimulation.

However, intratracheal immunization of rabbits with a lyophilized pure culture thermophilic actinomycete (*M. faeni*) known to be an important source of antigen in "moldy" composts resulted in good antigen-induced in vitro peripheral lymph node cell ^3H -TdR incorporation at immunizing dose levels of 10 to 300 mg. Some of these animals also developed antiactinomycete serum precipitins and delayed skin reactions, but only after repeated intratracheal immunization with lyophilized actinomycete antigen (80 to 300 mg. total dose). These results demonstrate that sufficiently intense local respiratory tract immunization can initiate a systemic immune response as noted in human hypersensitivity pneumonitis.

Waldman and Henney^{15, 16} have demonstrated in the guinea pig that respiratory tract immunization with microgram quantities of dinitrophenolated human IgG gives rise to local but not systemic cell-mediated immunity. Their findings strongly suggest that in the respiratory tract there are locally "sensitized" lymphocytes which remain in this area for some time. Our current studies were not concerned with the local cell-mediated immune response to respiratory tract immunization, and gross differences in antigen composition and dosage preclude comparison between our results and those of Waldman and Henney. However, it is likely that the repeated administration of comparatively large intratracheal immunizing doses of *M. faeni* was sufficient for antigen "processing" and transport to peripheral immunologically competent cells by a population of mobile macrophages and/or for stimulation of locally "sensitized" lymphocytes as well as small numbers of systemic lymphocytes which migrated into the respiratory tract.

Thus our findings, while compatible with Gowans^{17, 18} concept of a highly mobile recirculating population of small lymphocytes, are not necessarily incompatible with Waldman and Henney's concept of local cellular hypersensitivity.

Recent immunofluorescence microscopy studies indicate that lungs from human patients with hypersensitivity pneumonitis contain actinomycete antigen in bronchial walls and antibody-forming cells plus mononuclear cells staining for $\beta_1\text{C}$ globulin.¹⁹ These findings suggest that at least part of the lung damage resulting from inhalation of composts rich in actinomycetes is mediated by a mechanism involving immune complexes and complement. The role of "local" pulmonary delayed hypersensitivity in the pathogenesis of pulmonary lesions following inhalation of actinomycete-laden composts remains to be determined.

Results of immunization with actinomycete antigen via other routes were also of interest. All rabbits immunized intramuscularly with Freund's adjuvant demonstrated good antigen-induced peripheral lymph node cell ^3H -TdR incorporation, circulating precipitins, and delayed hypersensitivity skin reactions, as has been noted for protein antigens in general with the use of this immunization route.

The intravenous route, however, resulted in no delayed skin reactivity with any of the immunization doses used. This route of immunization has previously been shown to produce neither antigen-induced peripheral lymph node cell ^3H -TdR incorporation nor peritoneal macrophage migration inhibition in the guinea

fig.^{20, 21} Nor has it lead to successful induction of systemic delayed hypersensitivity or circulating antibody against bovine serum albumin after primary immunization in the rabbit.²² However, the anamnestic response to an intravenous "booster" injection of bovine serum albumin in the rabbit is known to induce considerable circulating antibody in the absence of delayed hypersensitivity skin reactions.²² Repeated intravenous injections of sheep erythrocytes have also resulted in good in vitro peripheral blood lymphocyte transformation.²³ Our rabbits immunized intravenously with actinomyces antigen followed this immune response pattern in demonstrating no detectable immune response after a single 10 mg. dose but good precipitin formation and antigen-induced peripheral lymph node cell ³H-TdR incorporation after repeated high-dose immunization.

There is conflicting evidence concerning the correlation between in vitro lymphocyte transformation and either in vivo delayed hypersensitivity or antibody formation.^{20, 24-27} Our results suggest that with the use of actinomyces antigen, rabbits can demonstrate antigen-induced peripheral lymph node ³H-TdR incorporation in the absence of detectable delayed skin reactivity. In the rabbit, intravenous immunization with BCG in Bayol F adjuvant has induced pulmonary granulomas and alveolar macrophage migration inhibition by purified protein derivative in the absence of delayed skin reactivity.²⁸ Alveolar macrophage migration inhibition studies after immunization with actinomyces antigen by various routes should aid in determining the presence of "local" in vitro delayed hypersensitivity responses in the absence of dermal skin reactivity, and such studies are in progress.

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