Mitogenic response to *Micropolyspora faeni* cell walls

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Three cell wall fractions, prepared from Micropolyspora faeni, *were shown to be mitogenic for the splenic lymphocytes of unsensitized guinea pigs. This response peaked after 48 hr in culture and was dose dependent. Using enriched lymphocyte subpopulations obtained by column-separation techniques, it was shown that the most active cell wall fractions were primarily B cell mitogens. Physical, chemical, and enzymatic digestion studies indicated that the heat-stable mitogenic reactivity was associated with polypeptide and polysaccharide. The reactive material is, at least in part, peptidoglycan, as shown by the decrease in its mitogenic potential after lysozyme digestion. A possible host-related role for this mitogenic reactivity was discussed.*

One form of hypersensitivity pneumonitis, farmers' lung disease, is associated with exposure to moldy hay contaminated with *Micropolyspora faeni*.[1] Much effort has been directed toward the study of patient and experimental animal response in this disease in an effort to understand its mechanism of pathogenesis[2]; however, the mechanism is not fully understood. Another approach, one taken in our laboratory, is to investigate the biologic activity of components of the presumed etiologic agent. Many substances have been shown to stimulate lymphocytes to undergo blastogenesis. The list of such agents includes phytohemagglutinin, concanavalin A, gram-negative lipolysaccharide (LPS), staphylococcal protein A,[3] *Nocardia opaca* water-soluble mitogen,[4, 5] and cell walls of *Actinomyces viscosus*[6] and *Listeria monocytogenes*.[7] Further, various mitogens, including *Nocardia* water-soluble mitogen,[8] cell walls of *L. monocytogenes*[7] and *A. viscosus*,[6] and LPS have been found to preferentially stimulate B lymphocytes. Our laboratory recently reported the ability of *M. faeni* cell wall fractions to induce lymphocyte proliferation[9]; these findings prompted additional studies to (1) characterize the mitogen(s) involved in this stimulation and (2) determine which population of lymphocytes from unsensitized guinea pigs was stimulated by *M. faeni* cell wall fractions.

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MATERIALS AND METHODS

M. faeni culture

Cells were grown in M-Standard Methods broth (BBL, Cockeysville, Md.) in 10-L batches on a laboratory scale fermentor (Magnaferm Fermentor, New Brunswick, N. J.). After incubation under aeration for 48 hr at 55° C, the cells were harvested by centrifugation, washed with distilled water, and lyophilized.

Cell wall fractions

Cell wall fractions I, II, and III were prepared as described previously.[9] Briefly, *M. faeni* was disrupted by an MSK Braun cell homogenizer and cell wall fragments were recovered by centrifugation. The pellet was washed sequentially with 0.2 M NaCl, 1 M NaCl, and distilled water and termed fraction I. This pellet was further treated with trypsin and ribonuclease (RNase) to yield fraction II, which was then extracted with chloroform and methanol to yield fraction III. All fractions consisted of insoluble residues.

Mitogens

Cell wall fractions I, II, and III and treated products were suspended in pyrogen-free saline by sonication for 3 min using a 1-amp Ystrom Systems sonicator (Technic International, Bergenfield, N. J.). Working dilutions of these fractions were made daily in either phosphate-buffered saline (PBS, pH 7.4) or RPMI-1640 (GIBCO, Grand Island, N. Y.) supplemented with 9.53 gm of HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer/L,

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0.62 gm of $NaHCO_3$/L, penicillin (100 U/ml), and streptomycin (100 μg/ml). *Escherichia coli* 055B5 LPS and phytohemagglutinin (Difco Labs.) were used as control mitogens.

Lymphocyte preparation

Splenic lymphocytes were obtained from female Hartley guinea pigs (Camm Research Institute, Wayne, N. J.). The spleens were teased in RPMI-1640 until a single-cell suspension resulted. The lymphocytes were recovered from Ficoll-Hypaque gradients, washed with RPMI-1640, counted, and then stained for viability with trypan blue. The percentage of lymphocytes, defined by the absence of neutral red dye phagocytosis,[10] averaged 95%. Cells were resuspended to a count of 5×10^6/ml in RPMI-1640 with 10% heat-inactivated guinea pig serum.

A pool of lymphocytes from three guinea pigs was separated into B and T lymphocyte–enriched fractions using nylon wool columns.[11, 12] Briefly, columns were prepared using nylon wool from a LP-1 Leuko-Pak filter (Fenwal Labs., Morton Grove, Ill.). Before use, the columns were equilibrated for 1 hr at 37° C with RPMI-1640 containing 5% heat-inactivated guinea pig serum. Lymphocytes were resuspended to a count of 1 to 1.5×10^8/2 ml and were added to the column, which was left undisturbed for 45 min at 37° C. After incubation, 25 ml of warm medium were added to the column and effluent cells were collected. Cells obtained in this manner have been shown to contain 2% to 5% B cells and 85% to 95% T cells.[11] After removal of nonadherent cells, 50 ml of warm medium were flushed through the column and discarded. Four 5-ml quantities of medium were added to the column and the adherent cells were then recovered by compressing the nylon column with the plunger. Cells in this population consisted of about 86% B cells and 10% T cells.[13] Cells from both populations were recovered by centrifugation at 100 × g for 10 min and were resuspended to a count of 5×10^6/ml. T lymphocytes were identified by the ability of the thymus-derived lymphocytes to form spontaneous rosettes with rabbit erythrocytes.[14]

Lymphocyte transformation assay

Aliquots of 0.1 ml of lymphocytes were added to sterile microtiter plates. To these cultures was added 0.1 ml of RPMI-1640 without serum or RPMI-1640 containing the mitogen. The cultures were incubated at 37° C in 5% CO_2 for 48 hr unless stated otherwise. During the last 10 hr in culture, cells were pulsed with 0.5 μCi of ^{3}H-thymidine (specific activity, 20 Ci/mmole; New England Nuclear, Boston) and then harvested and washed on fiberglass strips using a Brandel multiwell harvester. The paper disks were dried and counted in a liquid scintillation counter. Each test was performed in triplicate and the results expressed as mean counts per minute or as a stimulation index. The stimulation index is the ratio of mean counts per minute of the mitogen-supplemented culture to the mean counts per minute of the untreated control. Each experiment was performed using lymphocytes from a single animal, except for the lymphocyte separation data, which required pooling of lymphocytes from three animals. The control (no mitogens) would monitor any changes due to lymphocyte reactivity, especially in samples from separation procedure.

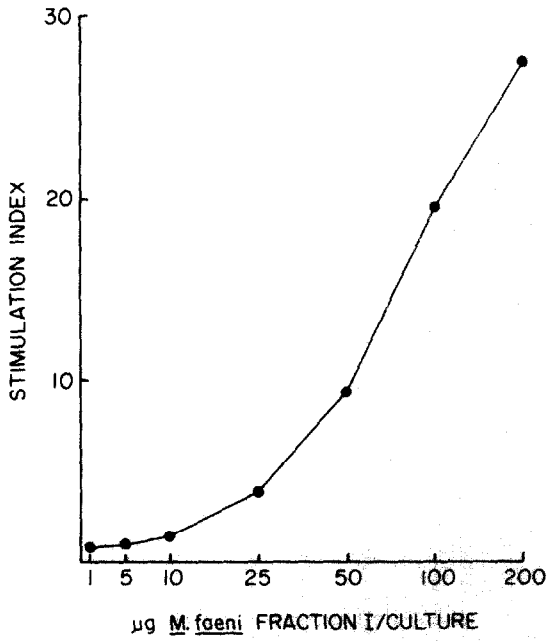

FIG. 1. Dose-response curve for thymidine uptake by guinea pig lymphocytes stimulated with *M. faeni* fraction I. Assays were performed in triplicate using lymphocytes from a single representative guinea pig at a concentration of 5×10^5/culture. Data are expressed as stimulation indices, i.e., the ratio of mean counts per minute of the mitogen-supplement culture to the mean counts per minute of the untreated control culture. SD < 10% for the three replicates.

Physical, enzymatic, and chemical modification of cell wall fractions

Heat stability of the mitogenic fractions was determined by heating the fraction suspended in PBS (pH 7.4) at 80° C for 30 min. The contribution of cell wall carbohydrates to the mitogenic activity was evaluated by treating samples with or without 0.1 N sodium metaperiodate[15] (Sigma Chemical Co., St. Louis, Mo.) in 0.2 M ammonium acetate buffer (pH 4.5) for 24 hr at 4° C. The periodate was removed by dialysis against distilled water for 2 days and then lyophilized.[6] The susceptibility of the mitogen to a nonspecific protease was determined[16] by suspending 10 mg of fraction I in 1 ml of PBS (pH 7.4) with 5 mg of protease (Pronase, grade B; Calbiochem, Los Angeles) in 1 ml of PBS. The mixtures were rotated for 8 hr at 37° C. *E. coli* LPS and PBS were treated similarly and used as controls. All tests including untreated samples were boiled for 60 min to allow for inactivation of the Pronase in the treated samples and to control for any changes due to heating. The effect of alkaline hydrolysis on the mitogenic activity of cell wall fractions was determined[17] by treating fraction I with 1 N NaOH at room temperature for 4 hr. The product was

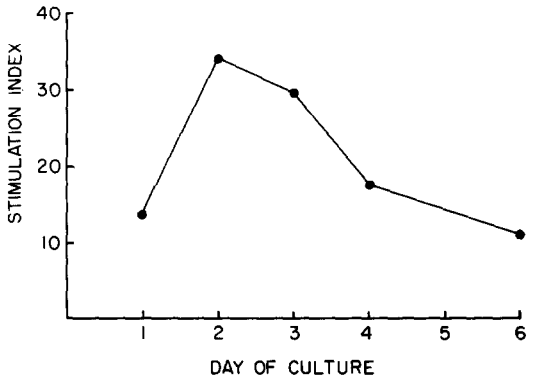

FIG. 2. Dose-response curve for thymidine uptake by guinea pig lymphocytes stimulated with *M. faeni* fraction I. Assays were performed in triplicate using lymphocytes from a single representative guinea pig at a concentration of 5 × 10^5/culture. Data are expressed as stimulation indices, i.e., the ratio of mean counts per minute of the mitogen-supplement culture to the mean counts per minute of the untreated-control culture. SD < 10% within each mean for all points until day 5, at which time it increased appreciably due to the decrease in viability of the cells.

neutralized with 1 N HCl, dialyzed for 2 days against distilled water, and then lyophilized. To determine if the mitogenic activity was due to peptidoglycan, the fractions were treated with crystalline egg white lysozyme (50,000 U/mg; Sigma). Cell wall fractions were suspended in 0.1 M PBS (pH 7.4) to a concentration of 2 mg dry weight/ml and mixed with an equal volume of lysozyme (200 μg dry weight/ml). The mixture was rotated at 37° C for 6 hr[18, 19] and the enzyme-treated preparations were used in lymphocyte studies.

RESULTS

Mitogenic stimulation of guinea pig splenic lymphocytes by a fraction of *M. faeni*

The incorporation of thymidine by splenic lymphocytes after culture with varying concentrations of *M. faeni* mitogen (fraction I), shown in Fig. 1, is the result of a representative experiment. No increase in the incorporation was observed at concentrations of 1 and 5 μg; however, the response increased with concentrations of 10 μg up to and including 200 μg per culture in 24 animals under observation (see reference 9 for further individual data).

A strongly reactive concentration of *M. faeni* mitogen, 100 μg, was employed to determine the time at which maximum thymidine incorporation occurred. A representative experiment is shown in Fig. 2. Higher concentrations of this material were too particulate to make even suspensions. With this concentration, maximum incorporation of labeled thymidine occurred by day 2 and then declined during the remaining 4 days in culture in 10 animals examined.

TABLE I. Mitogenic effect of *M. faeni* on various fractions of guinea pig splenic lymphocytes

Cell source*	Stimulation indices†				
	Viability at time 0 (%)	PHA (1 μg)	LPS (25 μg)	Fraction I (100 μg)	% E rosettes
Spleen unfractionated	97	54.4	5.6	16.0	14
T cell–enriched	98	27.6	2.5	3.3	34
B cell–enriched	90	11.7	28.0	35.7	5

*A pool of splenic lymphocytes from three unsensitized guinea pigs was used for the unfractioned spleen cell group and also for column-separation procedures. Cultures contained 5 × 10^5 lymphocytes and were incubated for 48 hr.

†Stimulation index = ratio of mean counts per minute of mitogen-supplemented culture to the mean counts per minute of untreated control culture. All cultures were done in triplicate. SD <10% for all triplicate values in each experiment.

Mitogenic stimulation of B cell–enriched lymphocytes

To determine the population of lymphocytes stimulated by the *M. faeni* mitogen, guinea pig splenic lymphocytes were separated into B and T cell–enriched populations using nylon wool columns. The number of erythrocyte rosettes was used to quantitate the T cells in the unfractionated spleen, the adherent population (B cell–enriched), and the nonadherent population (T cell–enriched). The results, recorded in Table I, show that the unfractionated spleen contained 14% rosette-forming cells, comparable to the data of Stadecker et al.[14] The designated T cell–enriched population contained a 2.5-fold increase in rosette-forming cells while the number of rosette-forming cells in the B cell–enriched population was decreased markedly as compared with the unfractionated spleen.

A pool of unfractionated splenic lymphocytes as well as aliquots of T and B cells separated from this pool were cultured with PHA, LPS, and *M. faeni* fraction I as well as without mitogen (control) for 48 hr. PHA, a T cell mitogen in guinea pigs,[12] stimulated the incorporation of thymidine in all cell groups (Table I); however, there was minimal stimulation of the B cell–enriched cultures, probably due to residual T cells in the population. In contrast, only the unfractionated spleen and the B cell–enriched populations were stimulated by LPS, a B cell mitogen. LPS resulted in a fivefold increase in the stimulation index of B cells as compared with that of the unfractionated spleen, suggesting increased numbers of B cells in this population. Fraction I from *M. faeni* stimulated both the unfractionated spleen and the B cell popula-

TABLE II. Lysozyme degradation of the mitogenic activity of cell wall fractions of *M. faeni*

Mitogen	Treatment: None cpm*	None S.I.†	Lysozyme cpm*	Lysozyme S.I.†	% change in S.I.‡
Fraction I (100 μg)	18,096	10.1	13,532	10.3	+2.0
Fraction II (100 μg)	13,041	7.3	5,550	4.4	−42.5
Fraction III (100 μg)	12,053	6.7	4,666	3.6	−46.3
PHA (1 μg)	37,117	20.7	25,370	19.4	−6.3
LPS (50 μg)	5,760	3.2	3,648	2.8	−12.5
Control	1,793	1.0	1,311	1.0	—

*Mean (cpm) of triplicate cultures having a standard deviation equal to or less than 10% of the mean counts per minute. All cultures were performed using lymphocytes from a single guinea pig at a concentration of 5×10^5/culture.

†S.I. (stimulation index) = ratio of mean counts per minute of mitogen-supplemented culture to mean counts per minute of untreated control culture. SD < 10% for all triplicate values in each experiment.

‡% change $= \frac{\text{S.I. (treated-untreated)}}{\text{S.I. untreated}} \times 100$

tion, but only minimally stimulated T cells. The lower stimulation index in the T cell population is probably due to residual B cells or to a very low level of T cell stimulation. These data indicate that the mitogen from *M. faeni* is most likely a B cell mitogen.

Effect of physical, chemical, and enzymatic treatments on the mitogenic activity

To partially characterize the mitogen(s), *M. faeni* fraction I was subjected to heat, periodate, protease, alkali, and lysozyme treatments. Fraction I, although crude, was selected for such studies because previous studies[9] indicated that purification resulted in greatly diminished activity. The mitogenic activity was stable to heat at 80° C for 30 min (Fig. 3, *A*). However, periodate oxidation abolished the mitogenic activity (Fig. 3, *B*). A parallel sample of mitogen treated with only buffer produced a strong mitogenic effect, thereby ruling out any loss of activity due to the buffer or to dialysis. These results suggest that carbohydrates are essential for mitogenic activity of *M. faeni* preparations. As shown in Fig. 3, *C*, treatment of *M. faeni* with Pronase markedly decreased the mitogenic activity. The effect of residual Pronase on the lymphocytes was monitored by including a Pronase cell control although the enzyme was inactivated by heating prior to use. The loss of activity by Pronase treatment suggests that proteins or peptides are important structures for the mitogenic activity of *M. faeni*. Sodium hydroxide abolished the mitogenic activity (Fig. 3, *D*). All of the studies in Fig. 3 were performed with cells from single animals.

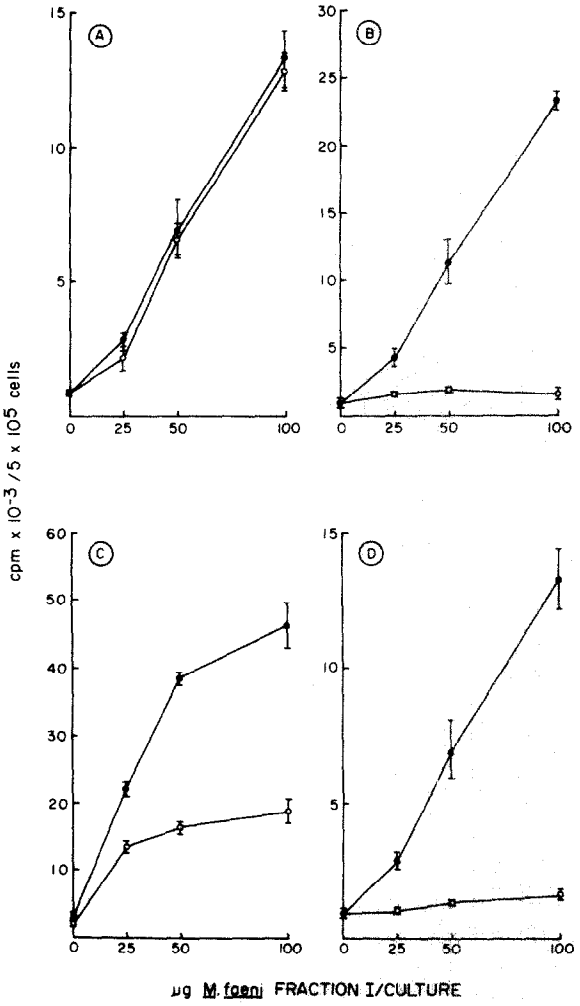

FIG. 3. The susceptibility of the mitogenic activity of *M. faeni* fraction I to *(A)* heat, *(B)* periodate, *(C)* protease, and *(D)* NaOH treatment. •---•, Untreated fraction I; o---o, treated sample. Each panel represents data obtained using the lymphocytes from a single representative guinea pig at a concentration of 5×10^5/culture. Data are expressed as the mean counts per minute of triplicate cultures ± SD.

Lysozyme, which specifically breaks the β-1,4 linkage of peptidoglycan, was also used to treat various fractions of *M. faeni*. Because lysozyme was not removed from the cell fractions, a lysozyme cell control was included to monitor any effect of the enzyme on the lymphocytes. The data, shown in Table II, are reported as both counts per minute and stimulation indices for untreated and lysozyme-treated samples. Lysozyme had no effect upon the mitogenic activity

of fraction I (crude cell walls); however, the mitogenic activity was decreased markedly by 42% and 46% in fractions II and III, respectively. This loss of activity suggests that peptidoglycan is an important portion of the mitogenic component of *M. faeni*. The small loss of activity in the lysozyme-treated LPS could be the result of peptidogylcan contamination in the phenol-water preparation of LPS.[20] PHA, as expected, was not affected by lysozyme treatment.

DISCUSSION

Previous work from our laboratory reported that cell wall fractions of *M. faeni* were mitogenic for splenic lymphocytes of unsensitized guinea pigs and rabbits and for peripheral blood lymphocytes of normal humans.[9] The data reported here further characterize the responsible component(s) and the response to these fractions.

Nonspecific mitogenicity is becoming recognized as an important characteristic of a number of microorganisms, many of which have adjuvant activity; *M. faeni* may be added to this list. Our findings are in agreement, in a number of ways, with similar studies performed on these other microorganisms.

That the mitogenicity was due to nonspecific activity rather than antigen-induced mitogenicity may be seen from the following. It is generally accepted in cellular immunology that nonspecific mitogens induce peak reactivity within 48 hr of culture, whereas antigen-induced mitogenicity does not reach maximum activity until after 4 days. Crude cell walls of *M. faeni* induced an early (2 day) stimulation of splenic lymphocytes of unsensitized guinea pigs. Our findings are also in agreement with those observed in unsensitized or even germfree animals with mitogens isolated from *A. viscosus* and *N. opaca*.[6, 21] Further, using B and T cell–enriched populations of lymphocytes obtained by nylon wool column separation techniques, crude cell walls of *M. faeni* (fraction I) were shown to be primarily a B cell mitogen. Although it was not the purpose of this paper to prepare isolated lymphocyte preparations in extreme purity, all indications using previously characterized,[11, 13] enriched populations point to the conclusion that the material is almost certainly a B cell mitogen. Mitogens from other gram-positive organisms (e.g., *L. monocytogenes, N. opaca, A. viscosus*) show similar patterns of lymphocyte stimulation.[4, 6, 7] Several of these organisms have been reported to possess adjuvant activity in addition to mitogenic activity.[4, 6, 7]

Hollingdale,[22] using a phenol-water extraction technique, reported isolation of an LPS-like molecule from *M. faeni*. However, peptidoglycan, as well as lipoteichoic acids and other cell wall–associated components, is known to contaminate preparations made by this techique.[20, 23] The possibility that the mitogenic potential of *M. faeni* is due to LPS appears unlikely for four reasons. First, this organism is gram-positive rather than gram-negative. Second, the stimulation indices reported in this study were higher with *M. faeni* cell wall fractions than with any concentration of *E. coli* LPS used. Third, the mitogenic activity of *M. faeni* was sensitive to lysozyme treatment, suggesting that the peptidoglycan polymer was responsible, at least in part, for the mitogenic activity and would rule out a major role for LPS in this activity. Fourth, a phenol-water extract of *M. faeni* prepared in similar manner to that of Hollingdale had lower *Limulus* reactivity as well as pyrogen activity than did a similar amount of *Salmonella typhimurium* LPS.[9]

Several approaches were used to characterize the mitogen(s) associated with *M. faeni*. Since fraction I was the most active in lymphocyte stimulation studies, it was often the only fraction utilized in some of the additional studies. The heat-stable mitogenic activity was abolished by metaperiodate oxidation, indicating the importance of a polysaccharide moiety in the mitogenic activity. The importance of proteins or peptides in the mitogenic component was indicated by the decrease in mitogenic potential after prolonged Pronase treatment. The necessity of proteins for activity was also in part supported by the fact that fraction II, which was prepared by treatment of fraction I with trypsin and RNAse, was less active than fraction I. These results indicate that both polypeptides and polysaccharides are important for the mitogenic activity associated with this organism. This would be in agreement with other workers who report the mitogenic activity of peptidoglycan.[24] The mitogenic activity was abolished by mild sodium hydroxide treatment which could alter carbohydrate, protein, ribonucleic acid (RNA), and lipid components. However, lipids appeared to have only a small role in the mitogenic activity since no major decrease in reactivity was observed during sequential purification of fraction II to fraction III unless they were already removed during earlier treatments.[9]

Since the peptidoglycan is a polymer of *N*-acetylglucosamine and *N*-acetylmuramic acid and sensitive to lysozyme degradation, cell wall fractions of *M. faeni* were subjected to lysozyme treatment and the mitogenic activity remaining after treatment was compared with untreated samples. Lysozyme markedly decreased the mitogenic potential of purified fractions II and III; however, the mitogenic activity of

crude cell walls (fraction I) was not affected by lysozyme treatment. This same observation has been made with cell walls of *A. viscosus*[6] and suggests that proteins, nucleic acids, and lipids attached to the cell walls may prevent lysozyme from gaining access to the peptidoglycan. Since the pH of the lysozyme solutions was not optimal for enzyme activity, the observed decreases in mitogenic activity may not be as great as could be obtained under optimal conditions.

The functional or host-related role of the *M. faeni* B cell mitogen in hypersensitivity pneumonitis is unknown. Fragmented mycelium and spores, both of which contain peptidoglycan, are in the respirable range (1 to 5 μ) and do reach the distal lung. It has been shown[25] that concentrations of *M. faeni* on the order of $1.6 \times 10^9/m^3$ can be found in a closed environment, therefore presenting the host with a significant number of organisms with mitogenic activity. Since the mitogen is resistant to lysozyme degradation, one could postulate that it might persist and concentrate in tissue, in a manner similar to that observed with streptococcal group A cell walls,[18] and cause release of hydrolytic enzymes from macrophages.[26] Along with continued nonspecific complement activation[9] and consequent production of biologically active substances, tissue damage ensues. Persistence of the B mitogen and resultant nonspecific lymphoid proliferation could contribute to the adjuvanticity of *M. faeni*, as reported by Bice et al.,[27] and thereby enhance the production of immunologic responses thought to be responsible for the later, chronic stages of hypersensitivity pneumonitis.[2] Pathologic evidence of hypersensitivity pneumonitis can not be produced in experimental animals to exogenous protein immunization unless Freund's adjuvant is used.[2] We are suggesting that the adjuvant in spontaneous, *M. faeni*–induced disease is in the organism itself. Current thinking suggests that cell-mediated immunity is important in the production of the pathology of hypersensitivity pneumonitis. Although T cells are usually associated with such tissue damage, it is becoming increasingly clear that B cells can also produce lymphokines, and such may be the case in the events described here.

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