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Macrophage secretion and the complement cleavage product C3a in the pathogenesis of infections by mycoplasmas and L-forms of bacteria and in immunity to these organisms

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SUMMARY

Mouse peritoneal macrophages in culture exposed to *Mycoplasma pulmonis* show marked biochemical changes. This micro-organism induces the release of hydrolytic enzymes from macrophages. 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. Secretory products of macrophages may play a role in the pathogenesis of chronic inflammatory responses elicited by mycoplasma infections. One of the products of activated macrophages is the complement cleavage product C3a. Purified C3a was incubated with *M. hominis*, *M. pulmonis*, *Proteus mirabilis* and an L-phase variant of this organism. All mycoplasmas and the L-phase variant were lysed by low concentrations of C3a, whereas the bacterial form of *Pr. mirabilis* was resistant.

INTRODUCTION

Several mechanisms may contribute to the recovery of animals from mycoplasma infections and resistance to reinfection. There is evidence that T-lymphocytes may play a role in recovery from and resistance to *M. pulmonis* respiratory infection in mice (Taylor & Taylor-Robinson, 1975). Serum antibody transferred from mice recovering from pneumonia to recipient mice provides some protection against the development of pneumonia in these animals following inoculation of *M. pulmonis* (Taylor & Taylor-Robinson, 1977). In human subjects (Brunner *et al.*, 1973) and hamsters (Fernald & Clyde, 1970) immunized with attenuated *M. pneumoniae*, resistance to reinfection was correlated with levels of IgA antibody in nasal secretions. The role of macrophages and of complement and its cleavage products in immunity to mycoplasma infections is less well understood. We have found that the complement cleavage product C3a kills mycoplasmas and L-forms of bacteria and this may be an important factor in immunity to infection by these micro-organisms. In this paper we discuss the details of this observation and the way in which it may be linked with the role of macrophages.

A feature of mycoplasma infections in the lungs and other sites is the associated mononuclear cell reaction. A variety of microbial and other agents eliciting chronic inflammatory reactions induce the secretion of hydrolytic enzymes from macrophages (Schorlemmer, Bitter-Suermann & Allison, 1977a). We have found that mycoplasmas and L-forms of bacteria have similar effects on macrophages.

MATERIALS AND METHODS

Experimental animals. Swiss mice (T.O. strain) were obtained from Scientific Agribusiness Consultants (International) Ltd., Brentwood, Essex.

Tissue culture materials. Tissue culture grade petri-dishes were obtained from Nunc Jobling Laboratories Division, Stone, Staffordshire; medium 199 from Burroughs Wellcome, Beckenham, Kent, and swine serum from Gibco Bio-Cult, Glasgow.

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Biochemical reagents. Penicillin, streptomycin and phenolphthalein glucuronic acid 0·01 M pH 7·0 were obtained from Sigma Chemical Co., Surbiton, Surrey; p-nitrophenyl-β-D′-deoxyglucopyranoside from Koch-Light Laboratories, Colnbrook, Bucks; heparin, preservative-free from Boots, Nottingham; pyruvate and nicotinamide adenine dinucleotide from Boehringer GmbH, Mannheim, Germany; Triton X-100 from British Drug Houses Ltd., Poole, Dorset; and ^{125}IUdR from Radiochemical Centre, Amersham, Bucks.

Macrophage collection and culture. Mouse macrophages were obtained by peritoneal lavage of T.O. mice with 5 ml of M 199 containing 100 iu/ml of penicillin and streptomycin and 10 iu/ml heparin. Five ml aliquots of the peritoneal exudate cell suspension containing 0·5–1·0 × 10^6 cells/ml were distributed into 50 mm petri-dishes and incubated in a humidified atmosphere of 5% carbon dioxide and 95% 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 M 199 containing 10% (v/v) inactivated swine serum. 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 released by adding saline containing 0·1 (w/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 using 2·5 mM pyruvate in 0·05 M phosphate buffer pH 7·5.

β-glucuronidase was assayed by the method of Talalay, Fishman & Huggins (1946). β-galactosidase was assayed by the method of Conchie, Findlay & Levvy (1959) using p-nitrophenyl-β-D-galactopyranoside as substrate.

N-acetyl-β-D-glucosaminidase was assayed by the method of Woollen, Heyworth & Walker (1961) using p-nitrophenyl-2-acetamido-2-β-D-glucopyranoside as substrate dissolved in 0·1 M acetate 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.

Micro-organisms. Mycoplasma pulmonis (JB), of murine origin, was a laboratory-passed strain received from Dr J. Tully (National Institutes of Health, Bethesda, USA). *Mycoplasma hominis* (PG26), of human origin, was supplied by Dr D.G.ff. Edward (Public Health Laboratory, Dulwich Hospital, London). The L-phase variant (L9) of *Proteus mirabilis* was received from Dr B.E. Andrews (Public Health Laboratory Service, Norwich, Norfolk). The bacterial form of this organism was a laboratory stock strain from the Microbiology Department, Northwick Park Hospital, Harrow.

Media. The mycoplasmas and L-phase variant were grown in a liquid medium which has been described previously (Manchee & Taylor-Robinson, 1968). This contained 0·1% glucose and was at pH 7·8 for *M. pulmonis* and the L-phase variant; 0·5% L-arginine was substituted in the case of *M. hominis* and the pH reduced to 7·0. *Pr. mirabilis* was grown in nutrient broth (Difco). Solidified medium for mycoplasmas and L-phase organism incorporated 1% mycoplasma agar (Oxoid) in the liquid medium, and colonies of *Pr. mirabilis* developed on MacConkey agar (Oxoid). All plates were dried for 2 hr at 37°C before use.

Propagation and preparation of organism suspensions. Each mycoplasma was grown in 400 ml of medium incubated for 72 hr at 37°C. The organisms were centrifuged at 105,000 *g* for 30 min and the pellets washed by resuspending in 50 ml of normal saline and recentrifuging. These pellets were resuspended in 2 ml of saline and aliquots stored at −70°C. The L-phase organisms were grown in 100 ml of medium, incubated for 72 hr at 37°C and centrifuged at 1363 *g* for 30 min. These pelleted organisms were washed with, and resuspended in, PPLO broth base (Difco) and, unlike the mycoplasmas, were used immediately, without freezing. Bacteria were grown by incubating in 10 ml nutrient broth cultures overnight at 37°C. These organisms were also tested immediately.

Labelling of L-phase variants with ^{125}IUdR. The L-phase variant of *Pr. mirabilis* was cultured for 96 hr in medium containing 0·1 μCi/ml ^{125}IUdR. The organisms were then washed three times and divided into two samples. One sample was incubated for 30 min with 10 μg/ml C3a in medium, the other in medium lacking C3a. The samples were centrifuged at 105,000 *g* for 30 min to deposit the organisms and radioactivity in the supernatant fluids and deposits was counted. More than 95% of ^{125}IUdR in the supernatant was incorporated into DNA, as shown by acid precipitation and DNAase release.

Preparation of complement components. Guinea pig C3 was prepared as previously reported (Bitter-Suermann *et al.*, 1970). For generation and purification of C3a, highly purified C3 was cleaved by trypsin (1 mg/ml) and, 1 min later, the reaction was stopped by the addition of soybean trypsin inhibitor (4 mg/ml). In other experiments, C3 was incubated with the C3 cleavage complex which is formed when cobra venom factor interacts with factor B, factor D and Mg^{++}. In both cases the reaction mixtures were passed through Sephadex G100 columns. The fractions which mediated contraction of isolated guinea-pig terminal ileum were pooled and concentrated.

C3a treatment of organisms. C3a at a concentration of 1 mg/0·1 ml was stored at −20°C. It was thawed rapidly, kept on wet ice, and dilutions made in normal saline. Equal volumes of diluted C3a and organism suspension were mixed thoroughly and incubated at 37°C for 30 min, or as indicated in the text. An aliquot of the same organism suspension mixed with an equal volume of PPLO broth base was similarly incubated and constituted the control.

Aliquots (0·025 ml) of the organism-C3a mixture and untreated organism control were inoculated onto the same plate of agar medium, as were ten-fold dilutions of these suspensions. Thus, the numbers of organisms were estimated by the

technique of Miles & Misra (1938). The media were incubated at 37°C in an atmosphere of 5% CO_2 + 95% N_2. Colonies of *Pr. mirabilis* were counted the next day, while those of the other micro-organisms were counted after 3–4 days when further colonies had ceased to appear.

Electron microscopy. A suspension of the organisms was made in 0·1 M phosphate buffered 2% glutaraldehyde. pH 7·4 and ultracentrifuged at 20,000 *g* for 30 min. The total time in glutaraldehyde was 90 min. The pellet was cut up and washed × 3 for 15 min with phosphate buffered 7·5% sucrose. The fragments were post-fixed in 1% osmium tetroxide in phosphate buffered 3·75% sucrose for 1 hr, rinsed in water and then dehydrated through ascending grades of alcohol, cleared in epoxypropane impregnated with araldite, and polymerized at 60°C for 48 hr.

The blocks were cut using a Ge-Fe-Ri diamond knife on a Sorval Porter-Blum MT-2 ultramicrotome. Sections were mounted on copper grids, stained with lead citrate for 2 min (Reynolds, 1963) and aqueous 2% uranyl acetate for 15 min, and then examined in an AEI EM6B electron microscope.

RESULTS

The selective release of acid hydrolases from cultured macrophages by Mycoplasma pulmonis

Marked changes in the levels and distribution of the activities of acid hydrolases were induced in mouse macrophages by *Mycoplasma pulmonis*. Two types of experiment were performed. In the first, the effect of various concentrations of *M. pulmonis* on the level and distribution of enzyme activity in mouse peritoneal macrophage cultures after 48 hr was measured. Secondly, the effect of a single concentration of *M. pulmonis* on the time course of changes in enyzme level and distribution in mouse macrophage cultures was determined. The organisms of *M. pulmonis* are rapidly taken up by phagocytosis and can be seen inside cultured macrophages after 3 hr incubation at 37°C. The ingestion is followed by marked selective release of lysosomal hydrolases into the culture medium. In cultures grown in the presence of various concentrations of *M. pulmonis* for a period of 48 hr the total lysosomal enyzme activity was not changed (Fig. 1). There was, however, a marked decrease in the intracellular acid hydrolase content with a reciprocal and concurrent increase in the extracellular enzyme activity in the culture media (Figs 1 and 2). This redistribution of activity was observed for all the lysosomal hydrolases measured; these included β-glucuronidase, N-acetyl-β-D-glucosaminidase and β-galactosidase. After 48 hr incubation in medium containing 25 μg/ml of *M. pulmonis*, the concentration of β-galactosidase in the medium was significantly greater than in the controls ($P < 0{\cdot}01$). There was a dose-dependent release of enzyme rising to 60% with 400 μg/ml of *M. pulmonis* (Fig. 2). The selective nature of the lysosomal enzyme release by *M. pulmonis* is also illustrated in Fig. 2. *M. pulmonis*, at concentrations up to 400 μg/ml, caused no morphologically detectable cell death and no significant increase in the amount of lactate dehydrogenase in the culture medium.

Time course of hydrolase secretion

The time course of enzyme production and release was studied in mouse macrophage cultures main-

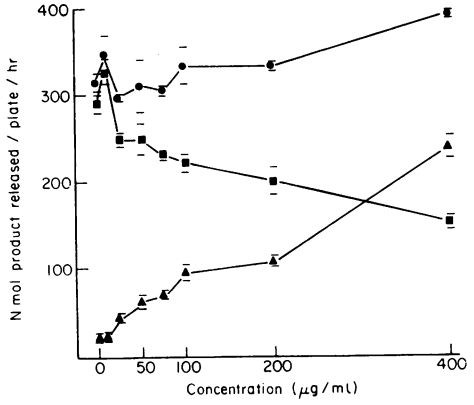

FIG. 1. The dose-dependent effect of *M. pulmonis* on the levels of β-glucuronidase activity in the total culture (●); in cells (■); and in the medium (▲). The cultures were assayed after 48 hr incubation with the organism.

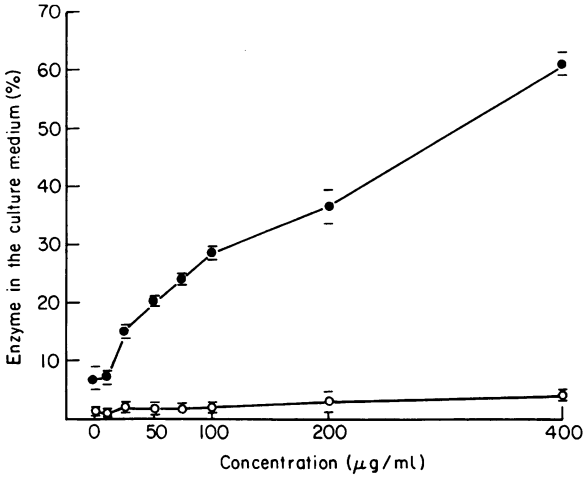

FIG. 2. Effects of 48 hr incubation with various concentrations of *M. pulmonis* on the release of β-galactosidase (●) and lactate dehydrogenase (○) from macrophages into the culture medium.

tained for various lengths of time up to 72 hr, in the presence of a single dose of 50 μg/ml of *M. pulmonis*. The time-dependence of the selective release of lysosomal enzymes caused by this stimulus is shown in Fig. 3. Macrophage cultures exposed to *M. pulmonis* showed a change in the distribution of a representative lysosomal enzyme, N-acetyl-β-D-glucosaminidase, between cells and culture medium. By 9 hr there was a highly significant increase in the amount of N-acetyl-β-D-glucosaminidase in the medium. Subsequently, a rapid rise in the release of enzyme into the culture medium occurred so that by 72 hr approximately 60% of the total enzyme activity was found in the culture medium. This time-dependence of the selective release of lysosomal enzymes caused by *M. pulmonis* occurs with no detectable release of cellular lactate dehydrogenase into the culture medium.

Treatment of mycoplasmas with C3a

Effect on viability. The results of incubating *M. pulmonis* with 10 μg or 50 μg of C3a for various periods of time are shown in Table 1. About 90% of the organisms were killed by 10 μg of C3a after 15 min. There was a further loss of viability up to 30 min but not significantly after this time. More organisms were killed by using 50 μg of C3a, 99% of them within 15 min and practically all after 2 hr.

The effect of C3a on the viability of *M. hominis* organisms was even greater than on *M. pulmonis* organisms. Thus, as shown in Table 2, there was a complete loss of viability of *M. hominis* organisms after they had been incubated with 10 μg of C3a for 30 min.

Effect on structure. Evidence for the killing of mycoplasma organisms through lysis was sought by electron microscopic observation. *M. hominis* organisms before and after treatment with 10 μg of C3a for 30 min were centrifuged at 20,000 *g* and the deposits observed. Intact organisms, which had a characteristic unit membrane and cytoplasmic structure before C3a treatment (Fig. 4) were never seen after treatment. Only membrane fragments remained (Fig. 5), indicating that lysis had occurred.

Treatment of Pr. mirabilis and L-phase variant with C3a

Effect on viability. As in the case of *M. hominis*, incubation of *Pr. mirabilis* L-phase variant with 25 μg of C3a for 30 min resulted in the killing of all the organisms (Table 3). In contrast, the bacterial form was completely unaffected by the same treatment.

Release of ^{125}IUdR-labelled DNA. Evidence that C3a lysed the L-phase variant organisms rather than merely inhibiting their growth, was obtained in experiments showing release of DNA pre-labelled with ^{125}IUdR from these cells by C3a. When the L-phase organisms were incubated with 10 μg/ml of C3a for 30 min, 92% of the label was released. On the other hand, when organisms were incubated with medium alone, only 6% of the label was released.

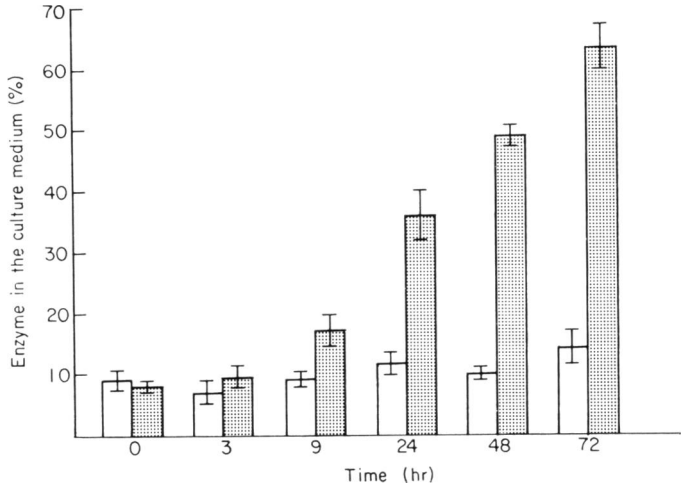

FIG. 3. The time-dependent release of N-acetyl-β-D-glucosaminidase from macrophages exposed to 50 μg/ml of *M. pulmonis*. (□), Control; (▦), *M. pulmonis*.

TABLE 1. The viability of *M. pulmonis* after treatment with two concentrations of C3a for various periods of time

Incubation time (min)	Number of organisms (c.f.u.)* after incubation at 37°C with		
	Medium	C3a (10μg)	C3a (50μg)
15	$1{\cdot}6 \times 10^4$	$1{\cdot}6 \times 10^3$	$1{\cdot}4 \times 10^2$
30	$1{\cdot}7 \times 10^4$	$5{\cdot}7 \times 10^2$	$5{\cdot}3 \times 10^1$
60	$1{\cdot}4 \times 10^4$	$5{\cdot}8 \times 10^2$	$1{\cdot}9 \times 10^1$
120	$1{\cdot}0 \times 10^4$	$3{\cdot}4 \times 10^2$	2·0

* Colony forming units.

TABLE 2. The effect of various concentrations of C3a on two different mycoplasma species

Concentration of C3a	Numbers of organisms (c.f.u.)* before and after incubation with C3a for 30 min at 37°C			
	M. pulmonis		*M. hominis*	
	Before	After	Before	After
10μg	$1{\cdot}7 \times 10^4$	$5{\cdot}7 \times 10^2$	$8{\cdot}5 \times 10^3$	0
25μg	$3{\cdot}1 \times 10^4$	$5{\cdot}5 \times 10^2$	$1{\cdot}8 \times 10^4$	0
50μg	$1{\cdot}7 \times 10^4$	$5{\cdot}3 \times 10^1$	$8{\cdot}5 \times 10^3$	0

* Colony forming units.

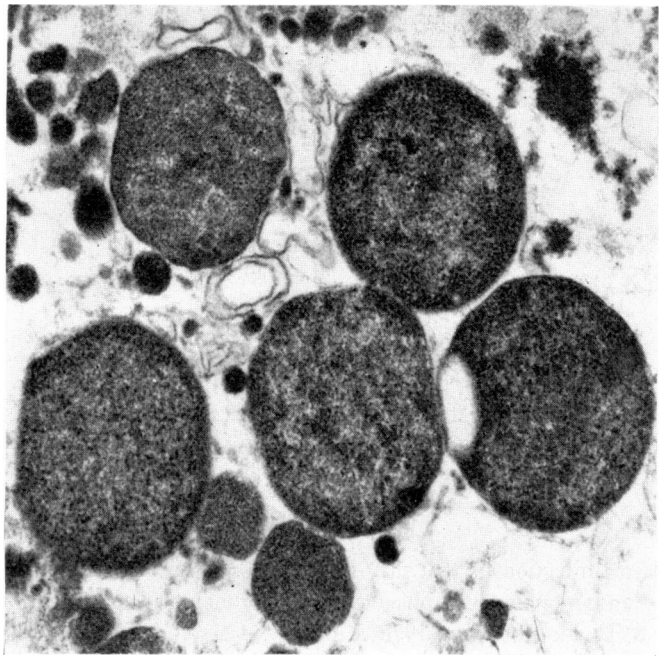

FIG. 4. Electron micrograph of *Mycoplasma hominis* incubated in growth medium showing intact organisms with electron-dense cytoplasm. (Magnification × 27,000.)

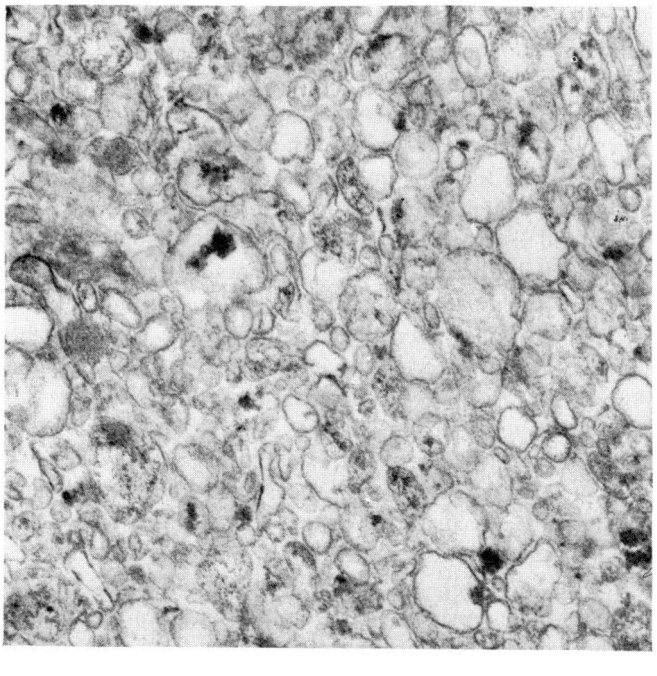

FIG. 5. Electron micrograph of *M. hominis* incubated for 1 hr with 10 μg/ml C3a. The organisms are disrupted and only membranous microvesicles remain. (Magnification × 27,000.)

TABLE 3. The effect of C3a on mycoplasmas, L-phase organisms and bacteria

Micro-organism	Number of organisms (c.f.u.) * after incubation for 30 min at 37°C with: Medium	C3a (25μg)
M. pulmonis	$3{\cdot}1 \times 10^4$	$5{\cdot}5 \times 10^2$
M. hominis	$1{\cdot}8 \times 10^4$	0
Proteus mirabilis L-phase	$3{\cdot}8 \times 10^6$	0
Proteus mirabilis bacterial form	$1{\cdot}1 \times 10^7$	$1{\cdot}0 \times 10^7$

* Colony forming units.

DISCUSSION

A feature of mycoplasma infections is that they elicit mononuclear cell reactions. The infiltrating cells include lymphocytes and mononuclear phagocytes. In bronchiolar exudates of mice infected with *M. pulmonis* (Taylor & Taylor-Robinson, 1975; Lindsey & Cassell, 1973) and of hamsters infected with *M. pneumoniae* (Taylor & Taylor-Robinson, 1975; Fernald & Clyde, 1970) macrophages are common. Normal macrophages have limited cytolytic and cytostatic activity, but activated macrophages acquire these capacities. Products of activated T-lymphocytes stimulate macrophages to increased phagocytic activity and capacity to kill intracellular bacteria (Mackaness, 1971).

Moreover, many bacterial or fungal products are able to activate complement by the alternative pathway, generating C3b which activates macrophages to secrete hydrolytic enzymes (Schorlemmer *et al.*, 1977a and d). In addition, the complement cleavage product C3a in intermediate concentrations lyses macrophages (Schorlemmer, Davies & Allison, 1976; Schorlemmer & Allison, 1976) and in lower concentrations lyses various tumour cells and lymphoblasts (Ferluga *et al.*, 1976; Schorlemmer, Ferluga & Allison, 1977e; Schorlemmer *et al.*, 1977f). The possibility therefore arose that mycoplasmas and L-phase variants of bacteria might be able to stimulate the secretion of hydrolytic enzymes and complement cleavage products by macrophages, and that these micro-organisms might be susceptible to the lytic effect of C3a. The results of the experiments now reported show that mouse peritoneal macrophages, exposed *in vitro* to *M. pulmonis*, release large amounts of lysosomal hydrolases. 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 release of enzymes may cause tissue damage and be a factor in the pathogenesis of some mycoplasmal diseases such as *M. pulmonis*-induced chronic arthritis.

In addition, the two species of mycoplasma and the bacterial L-phase variant are sensitive to C3a. The effect was a cytolytic rather than a cytostatic one, as shown by electron microscopy and release of labelled DNA. In contrast, intact bacteria were resistant to C3a. The latter is a basic polypeptide of molecular weight about 9000 daltons (Hügli, Vallota & Müller-Eberhard, 1975), and presumably interaction with acidic components of the bacterial cell wall prevents access to the sub-adjacent plasma membrane. Thus, the capacity of C3a to lyse cells is very widespread, from mycoplasmas and L-phase variants of bacteria on the one hand, to mammalian cells on the other. We have shown that several agents which induce chronic inflammation, including streptococcal cell walls (Davies, Page & Allison, 1974), dental plaque (Page, Davies & Allison, 1973), dextran sulphate (Schorlemmer *et al.*, 1977b) and zymosan (Schorlemmer *et al.*, 1977c) induce secretion of hydrolytic enzymes from macrophages. In seeking a common factor among the agents that induce enzyme secretion from macrophages, we have found that all have the capacity to activate complement by the alternative pathway (Schorlemmer *et al.*, 1977a). This results in cleavage of C3, generating C3b, which we have also found to induce hydrolase secretion

from macrophages (Schorlemmer *et al.*, 1976). The enzymes released from stimulated macrophages can themselves cleave C3 (Schorlemmer & Allison, 1976). It is remarkable that some complement components and proteolytic enzymes synthesized by macrophages together comprise a self-activating system. The larger cleavage product of the third complement component, C3b, stimulates macrophages to secrete hydrolytic enzymes without loss of viability, whereas incubation of the smaller cleavage product, C3a, with several cell types results in the death of these cells (Ferluga *et al.*, 1976; Schorlemmer *et al.*, 1977c.)

Our results should be considered in relation to other *in vitro* observations and to their possible relevance to the protection of animals against infections by mycoplasmas and bacterial L-phase variants, and recovery from these infections. McGee *et al.* (1972) reported that L-phase organisms can be killed by normal serum. This was attributed to the presence of small amounts of antibody acting with complement. In the case of mycoplasmas, the role of antibody and several aspects of cell-mediated response have been studied in some detail (Taylor-Robinson *et al.*, 1972; Taylor & Taylor-Robinson, 1975). However, the ways in which macrophages and the components of complement may aid in recovery from infection are less well understood, but an important factor may be macrophage activation.

Several factors could trigger this process. Bredt (1975) found that peritoneal macrophages were inefficient in phagocytosing *M. pneumoniae* organisms, but complement, activated by either the classical or alternative pathways, induced phagocytosis which might have been due to C3b on the surface of the organisms. *M. pneumoniae* cells are able to activate the alternative pathway of complement (Bredt & Bitter-Suerman, 1975). Activation could occur also through products of sensitized T-lymphocytes reacting with mycoplasma or L-phase antigens, or through complexes of organism antigens and antibodies. In the lungs of thymectomized hamsters, mononuclear reactions to *M. pneumoniae* are much less marked than in intact animals, even though the number of organisms is higher (Denny, Taylor-Robinson & Allison, 1972). The activated macrophages could terminate mycoplasma or bacterial L-phase infections by ingesting the organisms, a process favoured by having antibodies and/or C3b on their surface, and then killing and digesting them. Alternatively, the activated macrophages might release C3a to lyse the organisms. The relative importance of these several mechanisms in recovery from different mycoplasma and bacterial L-phase infections has yet to be established.

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REFERENCES

BITTER-SUERMANN, D., HADDING, U., MELCHERT, F. & WELLENSIEK, H.J. (1970) Independent and consecutive action of C5, C6 and C7 in immune hemolysis. I. Preparation of EAC1-5 with purified guinea pig C3 and C5. *Immunochemistry*, **7**, 955.

BREDT, W. (1975) Phagocytosis by macrophages of *Mycoplasma pneumoniae* after opsonization by complement. *Infect. Immunity*, **12**, 695.

BREDT, W. & BITTER-SUERMANN, D. (1975) Interactions between *Mycoplasma pneumoniae* and guinea pig complement. *Infect. Immunity*, **11**, 497.

BRUNNER, H., GREENBERG, H.B., JAMES, W.D., HORSWOOD, R.L., COUCH, R.B. & CHANOCK, R.M. (1973) Antibody to *Mycoplasma pneumoniae* in nasal secretions and sputa of experimentally infected human volunteers. *Infect. Immunity*, **8**, 612.

CONCHIE, J., FINDLAY, J. & LEVVY, G.A. (1959) Mammalian glucosidases. Distribution in the body. *Biochem. J.* **71**, 318.

DAVIES, P., PAGE, R.C. & ALLISON, A.C. (1974) Changes in cellular enzyme levels and extracellular release of lysosomal acid hydrolases in macrophages exposed to group A streptococcal cell wall substance. *J. exp. Med.* **139**, 1262.

DENNY, F.W., TAYLOR-ROBINSON, D. & ALLISON, A.C. (1972) The role of thymus-dependent immunity in *Mycoplasma pulmonis* infections of mice. *J. med. Microbiol.* **5**, 327.

FERLUGA, J., SCHORLEMMER, H.U., BAPTISTA, L.C. & ALLISON, A.C. (1976) Cytolytic effects of the complement cleavage product C3a. *Brit. J. Cancer*, **34**, 626.

FERNALD, G.W. & CLYDE, W.A. JR. (1970) Protective effect of vaccines in experimental *Mycoplasma pneumoniae* disease. *Infect. Immunity*, **1**, 559.

HÜGLI, T.E., VALLOTA, E.H. & MÜLLER-EBERHARD, H.J. (1975) Purification and partial characterization of human and porcine C3a anaphylatoxin. *J. biol. Chem.* **250**, 1472.

LINDSEY, J.R. & CASSELL, G. (1973) Experimental *Mycoplasma pulmonis* infection in pathogen-free mice: models for studying mycoplasmosis of the respiratory tract. *Amer. J. Path.* **72**, 63.

MACKANESS, G.B. (1971) Resistance to intracellular infection. *J. infect. Dis.* **123**, 439.

MANCHEE, R.J. & TAYLOR-ROBINSON, D. (1968) Haemadsorption and haemagglutination of mycoplasmas. *J. gen. Microbiol.* **50**, 465.

McGee, Z.A., Ratner, H.B., Bryant, R.E., Rosenthal, A.S. & Koenig, M.G. (1972) An antibody-complement system in human serum lethal to L-phase variants of bacteria. *J. Infect. Dis.* **125**, 231.

Miles, A.A. & Misra, S.S. (1938) The estimation of the bactericidal power of blood. *J. Hyg. (Lond.)* **38**, 732.

Page, R.C., Davies, P. & Allison, A.C. (1973) Effects of dental plaque on the production and release of lysosomal hydrolases by macrophages in culture. *Archs. oral Biol.* **18**, 1481.

Reynolds, E.S. (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. cell Biol.* **17**, 208.

Schorlemmer, H.U. & Allison, A.C. (1976) Effects of activated complement components on enzyme secretion by macrophages. *Immunology*, **31**, 781.

Schorlemmer, H.U., Davies, P. & Allison, A.C. (1976) Ability of activated complement components to induce lysosomal enzyme release from macrophages. *Nature (Lond.)* **261**, 48.

Schorlemmer, H.U., Bitter-Suermann, D. & Allison, A.C. (1977a) Complement activation by the alternative pathway and macrophage enzyme secretion in the pathogenesis of chronic inflammation. *Immunology*, **32**, 929.

Schorlemmer, H.U., Burger, R., Hylton, W. & Allison, A.C. (1977b) Induction of lysosomal enzyme release from cultured macrophages by dextran sulphate. *Clin. immunol. Immunopath.* **7**, 88.

Schorlemmer, H.U., Davies, P., Hylton, W., Gugig, M. & Allison, A.C. (1977c) The selective release of lysosomal acid hydrolases from mouse peritoneal macrophages by stimuli of chronic inflammation. *Brit. J. exp. Path.* **58**, 315.

Schorlemmer, H.U., Edwards, J.H., Davies, P. & Allison, A.C. (1977d) Macrophage responses to mouldy hay dust, *Micropolyspora faeni* and zymosan, activators of complement by the alternative pathway. *Clin. exp. Immunol.* **27**, 198.

Schorlemmer, H.U., Ferluga, J. & Allison, A.C. (1977e) Interactions of macrophages and complement component in the pathogenesis of chronic inflammation. *Perspectives in Inflammation* (ed. by D. A. Willoughby), J. P. Giroud and G. P. Velo), p. 191. MTP Press Ltd. International Medical Publishers, Lancaster, England.

Schorlemmer, H.U., Hadding, U., Bitter-Suermann, D. & Allison, A.C. (1977f) The role of complement cleavage products in killing of tumour cells by macrophages. *Proceedings of the European Reticuloendothelial Society on The Macrophage and Cancer* (ed. by K. James, B. McBride and A. Stuart), Section II, p. 68. Edinburgh.

Talalay, P., Fishman, W.M. & Huggins, C. (1946) Chromiogenic substrates. II. Phenolphthalein glucoronic acid as substrate for the assay of glucoronidase activity. *J. biol. Chem.* **166**, 757.

Taylor, G. & Taylor-Robinson, D. (1975) The part played by cell-mediated immunity in mycoplasma respiratory infections. *Develop. biol. Standard*, **28**, 195.

Taylor, G. & Taylor-Robinson, D. (1977) Factors involved in resistance to and recovery from *Mycoplasma pulmonis* induced arthritis in mice. *Ann. rheum. Dis.* **36**, 232.

Taylor-Robinson, D., Denny, F.W., Allison, A.C., Thompson, G.W. & Taylor, G. (1972) Cell-mediated immunity and the response to mycoplasma infections. '*Pathogenic Mycoplasmas*', *Ciba Foundation Symposium.* p. 349. Associated Scientific Publishers, North Holland, Amsterdam.

Woollen, J.W., Heyworth, R. & Walker, P.G. (1961) Studies on glucosaminidase and N-acetyl-β galactosaminidase. *Biochem. J.* **78**, 111.