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Macrophage responses to mouldy hay dust, *Micropolyspora faeni* and zymosan, activators of complement by the alternative pathway

H. U. SCHORLEMMER,* J. H. EDWARDS,† P. DAVIES‡ & A. C. ALLISON*
**Division of Cell Pathology, MRC Clinical Research Centre, Watford Road, Harrow, Middlesex*
†MRC Pneumoconiosis Unit, Llandough Hospital, Penarth and ‡ Merck Sharp and Dohme, Research Laboratories, Rahway, New Jersey, U.S.A.

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SUMMARY

Mouse peritoneal macrophages in culture exposed to mouldy hay dust, *Micropolyspora faeni* or glycopeptide or protein/glycoprotein fractions from this organism show marked biochemical changes. For comparison the interaction of cultured macrophages with zymosan has been investigated. All these agents induce the release of hydrolytic enzymes from macrophages, even in the absence of serum in the medium. The release is time- and dose-dependent and is not associated with loss of the cytoplasmic enzyme lactate dehydrogenase or any other sign of cell death. The parallelism between the capacity of these agents to activate the complement system via the alternative pathway and to induce inflammatory responses *in vivo* and selective lysosomal enzyme secretion from cultures of macrophages is discussed. The *in vitro* phenomena seen with mouldy hay dust, *M. faeni*, the protein/glycoprotein and the glycopeptide derived from it, may be relevant to understanding the role of mononuclear phagocytes in the disease farmer's lung and other inflammatory reactions.

INTRODUCTION

Inhalation of mouldy hay dust (MHD) produces the disease farmer's lung, an acute pulmonary inflammatory reaction that may develop into a chronic phase with permanent respiratory impairment. The main active agent in MHD is the thermophilic actinomycete *Micropolyspora faeni* (Wenzel *et al.*, 1965). For some time complexes of *M. faeni* antigens and antibodies have been thought to be responsible for the pathogenesis of farmer's lung (Wilkie, Pauli & Gygax, 1973). More recently, evidence has accumulated that farmer's lung may be seen in individuals lacking precipitating antibodies to *M. faeni* antigens (Edwards, Baker & Davies, 1974). Mouldy hay dust and *M. faeni* in particular are efficient activators of the complement system via the alternative pathway (Edwards *et al.*, 1974, Edwards, 1976). When *M. faeni*, MHD or zymosan, a well-known activator of complement by the alternative pathway, were administered to rabbits by i.t. injection, marked inflammatory reactions were observed in the lungs comparable to those in farmer's lung (Edwards, 1974; Edwards, Wagner & Seal, 1976). These reactions to *M. faeni* were as severe following first exposure as in animals previously immunized with the organism. These results suggest that activation of complement system via the alternative pathway may play an important role in the pathogenesis of farmer's lung.

Since *M. faeni* constituents are ingested by alveolar macrophages soon after their inhalation, and these cells are involved in all stages of the disease, effects of mouldy hay dust, *M. faeni*, and fractions derived

Correspondence: Dr H. U. Schorlemmer, Division of Cell Pathology, MRC Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ.

from it, on cultures of macrophages have been investigated. For comparison, the interactions of cultured macrophages with zymosan are presented.

MATERIALS AND METHODS

Experimental animals. Swiss mice (T.O. strain) were obtained from SAC1, Brentwood, Essex.

Tissue culture materials. Tissue culture grade Petri dishes were obtained from Nunc Joblin Laboratories Division, Stone, Staffordshire, U.K. and M119 from Burroughs Wellcome, Beckenham, Kent, U.K., swine serum from Bio Cult Laboratories Ltd, Glasgow, Scotland.

Biochemical reagents. Bovine serum albumin, penicillin, streptomycin, phenolphthalein glucuronic acid 0·01 M pH 7·0 and zymosan prepared from *S. cerevisiae* yeast were from Sigma Chemical Co., Surbiton, Surrey, p-nitrophenyl-β-D-galactopyranoside and p-nitrophenyl-2-acetamido-β-D-deoxyglucopyranoside from Koch-Light Laboratories, Colnbrook, Buckinghamshire, heparin, preservative-free, from Boots, Nottingham, pyruvate and nicotinamide adenine dinucleotide from Boehringer Mannheim GmbH Germany; Triton X-100 from British Drug Houses Ltd, Poole, Dorset.

Macrophage collection and culture. Mouse macrophages were obtained by peritoneal lavage of Swiss mice with 5 ml M199 containing 100 u/ml of penicillin and streptomycin and 10 iu/ml heparin. Five-millilitre aliquots of the peritoneal exudate cell suspension containing $0{\cdot}5-1{\cdot}0\times10^6$ cells/ml were distributed into 50-mm Petri dishes and incubated in a humidified atmosphere of 5% carbon dioxide and air at 37°C for 1–2 hr to allow attachment of adherent cells. Non-adherent cells were removed by washing four times with phosphate-buffered saline. After washing, the cells were cultured in M199 containing 10% (v/v) swine serum. The serum was heated at 56°C for 30 min before use. Cultures prepared in this way give a sheet of well-spread cells within 24 hr.

In all experiments quadruplicate cultures were used and biochemical results are expressed as the mean and standard deviation.

At the end of each incubation period the medium was removed and the adherent cells were washed once with phosphate-buffered saline. The cells were then released by adding saline containing 0·1% (v/v) Triton X-100 and 0·1% (w/v) bovine serum albumin and scraping with sterile silicone rubber bungs. The activities of various enzymes were assayed in both the media and cell-containing fractions.

Enzyme assays. All assays were conducted under conditions giving linear release of product in relation to the amount of sample used and the time of incubation. Lactate dehydrogenase was assayed by determining the rate of oxidation of reduced nicotinamide adenine dinucleotide at 340 nm.

β-glucuronidase was assayed by the method of Talalay, Fishman & Huggins (1946). β-galactosidase was assayed by the method of Conchie, Findley & Levvy (1959) using p-nitrophenyl-β-D-galactopyranoside as substrate. N-acetyl-β-D-glucosaminidase was assayed by the method of Woolen, Heyworth & Walker (1961) using p-nitrophenyl-2-acetamido-2-β-D-glucopyranoside as substrate dissolved in 0·1 M citrate-phosphate buffer pH 4·5.

Statistical tests. Means and standard deviation were calculated after samples were shown to be homogeneous by calculation of coefficients of variance. The significance of differences was established by the Student's *t*-test.

Mouldy hay dust. A sample of mouldy hay was obtained locally and a fraction of the dust collected by shaking the hay within a polythene bag connected to a Casella Hexlet gravimetric sampler drawing 50 l air/min. The dust was collected in an extraction thimble and kept at 4°C until used. The concentrations were made up in saline using gentle ultrasonication to facilitate suspension.

Propagation of M. faeni and preparation of fractions. M. faeni organisms and antigens were prepared by the double dialysis technique (Edwards, 1971). Organisms were removed from the culture using a fine, sterile wire mesh and lyophilized.

Protein/glycoprotein antigens were precipitated by adding an equal volume of ethyl alcohol, washed with 50% alcohol and redissolved in deionized water. Trichloracetic acid was added to the alcohol supernatant and further precipitate discarded. The resulting solution was dialysed against running water and concentrated by air dialysis.

Both protein/glycoprotein and glycopeptide solutions were freed from traces of the other by elution from a DEAE column equilibrated with 0·01 M Tris-HCl buffer pH 8·0 using a continuous gradient of 0–0·5 M NaCl. Eluting fractions were monitored by immunoelectrophoresis using farmer's lung serum and appropriate fractions pooled, dialysed and filtered through sterile 220 mμ filters before lyophilization. Prior to use they were redissolved in phosphate-buffered saline and filtered through sterile 220 mμ filters.

RESULTS

Effects of zymosan and mouldy hay dust on macrophages

Particles of zymosan and mouldy hay dust are rapidly taken up by phagocytosis, and can be seen inside cultured macrophages after 1 hr incubation at 37°C. The ingestion is followed by marked, selective release of lysosomal hydrolases into the culture medium. This effect depends on the nature and concentration of the particles used and the time of incubation (Fig. 1). After 24 hr incubation in medium

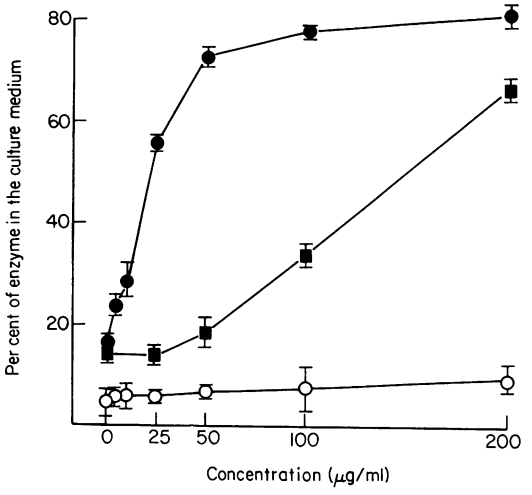

FIG. 1. Effects of 24 hr incubation with various concentrations of zymosan (●) and mouldy hay dust (■) on the release of N-acetyl-β-D-glucosaminidase and lactate dehydrogenase (○) from macrophages into the culture medium.

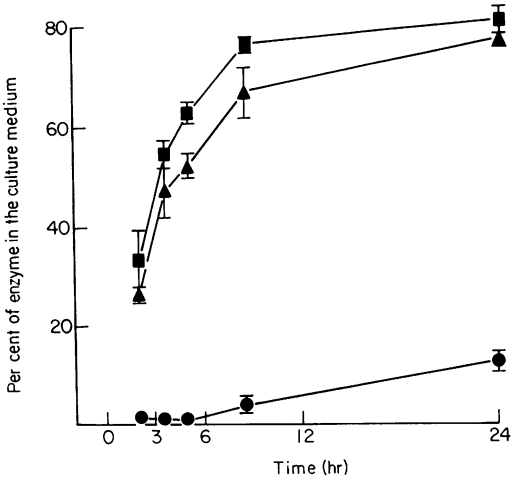

FIG. 2. The time-dependent release of N-acetyl-β-D-glucosaminidase from macrophages exposed to 50 μg/ml of zymosan (■) and 200 μg/ml of mouldy hay dust (▲) in a serum-free medium. Controls in the absence of added particles (●).

containing 25 μg/ml of zymosan the concentration of N-acetyl-β-D-glucosaminidase in the medium is significantly greater than in the controls ($P<0{\cdot}01$). This dose-dependent release of enzyme rises up to about 80% with 200 μg/ml of zymosan and to about 70% with 200 μg/ml of mouldy hay dust. The selective nature of the lysosomal enzyme release is shown by the absence of significant increase in the amount of lactate dehydrogenase, a marker of cytoplasmic enzyme activity in the culture medium. Zymosan and mouldy hay dust at concentrations up to 200 μg/ml cause no morphologically detectable cell death, even in the absence of serum in the medium.

Time course of hydrolase secretion

The time course of enzyme production and release was studied in mouse macrophage cultures maintained for various times up to 24 hr in the presence of a single dose of 50 μg/ml of zymosan and 200 μg/ml of mouldy hay dust. This time dependence of the selective release of lysosomal enzymes caused by the

two stimuli is shown in Fig. 2. Macrophage cultures exposed to zymosan or hay dust show a change compared to control cultures, in the distribution of a representative lysosomal enzyme, N-acetyl-β-D-glucosaminidase, between cells and culture medium. By 2 hr a highly significant increase ($P<0{\cdot}01$) in the amount of N-acetyl-β-D-glucosaminidase in the medium is seen. Subsequently a rapid rise in the release of enzyme into the culture medium occurs so that by 24 hr approximately 80% of the total enzyme activity is found in the culture medium. While data are presented only for N-acetyl-β-D-glucosaminidase, the same pattern of release was observed for other hydrolases measured including β-galactosidase and β-glucuronidase (Table 1). As in the experiments described in Fig. 2 and Table 1 this release occurs with no detectable loss of cellular lactate dehydrogenase into the culture medium and there is no decrease of cell viability at 24 hr even in the absence of serum in the culture medium.

TABLE 1. Enzyme levels and distribution between cells and culture medium in macrophage cultures exposed to zymosan and hay dust for various times

	β-glucuronidase (nmol product/plate/hr)					
	Control		Zymosan		Hay dust	
Time (hr)	Total	Per cent in medium	Total	Per cent in medium	Total	Per cent in medium
2	182±1·2	1·6±0·6	121±4·7	46·0±2·6	116±9·7	39·6±1·5
3½	187±8·7	1·7±0·7	107±4·3	65·4±0·9	116±5·7	64·5±0·4
5	186±1·4	3·9±0·6	114±3·8	74·5±0·9	100±8·1	68·7±5·1
8½	191±1·7	4·8±1·2	107±4·3	85·0±1·8	119±4·2	77·9±2·1
24	189±1·0	13·8±1·0	120±2·7	91·3±7·3	133±7·3	88·3±0·3
	β-galactosidase (nmol product/plate/hr)					
	Control		Zymosan		Hay dust	
Time (hr)	Total	Per cent in medium	Total	Per cent in medium	Total	Per cent in medium
2	93·7±6·8	2·8±1·0	47·7±1·2	36·6±0·8	64·6±1·2	23·7±1·6
3½	96·1±3·1	3·5±0·8	46·2±3·4	56·9±1·7	52·1±1·9	42·5±2·0
5	90·3±6·8	5·3±0·5	44·0±0·5	64·0±3·1	65·5±1·2	54·2±3·3
8½	89·9±2·6	11·7±4·9	46·8±1·3	68·9±3·1	58·6±1·6	64·6±5·5
24	92·8±3·7	12·7±2·2	45·3±2·2	79·2±6·8	69·6±0	78·5±4·5
	Lactate dehydrogenase (mu/plate)					
	Control		Zymosan		Hay dust	
Time (hr)	Total	Per cent in medium	Total	Per cent in medium	Total	Per cent in medium
2	487±14·1	3·2±1·3	513±7·8	4·3±2·2	481±4·8	3·8±0·7
3½	539±36·8	4·9±2·5	503±14·1	6·1±3·4	490±3·5	6·4±0·1
5	525±6·4	6·9±0·1	527±3·9	9·9±2·2	527±4·2	9·9±2·7
8½	534±6·4	8·7±2·6	540±7·8	11·7±2·6	563±3·2	10·9±4·5
24	491±10·3	13·4±5·8	546±14·8	13·5±2·3	546±0·7	12·6±1·3

Induction by M. faeni of macrophage hydrolase secretion

Marked changes in the levels and distribution of the activities of acid hydrolases were induced in mouse macrophages by *M. faeni*. Two types of experiments were performed. Firstly the effect of various concentrations of *M. faeni* on the levels and distribution of enzyme activity in macrophage cultures after

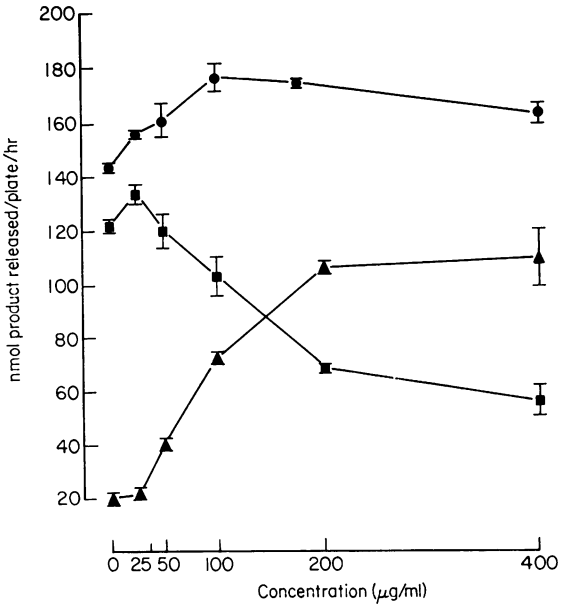

FIG. 3. The dose-dependent effect of *M. faeni* on the levels of β-glucuronidase activity in the total culture (●); in cells (■); and in the medium (▲). The cultures were assayed after 24 hr incubation with the organism.

TABLE 2. Lysosomal enzyme levels and distribution between cells and culture medium in mouse macrophage cultures exposed to various concentrations of *M. faeni* for 24 hr

Concentration of *M. faeni* (μg/ml)	N-acetyl-β-D-glucosaminidase nmol product/plate/hr		β-glucuronidase nmol product/plate/hr		β-galactosidase nmol product/plate/hr	
	Total	Per cent in medium	Total	Per cent in medium	Total	Per cent in medium
0	2746±39·8	8·6±0·6	143·0±1·3	14·2±1·5	95·4±0	8·5±0
25	2474±37·4	15·0±1·3	155·8±0·3	14·5±0·4	88·9±0	10·5±0
50	2626±65·5	15·7±0·7	160·6±5·7	25·3±1·2	81·4±1·9	11·9±3·5
100	2648±25·8	28·5±1·6	175·6±5·1	41·5±2·3	77·7±4·3	26·0±6·6
200	2500±53·8	46·4±0·8	174·6±0·3	60·8±0·5	96·1±3·4	41·5±5·8
400	2655±86·5	50·1±2·7	162·5±3·2	67·9±2·3	81·1±0·3	52·5±0·4

24 hr was measured. Secondly, the effect of a single concentration of *M. faeni* on the time course of changes in enzyme level and distribution was determined. In cultures grown in the presence of various concentrations of *M. faeni* for a period of 24 hr the total lysosomal enzyme activity was not changed (Fig. 3). However, there was a marked decrease in the intracellular hydrolase content, with a reciprocal and concurrent increase in the extracellular enzyme activity in the culture media. This redistribution of activity was observed for all of the lysosomal hydrolases measured; these included β-glucuronidase, β-galactosidase and N-acetyl-β-D-glucosaminidase, as shown in Table 2. The release of the acid hydrolases is also time dependent. Release is demonstrable 3 hr after contact with the organism and continues over the subsequent 21 hr. At no time during the 24-hr period is there a significant decrease in cellular lactate dehydrogenase levels in cultures exposed to *M. faeni* compared to controls (Table 3).

TABLE 3. The cellular levels of lactate dehydrogenase in mononuclear phagocytes exposed to *M. faeni* and fractions derived from it for various periods of time

Time (hr)	Controls	*M. faeni* (200 μg/ml)	Glycoprotein (200 μg/ml)	Glycopeptide (200 μg/ml)
3	253·2±16·9	270·1±12·6	227·3±20·8	238·1±26·7
5	286·9±67·5	270·1±33·8	239·4±8·4	253·9±47·8
7	241·7±23·8	236·3±33·8	253·7±6·2	272·2±8·4
9	193·6±48·2	268·8±50·6	238·1±16·4	202·6±13·7
24	219·4±0	236·8±13·7	244·8±25·3	198·8±27·5

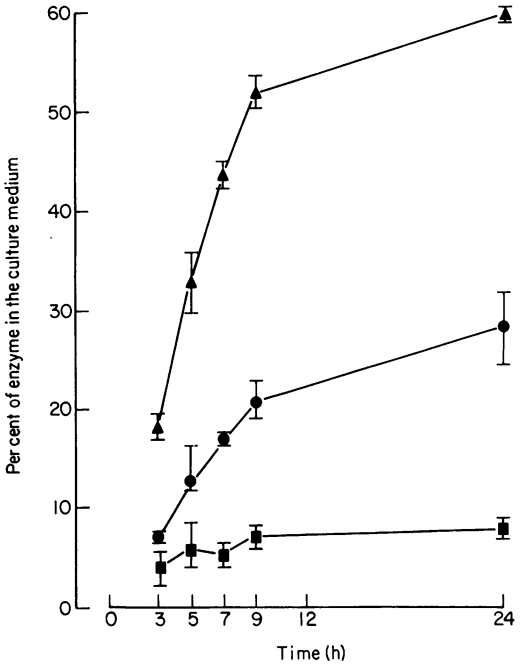

FIG. 4. The time-dependent release of β-glucuronidase from macrophages exposed to 200 μg/ml *M. faeni* (▲) and 200 μg/ml of a protein/glycoprotein fraction from this organism (●). The control (■) was incubated without added materials.

Influence of M. faeni constituents on the enzyme release

Effects of 200 μg/ml *M. faeni* and of comparable weights of protein/glycoprotein and glycopeptide fractions derived from this organism are shown in Fig. 4. *M. faeni* caused enzyme release significantly ($P<0·01$) different from control at 3–24 hr, the protein/glycoprotein caused enzyme release significantly from controls at 5–24 hr and the glycopeptide caused enzyme release significantly different from controls at 7 ($P<0·05$) and 24 hr ($P<0·01$). The time-dependent release of acid hydrolases by *M. faeni* and fractions derived from it was selective since lactate dehydrogenase levels in culture medium did not differ significantly from those in control cultures (Table 3). The results shown in Fig. 5 demonstrate that the release of lysosomal enzymes is directly proportional to the dose of the used material. Under these conditions hydrolase secretion was not accompanied by a loss of cell viability. After 24 hr exposure to the material there was no significant increase of the cytoplasmic enzymelactate dehydrogenase, in the culture medium.

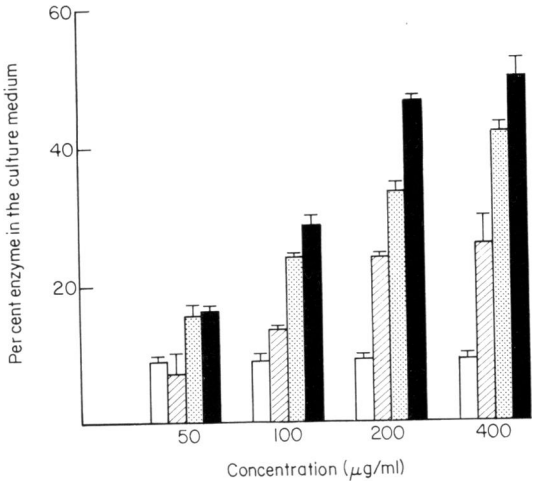

FIG. 5. The release of N-acetyl-β-D-glucosaminidase from macrophages after 24 hr incubation with various concentrations of *M. faeni*, the glycopeptide and the protein/glycoprotein from this organism. Open columns, control; hatched columns, glycopeptide; stippled columns, glycoprotein; solid columns, *M. faeni*.

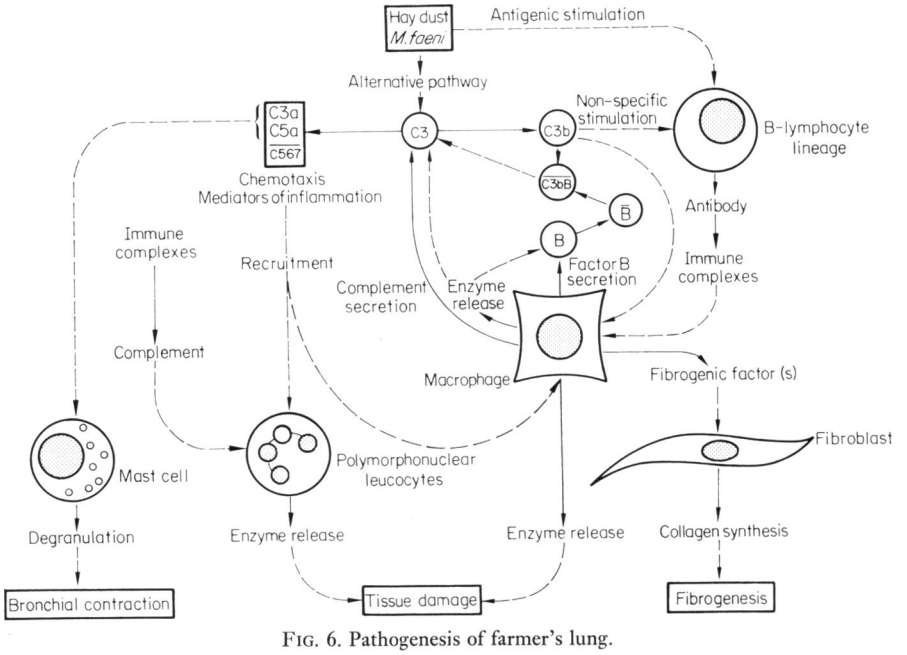

FIG. 6. Pathogenesis of farmer's lung.

DISCUSSION

Mouldy hay dust, *M. faeni*, and the glycopeptide and protein/glycoprotein from this organism all induce the release of hydrolytic enzymes from macrophages in culture. The release is time- and dose-dependent and is not associated with loss of the cytoplasmic enzyme lactate dehydrogenase or any other sign of cell death. Zymosan is also a potent releaser of macrophage hydrolases, as previously mentioned by Weissmann, Dukor & Zurier (1971), even in the absence of serum in the medium. We have shown that several agents which induce chronic inflammation, including streptococcal cell walls (Davies, Page & Allison, 1974), immune complexes (Cardella, Davies & Allison, 1974), dental plaque (Page, Davies & Allison, 1973) and carrageenan (Davies & Allison, 1976), induce secretion of hydrolytic enzymes from macrophages.

All these agents are efficient activators of complement by the alternative pathway (Bitter-Suermann *et al.*, 1975). The parallelism between these two activities is emphasized by comparing the effects of closely related compounds such as dextran sulphates. Here the capacity to activate complement and induce enzyme release are both correlated with molecular weight and degree of sulphation in similar manner (Schorlemmer *et al.*, 1976a). Activation of the alternative pathway results in cleavage of C3, generating C3b, which we have also found to induce hydrolase secretion from macrophages (Schorlemmer, Davies & Allison, 1976b). The enzymes released from stimulated macrophages can themselves cleave C3 (Schorlemmer & Allison, 1976), generating C3b, so that an amplification loop involving macrophages is possible. In addition, a humoral amplification loop has been described. Activated factor B ($\overline{B}$) forms a complex with C3b which is able to cleave C3, generating more C3b (Nicholson *et al.*, 1975). Several proteinases can activate factor B, including enzymes released from polymorphonuclear leucocytes (Brade *et al.*, 1974, Goldstein & Weissmann, 1974). The identification of enzymes secreted from macrophages which can activate factor B is in progress in this laboratory.

Since mouldy hay dust and zymosan induce enzyme release in a serum-free medium the components involved are synthesized by or associated with the macrophages. Synthesis of C3 and factor B of the alternative pathway by macrophages has been demonstrated (Lai A Fat & van Furth, 1975; Bentley *et al.*, 1976). Hence the system for activating macrophages by *M. faeni* or other agents inducing chronic inflammation is produced by the macrophages themselves and is not completely dependent on serum constituents. This could be relevant to the pathogenesis of granulomas containing large numbers of macrophages with ingested *M. faeni* or other materials. These could form a self-activating system generating chemotactic factors, which recruit more mononuclear phagocytes in the lesion and function independently of serum. Indeed, serum contains an inactivator of C3b (Tamura & Nelson, 1967; Lachmann & Müller-Eberhard, 1968) and several proteinase inhibitors (Davies, 1975) which might limit the progress of the lesion.

A tentative interpretation of the pathogenesis of farmer's lung can be offered (Fig. 6). *M. faeni* is inhaled and in the alveolar walls activates the alternative pathway of the complement system. Complement cleavage products (C3a, C5a, $\overline{C567}$) are chemotactic initially for polymorphonuclear leucocytes (Bokisch, Müller-Eberhard & Cochrane, 1969; Ward, 1958; Ward, Cochrane & Müller-Eberhard, 1966) which are involved in the acute disease. *M. faeni* particles are ingested by alveolar macrophages and activate these to secrete hydrolytic enzymes which can cleave C3, directly or through the complex with activated factor B. The C3b generated can stimulate the macrophages to secrete enzymes. Less is known about chemotaxis of macrophages than about this process in polymorphonuclear leucocytes, but Snyderman, Shin & Dannenberg (1972) have shown that a macrophage proteinase can cleave C5, thereby generating a factor chemotactic for macrophages. In this way, provided that enough *M. faeni* is inhaled, there would be a continuing chemotactic stimulus and accumulation of mononuclear phagocytes to produce a granuloma. After inhalation of small amounts of *M. faeni* macrophages carrying the organisms would disperse, so that the reaction would be self-limited. *M. faeni*, constituents remain demonstrable in macrophages *in vivo* and *in vitro* for a long time, so that they may not be degradable by macrophage enzymes, in this respect resembling group A streptococcal cell walls (Davies *et al.*, 1974; Lahav *et al.*, 1974), mycobacteria and other agents inducing chronic inflammation.

Two other processes contribute to the lesions of farmer's lung. One is the presence of stimulated lymphocytes of the B lineage, including plasma cells. These may be responding to both specific antigenic stimulation and non-specific stimulation, resulting from various mechanisms, including stimulation mediated by C3b itself (Dukor & Hartmann, 1973), and products of macrophages activated by C3b that increase the proliferation of lymphocytes (Gery & Waksman, 1972). The antibodies formed may combine with antigens to generate immune complexes which help to perpetuate the lesions. Immune complexes can themselves stimulate enzyme secretion by macrophages (Cardella *et al.*, 1974). The second is the stimulation of fibrogenesis. There is evidence that this can be mediated by a two-stage mechanism in which activated macrophages liberate a factor or factors stimulating proliferation or collagen synthesis by fibroblasts (Allison, Clark & Davies, 1976).

Another factor to be borne in mind is that C3a, generated by complement activation as described

above, could bring about mast cell degranulation and bronchoconstriction. The observations of Edwards *et al.* (1976) and those here reported provide evidence that inhalation of sufficient MHD in persons not previously sensitized can produce an inflammatory reaction because of complement activation by the alternative pathway. If an individual is already sensitized to MHD, allergic reactions due to acquired immune responses would be superimposed on this basic response. Under these circumstances inhalation of smaller amounts of MHD might be sufficient to produce disease.

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