Immunology 1981 **44** 29

Studies on the possible involvement of complement component C3 in the initiation of acid hydrolase secretion by macrophages

I. CORRELATION BETWEEN ENZYME-RELEASING AND COMPLEMENT-ACTIVATING CAPACITIES OF SEVERAL SECRETAGOGUES

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Accepted for publication 5 March 1981

Summary. A possible relationship between activation of the alternative pathway of complement and acid hydrolase secretion by macrophages has been investigated *in vitro* by examining the dose–response characteristics of several immunological and non-immunological stimuli of these two processes. Zymosan particles, insoluble immune complexes, methylamine and several other primary aliphatic monoamines were all found to elicit the selective release of lysosomal enzymes from macrophages by a process that correlated well with the ability of these agents to bring about consumption of haemolytically-active components of the alternative complement pathway. By contrast, substances which failed to activate the alternative complement pathway, i.e. soluble aggregated immunoglobulin and several primary aliphatic diamines, were found to be likewise incapable of inducing the selective release of lysosomal glycosidases from macrophages. These observations are interpreted as further evidence for imputing a role for complement C3 in the initiation of lysosomal enzyme release from macrophages.

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0019-2805/81/0900-0029$02.00

INTRODUCTION

The *in vitro* secretion of lysosomal acid hydrolases from macrophages has been studied extensively in recent years. However, despite the identification of many different substances capable of initiating this response, e.g. streptococcal cell walls (Davies, Page & Allison, 1974), immune complexes (Cardella, Davies & Allison, 1974) and several weak bases (Riches & Stanworth, 1980a), the biochemical mechanisms underlying the process remain imperfectly understood. In view of both the structural and chemical diversity of lysosomal enzyme secretagogues it would seem unlikely that specific cell-surface receptors exist for each stimulus. This implies, therefore that the recognition of such substances by the macrophage is mediated via a common pathway. Seeking such a common factor amongst the agents which induce lysosomal acid hydrolase secretion from macrophages, Schorlemmer, Bitter-Suermann & Allison (1977a) have drawn attention to the fact that all such substances so far

investigated also activate the complement cascade by the alternative pathway. Such activation results in the generation of component C3b, which itself has been shown to be a stimulator of enzyme secretion (Schorlemmer, Davies & Allison, 1976) and, since cells of the macrophage/monocyte lineage are capable of the synthesis and secretion of all the known components of the alternative pathway activation unit (Bentley, Fries & Brade, 1978; Whaley, 1980), it has been suggested that the macrophage could comprise a self-activating unit.

We have investigated the relationship between alternative complement pathway activation and acid hydrolase secretion from macrophages by examining quantifiably those factors which influence initiation of these processes by a number of previously described lysosomal enzyme secretagogues. Our findings, of highly significant correlations between lysosomal enzyme release and complement activation by the alternative pathway for zymosan, insoluble immune complexes and a variety of weak bases, are taken as further reasons for implicating complement component C3 in the initiation of acid hydrolase secretion from macrophages.

MATERIALS AND METHODS

Complement reagents

Complement fixation-test diluent tablets were obtained from Oxoid Ltd, Basingstoke, Hants. Alternative pathway complement fixation-test diluent (AP-CFTD) was prepared by adding 10 vol of 0·1 M EGTA and 7 vol of 0·1 M $MgCl_2$ to 83 vol of veronal-buffered saline (pH 7·2).

Human serum, obtained from normal healthy volunteers, was stored at −70° for up to 3 months.

Rabbit erythrocytes were obtained from normal adult half-lop rabbits by venipuncture of the marginal ear vein. Acid citrate dextrose was added to a final concentration of 20% v/v to prevent coagulation of the blood. The cells were washed three times in 20% acid citrate dextrose, three times in AP-CFTD and were finally resuspended in AP-CFTD at a concentration of 1% v/v.

Animals

Adult male BALB/c mice, weighing approximately 25 g, were obtained from the Department of Immunology animal house, University of Birmingham.

Tissue culture media

Tissue culture medium 199, HEPES [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], streptomycin, penicillin, fungizone and heat-inactivated foetal calf serum were obtained from Gibco Bio-Cult. Ltd, Paisley, Scotland. Plastic tissue-culture grade Petri dishes (30 mm diameter) were obtained from Sterilin Ltd, Teddington, Middx,

Enzyme substrates

B-NAD^+ (grade IV), B-NADH (grade IV), DL-lactic acid, 4-methylumbelliferone, 4-methylumbelliferyl-β-D-glucuronide and 4-methylumbelliferyl-β-D-galactoside were obtained from Sigma Chemical Co., Poole, Dorset.

Stimulating agents

Methylamine hydrochloride, pentylamine, hexylamine, 1,4-diaminobutane 1,5-diaminopentane, zymosan-A and bovine serum albumin (BSA) were obtained from Sigma Chemical Co., Poole, Dorset. Ethylamine hydrochloride, propylamine, butylamine, 1,2-diaminoethane, 1,3-diaminopropane and 1,6-diaminohexane were obtained from BDH, Poole, Dorset. Insoluble immune complexes comprising rabbit IgG antibodies were prepared by incubating heat-inactivated hyperimmune rabbit anti-BSA antiserum with BSA at the point of antigen–antibody equivalence for 60 min at 37°. The precipitated complexes were washed four times in saline before being used.

Macrophage collection and culture

Mouse macrophages were obtained from inbred male BALB/c mice by lavage of the peritoneal cavity with 3 ml portions of medium 199 buffered with 20 mM HEPES, pH 7·2, and supplemented with 100 μg ml^{-1} of streptomycin, 60 μg ml^{-1} of penicillin 25 μg ml^{-1} of fungizone, and 10% v/v of heat-inactivated foetal calf serum. The cell suspension, which comprised about 65% macrophages was adjusted to a concentration of 2×10^6 cells ml^{-1} and 2 ml portions were distributed into 30 mm diameter plastic Petri dishes. After incubation at 37° for 2 hr, the non-adherent cells were removed, and the remaining macrophage monolayers rinsed three times with medium 199, before adding fresh medium 199 supplemented with 10% v/v heat-inactivated foetal calf serum. The macrophages were then cultivated overnight to give a uniform layer of well-spread cells composed of more than 95% macrophages as judged by nuclear staining with buffered Giemsa's stain, and by their ability to phagocytose

zymosan particles. The cells were then rinsed three times with medium 199 (without serum) before being exposed to various stimuli in serum-free medium 199. At the end of the incubation period, the supernatants were removed and the cells lysed with 2 ml portions of 0·1% v/v Triton X-100 in 0·9% w/v NaCl. Both supernatant and cell fractions were analysed for activities of various lysosomal and cytoplasmic enzymes.

Enzyme assays

Lactate dehydrogenase was assayed spectrophotometrically by measuring the rate of NAD^+ reduction at 340 nm; using 100 mM lactic acid and 2·5 mM NAD^+ dissolved in 100 mM 2-amino-2-methylpropan-1-ol/HCl buffer pH 9·0. β-glucuronidase and β-galactosidase were assayed fluorimetrically (excitation wavelength = 368 nm; emission wavelength = 435 nm) using the glucuronic acid and galactose derivatives of 4-methylumbelliferone at a concentration of 0·8 mM in sodium acetate-acetic acid buffer, pH 4·0, and citric acid-sodium phosphate buffer pH 4·3 respectively. All enzyme assays were carried out using an automated continuous flow method (see Fig. 1 for manifold details) and were conducted under conditions which gave a linear release of reaction products with time.

Complement assay

Lysosomal secretagogues were tested for a capacity to activate the alternative pathway of complement using the method of Riches & Stanworth (1980b). Briefly, test substances are incubated with an optimal amount of normal human serum supplemented with 10 mM EGTA and 7 mM $MgCl_2$ (to inhibit the classical pathway). Any activation of the alternative pathway of complement will result in a reduction of the total available complement. The residual complement is then quantified by back-titration against unsensitized rabbit erythrocytes; the reduction in haemolysis, as compared with controls in the absence of complement activation, being proportional to the concentration of the activator.

RESULTS

Acid hydrolase secretion by various immunological and non-immunological stimuli

Zymosan particles. As previous investigators have shown (Weissmann, Dukor & Zurier, 1971; Schorlemmer, Edwards, Davies & Allison, 1979b) zymosan particles are rapidly endocytosed by mouse macro-

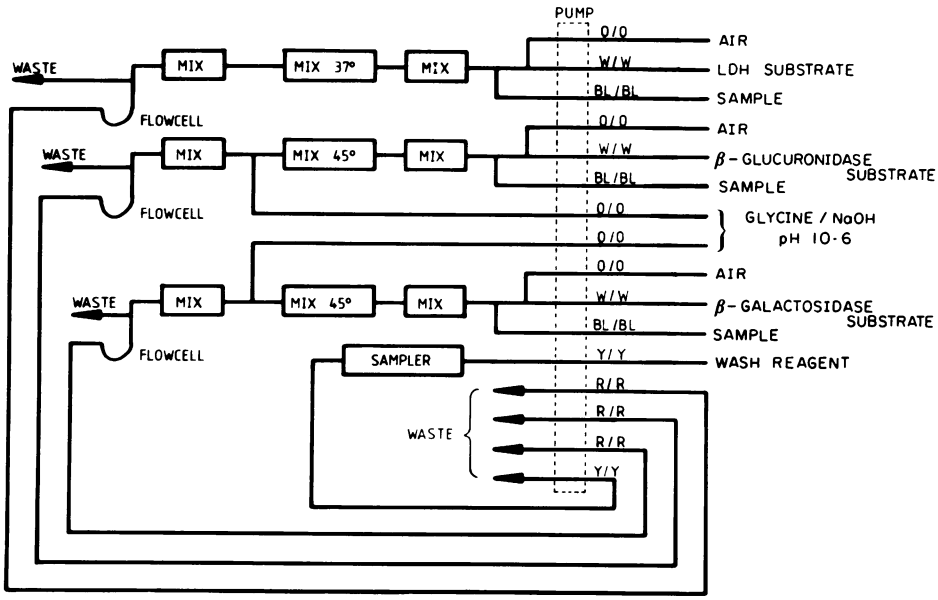

Figure 1. Manifold details for the continuous-flow analysis of activities of lysosomal β-glucuronidase and β-galactosidase and cytoplasmic lactate dehydrogenase.

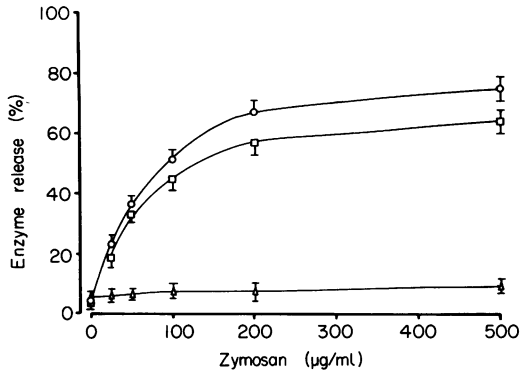

Figure 2. Dose-dependent release of lysosomal enzymes from mouse macrophages after exposure to zymosan particles for 24 hr in serum-free medium 199 at 37°. (o) β-glucuronidase; (□) β-galactosidase; (Δ) lactate dehydrogenase. Each point represents the mean ± standard deviation of four observations.

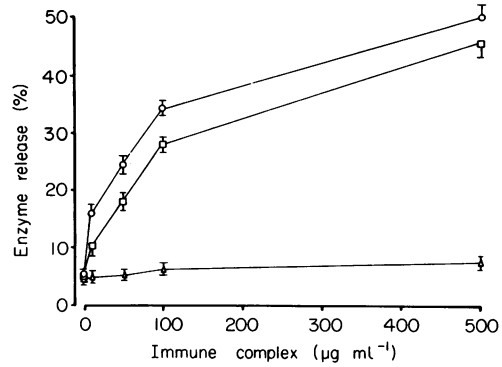

Figure 3. Dose-dependent release of lysosomal enzymes from mouse macrophages after exposure to insoluble immune complexes for 24 hr in serum-free medium 199 at 37°. (o) β-glucuronidase; (□) β-galactosidase; (Δ) lactate dehydrogenase. Each point represents the mean ± standard deviation of four observations.

phages in the absence of serum opsonins and by a process that is marked by the concomitant selective release of lysosomal acid hydrolases. As will be seen from Fig. 2, the release of β-glucuronidase and β-galactosidase from macrophage monolayers following exposure to increasing doses of zymosan for 24 hr at 37° rose steadily to almost 80% release of the total available cellular activities of these enzymes and occurred in the absence of cell death as evidenced by the failure to detect significant levels of cytoplasmic lactate dehydrogenase at any concentration of zymosan tested.

Insoluble immune complexes and soluble aggregated immunoglobulin. The effect of incubating fine suspensions of insoluble immune (IgG antibody-BSA antigen) complexes ranging in concentration from 10–200 μg ml^{-1} with cultures of mouse macrophages for 24 hr at 37° is shown in Fig. 3. Significant non-lytic release from the macrophages of lysosomal β-glucuronidase and β-galactosidase ($P < 0.01$) occurred following challenge with as little as 10 μg ml^{-1} of insoluble immune complex, and the amount of lysosomal enzymes released increased progressively before levelling off at about 40% release of the total available cellular activities following challenge with 200 μg ml^{-1} of immune complex.

By contrast, exposure of mouse macrophages to up to 1 mg ml^{-1} of soluble aggregated human IgG (isolated from out-dated stocks of prophylactic IgG preparations (Stanworth & Johns, 1977)) did not stimulate lysosomal acid hydrolase secretion at any of the concentrations that were studied (Table 1).

Methylamine. As we have shown in previous studies (Riches & Stanworth, 1980a) the simple weak base methylamine is also an effective stimulant of acid hydrolase secretion from macrophages. Figure 4 shows the effect of exposing macrophage monolayers to millimolar levels of methylamine for 5 hr at 37°. The release of β-glucuronidase and β-galactosidase from the cells into the culture supernatants was found to be highly significant ($P < 0.01$) following exposure to 10 mM methylamine; while maximum release, which was in the order of 80% of the total available cellular activities of these enzymes, was observed after exposure to 50 mM methylamine. The selectivity of the release process is indicated by lack of release of cytoplasmic lactate dehydrogenase at any of the concentrations of methylamine that were tested.

Table 1. Effect of soluble aggregated human IgG on the release of lysosomal acid hydrolases from macrophages

	Aggregated human IgG (mg ml^{-1})					
Release (%)	0	0·01	0·05	0·1	0·5	1·0
LDH release	10·5	14·8	14·0	12·9	14·0	15·0
β-glucuronidase release	7·8	5·5	7·7	9·2	13·5	16·2
β-galactosidase release	4·8	4·4	4·0	4·1	5·8	9·2

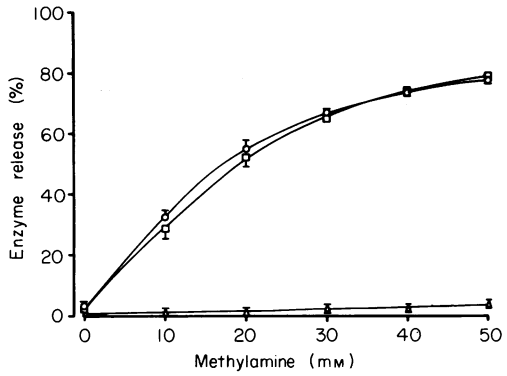

Figure 4. Dose-dependent release of lysosomal enzymes from mouse macrophages after exposure to methylamine for 5 hr in serum-free medium 199 at 37°. (o) β-glucuronidase; (□) β-galactosidase; (Δ) lactate dehydrogenase. Each point represents the mean ± standard deviation of four observations. (Reproduced by courtesy of the Biochemical Journal.)

Other weak bases. The selective release of lysosomal acid hydrolases from macrophages exposed to weak bases is highly dependent upon the chemical structure of the stimulating agent. As will be seen from Table 2, methylamine, ethylamine, propylamine and butylamine when employed at a concentration of 20 mM were all effective stimulants of selective lysosomal enzyme release; but further increases in the length of the aliphatic chain, as in pentylamine and hexylamine, resulted in the compounds becoming progressively lytic. By contrast, structurally-related primary aliphatic diamines of increasing molecular size from 1,2-diaminoethane, through to 1,6-diaminohexane, proved to be incapable of triggering either lytic or selective lysosomal enzyme release (Table 2).

Activation of the alternative complement pathway by various immunological and non-immunological stimuli

Zymosan particles. Zymosan particles in the same concentration range as was employed in the acid hydrolase release studies were tested for a capacity to activate the alternative complement pathway in EGTA-chelated human serum. As will be seen from Fig. 5, there was a dose-dependent consumption of alternative pathway activity that gradually levelled off at zymosan concentrations of greater than 50 $\mu g\ ml^{-1}$ and which becomes independent of zymosan concentration above 100 $\mu g\ ml^{-1}$.

Insoluble immune complexes and soluble aggregated immunoglobulin. The effects of incubating insoluble immune (IgG antibody–BSA antigen) complexes and soluble aggregated human serum are shown in Fig. 6. In many respects insoluble immune complexes behaved similarly to zymosan particles in that they induced a marked consumption of components of the alternative pathway with greater than 90% complement consumption being observed at complex concen-

Table 2. Ability of primary aliphatic monoamines and diamines to induce selective lysosomal enzyme release from macrophages and to activate the alternative complement pathway

Amine	Enzyme release (%)			Complement consumed (%)
	LDH	β-glucuronidase	β-galactosidase	
Methylamine	6·3	68·2	67·5	71·0
Ethylamine	5·8	54·5	50·6	65·4
Propylamine	5·5	49·7	42·7	55·0
Butylamine	5·0	64·5	62·5	43·0
Pentylamine	22·1	62·5	62·5	ND
Hexylamine	73·1	75·6	79·5	ND
1,2-diaminoethane	6·6	7·6	6·4	25·7
1,3-diaminopropane	2·7	3·9	3·0	12·7
1,4-diaminobutane	1·2	2·3	2·8	14·2
1,5-diaminopentane	3·6	4·5	1·8	7·8
1,6-diaminohexane	1·0	2·0	1·5	2·5

ND, not determined. All determinations were carried out with the various amines at 20 mM.

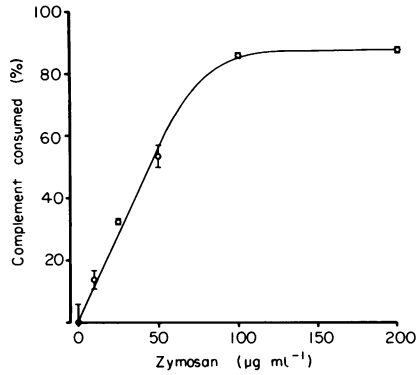

Figure 5. Dose-dependent activation of the alternative complement pathway in EGTA-chelated normal human serum by zymosan particles (Reproduced by courtesy of Immunology Letters).

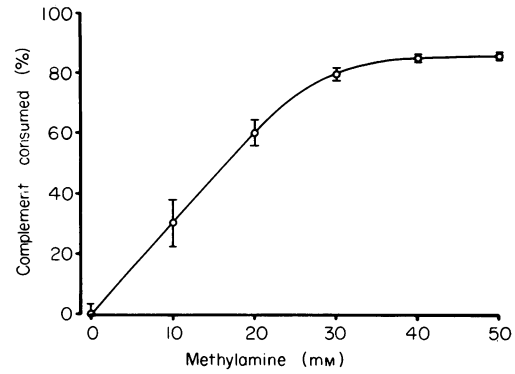

Figure 7. Dose-dependent consumption of haemolytically active components of the alternative complement pathway in EGTA-chelated normal human serum by methylamine.

trations greater than 50 μg ml^{-1}. By contrast, soluble aggregated immunoglobulin failed to activate the alternative pathway of complement even when employed at a concentration as high as 200 μg ml^{-1}.

Methylamine. In view of its potent capacity to release lysosomal enzymes from mouse macrophages, it was of interest to investigate whether this simple weak base would show similar dose-dependent effects on the complement system. As will be seen from Fig. 7, treatment of EGTA-chelated human serum with increasing concentrations of methylamine led to a dose-dependent inhibition of the ability of the serum to lyse the rabbit erythrocytes. Initially, the consumption of complement was linearly dependent upon methylamine concentration. However, at concentrations above 25 mM there was a progressive flattening of the dose–response curve until a plateau was reached at concentrations of methylamine greater than about 35 mM.

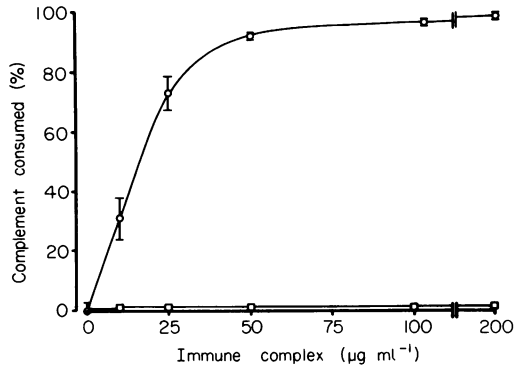

Figure 6. Effects of insoluble immune complexes (o) and soluble aggregated human IgG (□) on the activation of the alternative pathway of complement in EGTA-chelated human serum (Reproduced by courtesy of Immunology Letters).

Other weak bases. Ethylamine, propylamine, butylamine, pentylamine, hexylamine, 1,2-diaminoethane, 1,3-diaminopropane, 1,4-diaminobutane, 1,5-diaminopentane and 1,6-diaminohexane were all tested for a capacity to activate the alternative complement pathway at a concentration of 20 mM. The results are presented in Table 2 and indicate that ethylamine, propylamine and butylamine all induced activation of the alternative pathway as judged by their ability to inhibit the complement-dependent lysis of unsensitized rabbit enthrocytes. The capacity of pentylamine and hexylamine to induce alternative pathway activation could not be determined, since it was found that these compounds caused progressive lysis of the rabbit erythrocytes in the absence of serum. By contrast, none of the primary aliphatic diamines (including the classical pathway activator, 1,4-diaminobutane) induced activation of the alternative pathway to any great extent.

It must be mentioned that the assay employed in the present study to assess alternative complement-pathway activation measures the functional activity of the entire sequence and does not provide information about changes in the activity of individual components. Therefore from the data described above, one can only speculate about the mode of action of

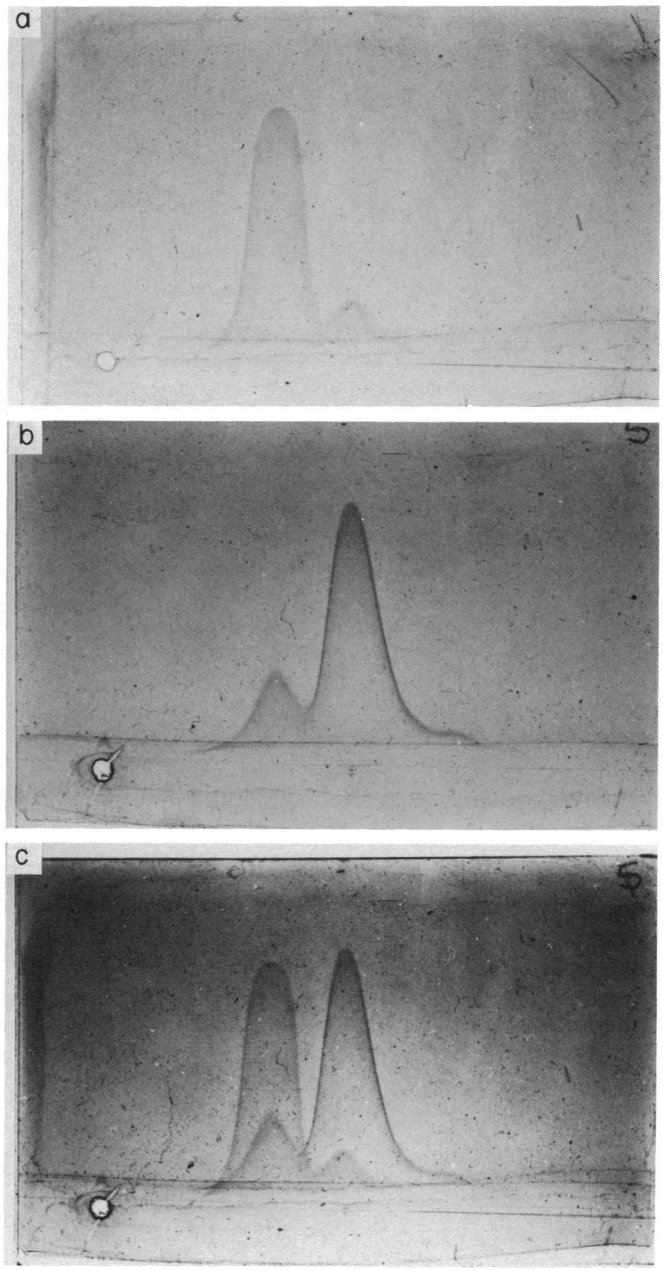

Figure 8. Two dimensional immunoelectrophoresis of zymosan-treated normal human serum, with anti-human C3 in the second dimension. (a) Normal human serum, (b) normal human serum + zymosan and (c) superimposition of (a) and (b) showing a reciprocal and concurrent redistribution of C3 fragments from the slow peak to the fast peak following complement activation.

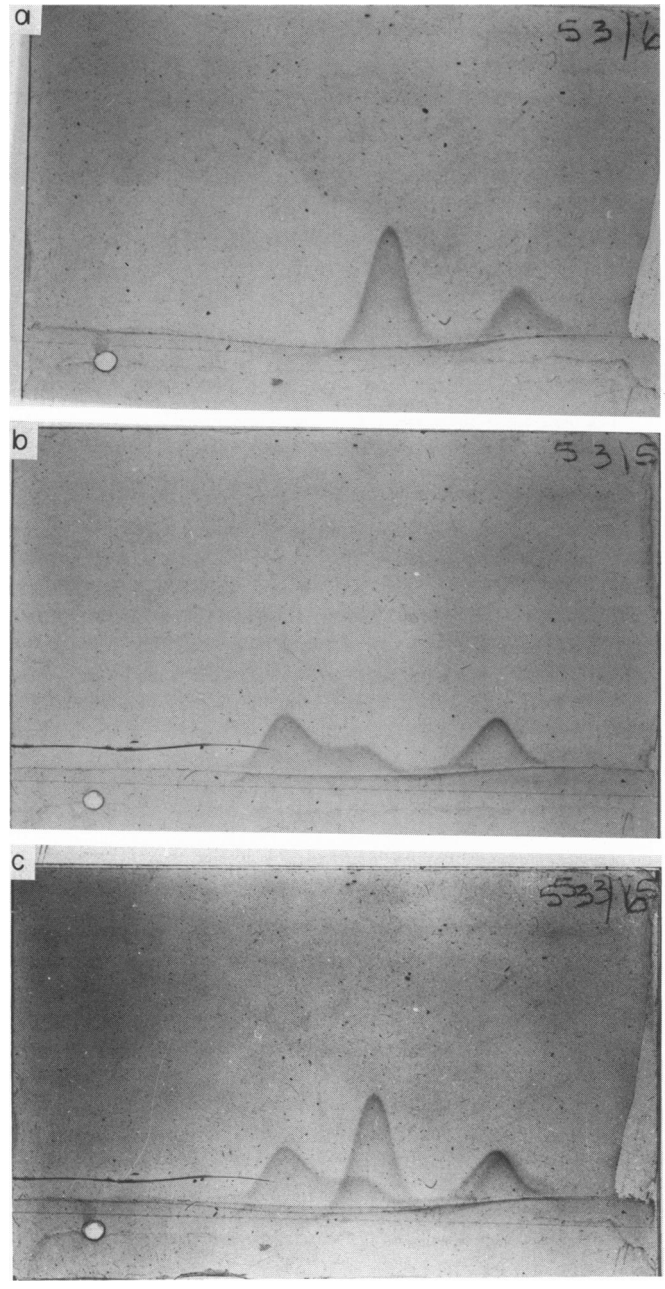

Figure 9. Two dimensional immunoelectrophoresis of methylamine-treated normal human serum, with anti-human C3 in the second dimension. (a) Normal human serum, (b) normal human serum + methylamine (50 mM) and (c) superimposition of (a) and (b) showing the redistribution of C3 fragments following methylamine treatment from the slow peak to the extra slow peak.

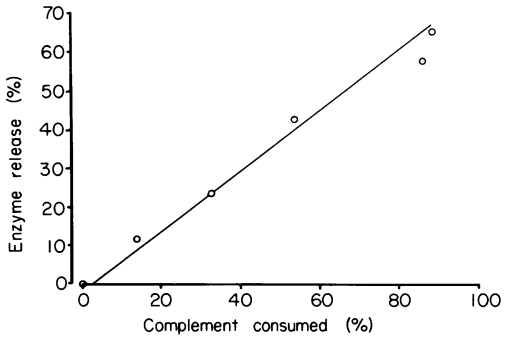

Figure 10. Correlation between the capacity of zymosan to release lysosomal enzymes from mouse macrophages and to activate the alternative pathway of complement in EGTA-chelated human serum.

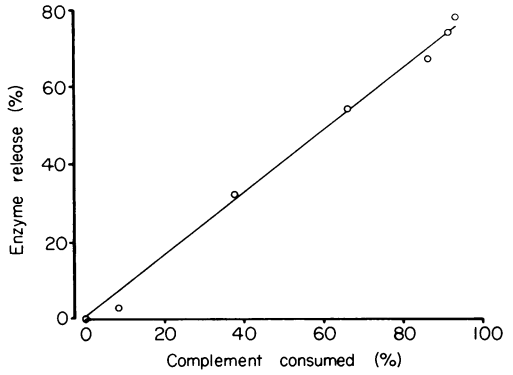

Figure 12. Correlation between the capacity of methylamine to release lysosomal enzymes from mouse macrophages and to activate the alternative pathway of complement in EGTA-chelated human serum.

methylamine on components of the alternative pathway. However, from the results of two dimensional immunoelectrophoretic analyses of C3 in methylamine- and zymosan-treated serum (Figs 8 and 9) we would conclude that action of methylamine on C3 is quite distinct from that of zymosan in that it produces an additional slower antigenic component not seen after zymosan treatment. Moreover, after activation with zymosan, the major antigenic C3 component appeared to be the fast-moving component; whereas after treatment with methylamine, the proportion of the fast component did not appear to be increased above control levels in the absence of treatment.

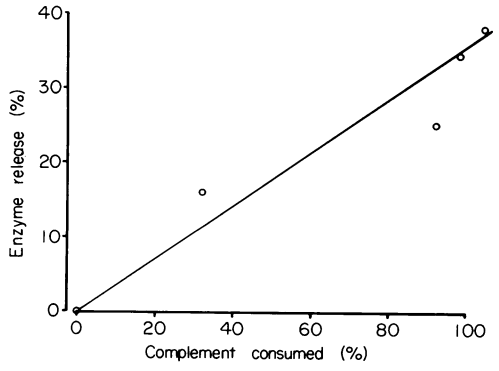

Figure 11. Correlation between the capacity of insoluble immune complexes to release lysosomal enzymes from mouse macrophages and to activate the alternative pathway of complement in EGTA-chelated human serum.

Correlation between lysosomal enzyme release from macrophages and activation of the alternative complement pathway. It was found that, quantitatively, the dose–response data for the induction of selective lysosomal enzyme release from macrophages by various immunological and non-immunological stimuli, correlated closely with those for the activation of the alternative pathway of complement. As will be seen from Figs 10, 11 and 12 highly-significant linear relationships exist between those two parameters for zymosan (correlation coefficient, $r=0{\cdot}995$, $P<0{\cdot}001$) insoluble immune complexes (correlation coefficient, $r=0{\cdot}975$, $P<0{\cdot}001$) and methylamine (correlation coefficient, $r=0{\cdot}997$, $P<0{\cdot}001$). By contrast, there was no correlation ($r=0$) between complement activation and lysosomal enzyme release initiated by soluble aggregated immunoglobulin G.

DISCUSSION

Complement activation products have been reported to elicit a variety of responses in different effector cell populations. For example, human C5a and $C5a_{desArg}$ are chemotactic for human neutrophils (Fernandez, Henson, Otani & Hugh, 1978), human C3a is an anaphylatoxin capable of mediating the release of vasoactive amines from homologous basophils (Glovsky & Hugli, 1979), and human C3b and C3d appear to act as opsonins for neutrophils, monocytes and macrophages (Mantovani, Rabinovitch & Nussenzweig, 1972; Ehlenberger & Nussenzweig, 1977). Moreover, Ferrone, Pellegrino & Cooper (1976) have

reported that C4, expressed on the surface of human lymphocytes, may be involved in the mixed lymphocyte reaction and in the mitogenic response towards phytohaemagglutinin; while Halbwachs & Lachmann (1976) have noted the existence of a factor B-like activity associated with the surfaces of both human and mouse lymphocytes. Our findings, of highly-significant correlations between the ability of a variety of agents to activate the alternative pathway of complement, on the one hand, and to induce the selective release of lysosomal enzymes from macrophages, on the other, provide further evidence to implicate the involvement of components of the alternative pathway, particularly C3, in the initiation of acid hydrolase secretion from macrophages as has been suggested by Schlorlemmer *et al.* (1977a). Furthermore, the inability of other substances (including classical pathway activators) to elicit either response is in support of this conclusion.

The interaction of insoluble immune complexes and zymosan with components of the alternative complement pathway has been well documented (Konig, Bitter-Suermann, Dierich & Hadding, 1973; Brade, Lee, Nicholson, Shin & Mayer, 1973) and would appear to be due to the stabilization of a factor D-dependent C3bBb convertase on the surfaces of these particles: an event that leads to the production of many molecules of C3b via a positive feedback amplification loop. It was the finding by Schorlemmer *et al.* (1976) that C3b produced in this way could stimulate the selective discharge of lysosomal glycosidases from macrophages, combined with the demonstration by Bentley *et al.* (1978) that mouse and guinea-pig macrophages could synthesize and secrete C3, factor B, factor D and properdin, that led Bitter-Suermann, Burger, Brade & Hadding (1976) and Schorlemmer *et al.* (1977a) to postulate that a similar sequence of events could take place at the macrophage plasma membrane during challenge of these cells with activators of the alternative pathway, thereby generating C3b which in turn could directly initiate the secretion of lysosomal enzymes. From the data obtained in the present study with regard to the effects of zymosan particles and insoluble immune complexes on the complement system and on isolated mouse macrophages, we believe that local alternative pathway activation at the macrophage plasma membrane level, with the generation of endogenous C3b could, quite conceivably, be a means of initiating the secretion of lysosomal enzymes.

Methylamine and other primary monoamines, on the other hand, show subtle differences to zymosan and insoluble immune complexes in their mode of activating components of the alternative complement pathway, and hence, probably, also in their mode of initiating lysosomal enzyme release. It is well known that methylamine, ammonia, hydrazine and a variety of other nucleophilic amino compounds interact with C3 and C4 in a non-enzymatic manner (Gordon, Whitehead & Wornell, 1926; Pillemer, Seifter & Ecker, 1940) to inhibit the functional activities of these complement proteins. More recently, it has been shown that this interaction results in the expression of a single sulphydryl group in the C3d domain of the α-chain of C3 (Tack, Janatova, Lorenz, Schechter & Prahe, 1980) after the hydrolysis of a postulated internal thiolester bond. Cleavage of this putative bond has been shown in other studies (Pangburn, 1980) to result in the total loss of C3 haemolytic activity and also to induce a marked change in the conformation of the molecule as indicated by the loss of the B-antigenic determinant found only to be present on the intact molecule (von Zabern, Nolte & Vogt, 1980). Moreover, methylamine-treated human C3 also becomes susceptible to cleavage by C3b-inactivator (Factor I) and β1H (Factor H; Pangburn, 1980). Thus antigenically and functionally methylamine (and other nucleophilic amine) treated C3 behaves in many respects like C3b although structurally it still has an intact α-chain (unlike C3b).

We believe that methylamine and the other amines, found in the present study and in previous studies to induce the selective release of lysosomal acid glycosidases from macrophages, could be interacting with C3 which is known to be expressed on the plasma membrane of mouse macrophages (D.W.H. Riches and D.R. Stanworth, unpublished observations) in an analogous way to produce a C3b-like molecule that is capable of initiating this response. Thus the action of methylamine in inducing lysosomal enzyme secretion from macrophages may be slightly different to insoluble immune complexes and zymosan in that it would appear to circumvent the involvement of other proteins of the alternative pathway such as factor B and factor D.

Clearly our evidence in favour of a role for C3 in the induction of lysosomal enzyme release is to some extent circumstantial. However it is suggested that the relationship between complement activation by the alternative pathway and lysosomal enzyme release from macrophages, both qualitatively and quantitatively, is too striking to be merely coincidental. The

work that is obviously required to delineate the precise molecular details of the initiating events involved in the induction of acid hydrolase release is now in progress.

ACKNOWLEDGMENTS

We wish to thank Miss Helen Evans for carrying out the two dimensional immunoelectrophoretic studies. This work was generously funded by the Arthritis and Rheumatism Council.

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