Immunology 1977 **32** 929

Complement activation by the alternative pathway and macrophage enzyme secretion in the pathogenesis of chronic inflammation

H. U. SCHORLEMMER, D. BITTER-SUERMANN* & A. C. ALLISON *Division of Cell Pathology, MRC Clinical Research Centre, Watford Road, Harrow, Middlesex and † Institut für Medizinische Mikrobiologie, Johannes-Gutenberg-Universität in Mainz, Germany*

Received 23 *September* 1976; *accepted for publication* 25 *November* 1976

Summary. A number of stimuli known to induce acid hydrolase secretion from cultured macrophages were examined for their ability to activate C3 via the alternative pathway of the complement system. Loss of haemolytically active C3 was checked in normal and C4-deficient guinea-pig serum. For comparison the interactions of cultured macrophages with other agents well known as potent activators of the alternative pathway of the complement system have been investigated. As judged by their activity in these assays, group A streptococcal cell walls, different carrageenan preparations, dental plaque and *Actinomyces viscosus* were all capable of initiating the alternative pathway but differed with respect to their potency and their ability to inhibit C3 turnover at high concentrations. Zymosan, some carrageenans, polyanethol sulphonate, and *Corynebacterium parvum* all induce the release of hydrolytic enzymes from macrophages in culture, 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 several agents to activate the complement system via the alternative pathway and to induce inflammatory responses *in vivo* and selective lysosoma enzyme secretion from cultures of macrophages is discussed.

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

INTRODUCTION

Chronic inflammation is of considerable medical importance in both temperate and tropical countries. Among the chronic inflammatory diseases prevalent in Europe and the United States are rheumatoid arthritis, periodontal disease, farmer's lung and related syndromes, regional ileitis and ulcerative colitis. Many of the parasitic diseases in tropical countries induce chronic inflammatory reactions and in some, e.g. schistosomiasis, these reactions play a major role in the pathogenesis of disease. The mechanisms underlying chronic inflammation are still imperfectly understood. Because the reactions *in vivo* are highly complex we have attempted to isolate components of the reactions for *in vitro* analysis. The predominant cells in chronic inflammatory responses are mononuclear phagocytes, so that the recruitment of these cells into lesions and their stimulation within the lesions is of central importance. A consequence of mononuclear phagocyte stimulation by a variety of agents that can induce chronic inflammation is secretion of hydrolytic enzymes (Davies & Allison, 1976) which can

degrade tissue constituents and interact with the complement system to generate mediators of inflammation (Schorlemmer & Allison, 1976; Goldstein & Weissmann, 1974). Moreover we have recently obtained evidence that complement cleavage products can themselves stimulate macrophages (Schorlemmer, Davies & Allison, 1976) thereby completing a chain of events that could perpetuate inflammatory responses.

However, the question still arises how complement is activated in the first instance. Recent studies have drawn attention to the parallelism between the capacity of different agents to activate complement by the alternative pathway and to induce chronic inflammation: this has been shown with mouldy hay dust (Edwards, Baker & Davies, 1974; Edwards, 1976) and the thermophilic actinomycete *Micropolyspora faeni* which is the active agent in farmer's lung (Schorlemmer, Edwards, Davies & Allison, 1977a), dextran sulphate (Burger, Hadding, Schorlemmer, Brade & Bitter-Suermann, 1975; Schorlemmer, Burger, Hylton & Allison, 1977b) and immune complexes (Cardella, Davies & Allison, 1974; König, Bitter-Suermann, Dierich & Hadding, 1973). For other material available information is incomplete. We have investigated further the correlation between the capacity of various materials to activate the alternative pathway and to stimulate hydrolase secretion from mononuclear phagocytes in culture. The results now presented confirm the striking parallelism between these properties and suggest that they are interrelated in the pathogenesis of chronic inflammation.

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 Jobling Laboratories Division, Stone, Staffordshire, M199 from Burroughs Wellcome, Beckenham, Kent, and swine serum from Bio Cult Laboratories Ltd, Glasgow.

Biochemical reagents

Bovine serum albumin, penicillin, steptomycin, phenolphthalein glucuronic acid 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; polyanethol sulphonate from Fluka, Switzerland; BCG from Glaxo Laboratories Ltd, Greenford; *Corynebacterium parvum* from Wellcome Reagents Ltd, Wellcome Research Laboratories, Beckenham, Kent. Native undegraded carrageenan from *Chondrus crispus* was manufactured by Marine Colloids Inc., Rockland, Maine, U.S.A. The sample of group A streptococcal cell walls used was that prepared for other studies (Davies, Page & Allison, 1974). Supragingival dental plaque was removed and used as described by Page, Davies & Allison (1973). Actinomyces viscosus (WVU /371/GB) was provided by Dr R.C. Page, School of Medicine, Department of Pathology, University of Washington, Seattle, Washington 98105, U.S.A. Polyanethol sulphonate and polyvinyl sulphate were a gift from Dr M. Loos, Institut für Medizinische Mikrobiologie, Johannes-Gutenberg-Universität, 65 Mainz, Germany. Dextran sulphates with different numbers of sulphate groups were kindly prepared by Dr R. Burger, Institut für Medizinische Mikrobiologie, Johannes-Gutenberg-Universtät, 65 Mainz, Germany.

Macrophage collection and culture

Mouse macrophages were obtained by peritoneal lavage of Swiss mice with 5 ml of M 199 containing 100 u/ml of penicillin and streptomycin and 10 iu/ml heparin. Five-millilitre aliquots of the peritoneal exudate cell suspension containing $0{\cdot}5–1{\cdot}0 \times 10^6$ cells/ml were distributed into 50-mm Petri dishes and incubated in a humidified atmosphere of 5% carbon dioxide and air at 37° for 1–2 h to allow attachement of adherent cells. Non-adherent cells were removed by washing four times with phosphate-buffered saline. After washing, the cells were normally cultured in M 199 containing 10% (v/v) swine serum. The serum was heated at 56° for 30 min before use. Cultures prepared in this way give a sheet of well-spread cells within 24 h.

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 & Higgins (1946).

β-galactosidase was assayed by the method of Conchie, Findlay & Levy (1959) using p-nitrophenyl-β-D-galactopyranoside as substrate.

N-acetyl-β-D-glucosaminidase was assayed by the method of Woolen, Hayworth & Walker (1961) using p-nitrophenyl-2-acetamido-2-β-D-glucopyranoside as substrate dissolved in 0·1 M acetate buffer pH 4·5.

Standard incubation mixture for C3 activation

Standard incubation mixture for C3 activation and assay for haemolytic C3 activation were described earlier by Bitter-Suermann, Hadding, Schorlemmar, Limbert & Dukor (1975). In brief it was performed as follows: equal volumes of either pooled normal guinea-pig serum or C4-deficient guinea-pig serum (C4-def GPS), frozen and stored at −80°, were incubated with appropriate dilutions of test materials for 30 min at 37° and cooled to 4° in an ice bath. The concentrations of the agents referred to in the text represent final concentrations in this standard incubation mixture (μg/ml). Controls were run with H_2O instead of test materials.

Assay for haemolytic C3 activity

Samples were withdrawn from the standard incubation mixture and subdiluted with veronal-buffered saline pH 7·5 (containing Ca^{2+} 10^{-3}M, Mg^{2+} 5×10^{-4}M and 1% gelatine) to a final serum concentration of 1:100, 1:500 and 1:1000. The amount of residual haemolytically active C3 (site-forming units; SFU) was determined and calculated as previously described, (Bitter-Suermann, Hadding, Melchert & Wellensieck, 1970). The values obtained were compared with that of the appropriate control and expressed as percentage of remaining haemolytically active C3. The C3 content of normal and C4-def GPS in the controls was in the range of $1–2 \times 10^{11}$ SFU C3/ml.

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.

RESULTS

The selective release of acid hydrolases from cultured macrophages by zymosan

Particles of zymosan are rapidly taken up by phagocytosis and can be seen inside cultured macrophages after 1 h incubation at 37°. The ingestion is followed by marked selective release of lysosomal hydrolases into the culture medium. This effect depends on the concentration of zymosan and the time of incubation. The time course of enzyme production and release was studied in mouse macrophage cultures maintained for various times up to 24 h in the presence of a single dose of 50 μg/ml of zymosan in a serum-free medium. The time dependence of the selective release of lysosomal enzymes caused by this stimulus is shown in Fig. 1. Macrophage cultures exposed to zymosan 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 h a highly significant increase ($P < 0·01$) 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 h approximately 80% of the total enzyme activity is found in the culture medium. In cultures grown in the presence of various concentrations of zymosan for a period of 24 h the total lysosomal enzyme activity was not changed; however there was a marked decrease in the intracellular acid hydrolase content with a reciprocal and concurrent increase in the extracellular enzyme activity in the culture media. After 24 h incubation in medium containing 25 μg/ml of zymosan the con-

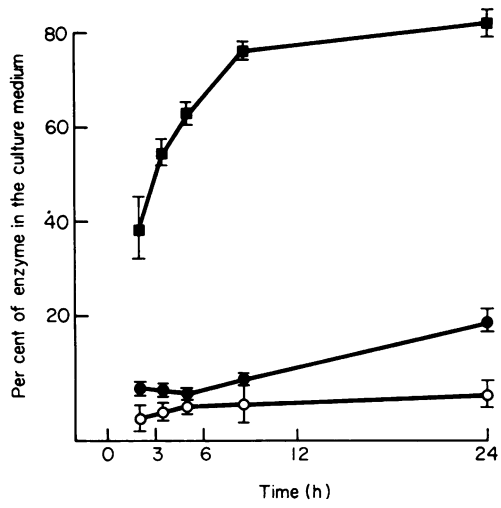

Figure 1. The time-dependent release of N-acetyl-β-D-glucosaminidase (■) and lactate dehydrogenase (○) from macrophages exposed to 50 μg/ml of zymosan cultivated in a serum-free medium. Controls show glucosaminidase release in the absence of added particles (●).

centration of lysosomal enzymes, e.g. β-glucuronidase in the medium is significantly greater than in the controls ($P < 0.01$). This dose-dependent release of enzymes rises up to about 80% with 200 μg/ml of zymosan. As in the experiments described, this dose- and time-dependent release occurs with no detectable loss of cellular lactate dehydrogenase into the culture medium, and there is no decrease of cell viability at 24 h, even in the absence of serum.

Induction by polyanions of macrophage hydrolase secretion

Marked changes in the levels and distribution of activities of acid hydrolases were induced in mouse macrophages by several polyanions e.g. dextran sulphate, polyvinyl sulphate, polyanetholsulphonate. Two types of experiments were performed. First the effect of various concentrations of polyanetholsulphonate on the levels and distribution of enzyme activity in macrophage cultures after 24 h was measured. Second the effect of a single concentration of polyanetholsulphonate on the time course of changes in enzyme level and distribution was determined. In macrophages cultured for 24 h in the presence of various concentrations of polyanetholsulphonate there was a dose-dependent,

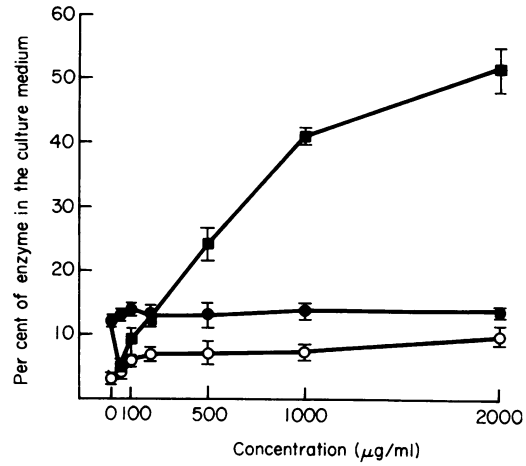

Figure 2. The release of β-glucuronidase (■) and lactate dehydrogenase (○) from macrophages after 24 h incubation with various concentrations of polyanethol sulphonate. Release of β-glucuronidase by heparin (●).

selective release of acid hydrolases from the cells into the culture medium (Fig. 2). This dose-dependent release of enzyme rises up to 51·6 ± 4·7% with 2000 μg/ml of polyanetholsulphonate. The release is demonstrable 6 h after contact with the stimulus and continues over the subsequent 18 h. At no time during the 24-h period is there a significant decrease in cellular lactate dehydrogenase levels in cultures exposed to polyanetholsulphonate compared to controls (Fig. 2). Polyanetholsulphonate at concentrations up to 2000 μg/ml causes no morphologically detectable cell death. As shown in Fig. 2 there is hardly detectable release of acid hydrolases after interaction of heparin with macrophages during an incubation time of 24 h; even 2000 μg/ml of heparin had absolutely no effect.

Effects of BCG on enzyme release from macrophages in culture

Mouse peritoneal macrophages exposed to BCG or *Corynebacterium parvum* show marked changes in the levels and distribution of acid hydrolase activities. The magnitude of these changes depends on the time of incubation and the concentration of the organisms used. In macrophage cultures grown in the presence of various concentrations of BCG for a period of 24 h, there was a dose-dependent, selective release of acid hydrolases from the cells into the culture medium with a reciprocal and concurrent decrease

in the intracellular acid hydrolase content; however the total lysosomal enzyme activity was not changed (Fig. 3). Exposure to concentrations of 1–25 μg/ml

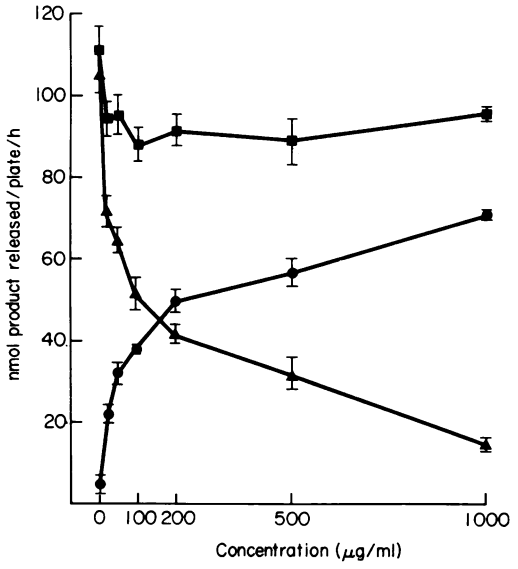

Figure 3. The dose-dependent effect of BCG on the levels of β-galactosidase activity in the total culture (■) in cells (▲) and in the medium (●). The cultures were assayed after 24 h incubation with the organism.

of *C. parvum* for a period of 24 h did not significantly alter the levels of N-acetyl-β-D-glucosaminidase in the culture medium, while in experiments carried out with concentrations of 50–1000 μg/ml there was a statistically significant increase ($P < 0.01$) of acid hydrolase activity in the medium after 24 h (Table 1). This dose-dependent increase in lysosomal enzyme activity was observed at concentrations up to 200 μg/ml of *C. parvum;* after having reached a maximum further increases in *C. parvum* diminished, and finally abolished, the inducing effect. In the dose range studied with BCG and *C. parvum* lactate dehydrogenase release into the culture medium was not detectable (Table 1).

Influence of carrageenan on enzyme release and complement activation

Recent experiments in our laboratory indicate that certain types of carrageenan induce intense chronic inflammatory lesions after i.m. injection in mice. The structure-activity relationships of various carrageenans have been examined. We have compared the ability of the different preparations of carrageenan to induce acid hydrolase secretion from cultured mouse peritoneal macrophages with their effect on the activation of the alternative pathway of the complement system. Native undegraded carrageenan (Gelcarin HMR, manufactured by Marine Colloids Inc., Rockland, Maine) induces the greatest increase in selective release of acid hydrolases. In contrast the calcium salt of carrageenan (obtained from Sigma Chemical Co., London) lacks the ability to induce selective release of acid hydrolases, while the potassium salt of carrageenan (Sigma) shows intermediate activity. Structure-activity relationships show that lambda-carrageenan, a linear polymer of D-galactose with α-1·3-linkages and sulphated on carbon-4 has the greatest activity. (Fig. 4). In macrophages cultured for 48 h in the presence of various concentrations of lambda-carrageenan there was a significant selective release of β-glucuronidase, a typical lysosomal hydrolase, which rises up to above 50% with 400

Table 1. Enzyme levels and distribution between cells and culture medium in macrophage cultures exposed to various concentrations of *C. parvum* for 24 h

Concentration of *C. parvum* (μg/ml)	N-acetyl-β-D-glucosaminidase nmol product/plate/h				Lactate dehydrogenase m u/plate			
	Cells	Medium	Total	Per cent in medium	Cells	Medium	Total	Per cent in medium
0	386·7±4·2	75·3±3·4	462·0±7·6	16·0±4·1	88·7±36·1	42·0±2·4	92·9±10·0	4·5±0·6
25	305·5±1·5	56·8±1·7	362·3±1·3	15·7±1·0	97·6±21·9	51·4±14·6	102·6±36·8	5·0±1·4
50	280·0±1·2	122·1±5·3	402·1±6·5	34·4±0·1	95·4±19·6	67·3±1·7	102·1±6·8	6·6±1·9
100	157·0±7·4	197·7±11·6	354·7±19·0	55·8±0·2	97·6±7·8	72·0±0	105·0±6·4	6·9±0·1
200	101·5±2·3	213·8±3·3	315·3±1·0	67·8±0·9	98·0±7·1	10·6±7·8	128·8±7·0	8·2±2·4
500	175·9±3·4	182·7±3·7	358·6±7·0	51·0±1·4	97·6±11·3	92·8±14·7	106·8±7·1	8·7±2·6
1000	220·1±7·9	154·6±1·7	374·7±6·3	41·3±1·1	96·0±9·8	12·8±4·6	128·8±14·8	9·9±1·2

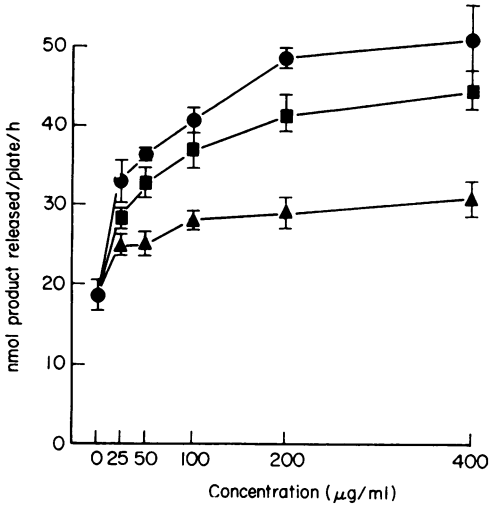

Figure 4. Effects of 48 h incubation with various concentrations of lambda-carrageenan (●), iota-carrageenan (■), kappa-carrageenan (▲) on the release of β-glucuronidase from macrophages into the culture medium.

μg/ml of lambda-carrageenan. Kappa-carrageenan, containing alternate units of 4-sulphated D-galactose and 3·6-dehydro-D-galactose with α-1·3 and β-1·4 linkages, does not induce release of β-glucuronidase from cultured macrophages. Iota-carrageenan shows intermediate activity in inducing release of acid hydrolases from mononuclear phagocytes. The release of the lysosomal enzymes was selective: lactate dehydrogenase levels in culture medium did not differ significantly from those in control cultures. Since the capacity of dextran sulphates to activate the alternative pathway of the complement system is dependent on the number of sulphate groups per molecule, we investigated the influence of the degree of substitution on the capacity of various carrageenans to activate the complement system. Varying amounts of lambda-carrageenan (9·6% sulphur), iota-carrageenan (8·9% sulphur) and kappa-carrageen (7·1% sulphur) were incubated with constant amounts of C4-deficient guinea-pig serum for 30 min at 37°. After the incubation, remaining haemolytic 3-activity was measured (Fig. 5). A dose-dependent C3-turnover was demonstrated; as shown in Fig. 5b the lambda-carrageenan was optimally active at a concentration of 40 μg/ml. A further increase in concentration led to a continuous decrease of the C3 turnover reaching at 600 μg/ml an almost complete inhibition of C3-consumption. The iota-carrageenan

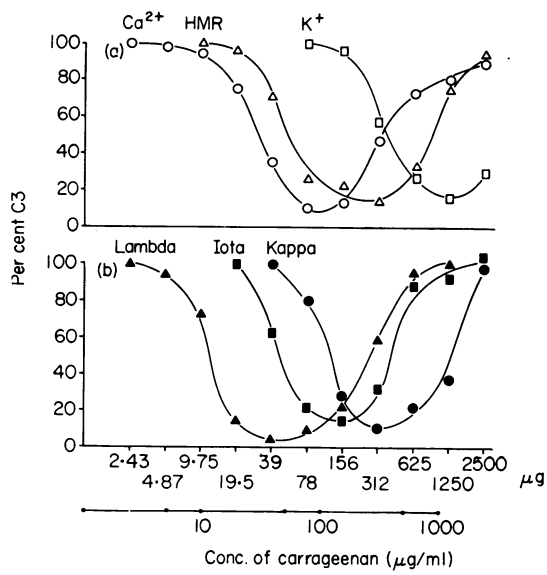

Figure 5. Dose-dependent C3-consumption by various purified preparations of carrageenan in C4-deficient guinea-pig serum. 100% refers to the H_2O-treated control and equals $2·2 \times 10^{11}$ SFU/ml C4-deficient guinea-pig serum.
(a) C3 consumption by various concentrations of the calcium salt of carrageenan (○), the native undegraded carrageenan, Gelcarin HMR (△) and the potassium salt of carrageenan (□). (b) The structure-activity relationships of various concentrations of lambda-carrageenan (9·6% sulphur, ▲), iota-carrageenan (8·9% sulphur, ■) and kappa-carrageenan (7·1% sulphur, ●).

had its optimum at 150 μg/ml and at higher concentrations showed the same phenomenon of inhibition. The kappa-carrageenan induced C3 turnover only at high concentrations with an optimum at 350 μg/ml. These findings demonstrated that each carrageenan preparation had a different optimum depending on the degree of sulphate substitution. In fact there is a striking parallelism between the capacity of carrageenans to activate the complement system and to induce selective lysosomal enzyme secretion from cultures of macrophages.

Effects of streptococcal A cell wall, dental plaque and Actinomyces viscosus on the activation of the alternative pathway of the complement system

Streptococcal A cell walls have been tested over a wide range of concentrations in the haemolytic assay using normal and C4-deficient guinea-pig serum. A significant C3 turnover could be induced in both

Table 2. C3 consumption by group A streptococcal cell wall in normal and C4 def GPS expressed as per cent C3 remaining after incubation

Strept. A cell wall (concentration μg/ml)	Normal guinea-pig serum	C4 deficient guinea-pig serum
10	100	93
20	100	84
80	96	71
310	86	55
625	66	48
1250	39	38
2500	28	34

sera (Table 2). The dose–response curve in C4-deficient guinea-pig serum was very similar to that obtained in normal serum. Hence, C3 activation must have occurred mainly via the alternative pathway of the complement system.

Various amounts of homogenates of dental plaque, a mixed bacteria growth on the sheltered areas of the teeth of humans and experimental animals and an active agent in dental plaque, *Actinomyces viscosus*, have been added to C4-deficient guinea-pig serum, resulting in a striking loss of C3-activity. As seen in Fig. 6 both preparations lead to a clear C3-consumption, although quantitative differences were observed. Increasing concentrations of dental plaque and *A. viscosus* induced increasing C3-consumption. Comparison of the two curves showed that the amount of dental plaque necessary for maximal C3-turnover in C4-deficient guinea-pig serum differed from that of *A. viscosus*; dental plaque required smaller amounts than *Actinomyces*. As can be seen the minimal dose required for 50% C3-consumption in C4-deficient guinea-pig serum is: dental plaque about 200 μg/ml, *A. viscosus* about 500 μg/ml.

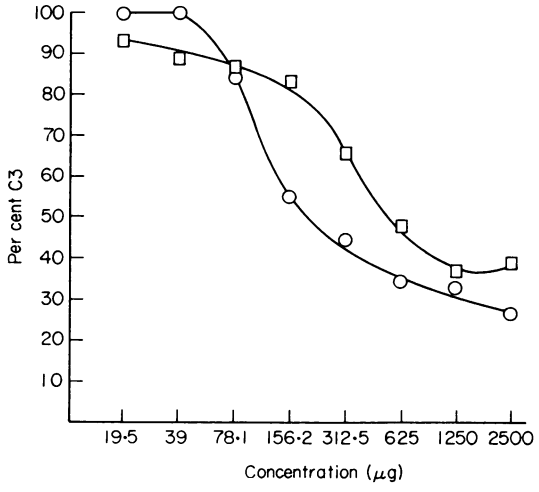

Figure 6. Dose-dependent C3-consumption by dental plaque (○) and *A. viscosus* (□). Experimental conditions were identical to those described in Fig. 5.

DISCUSSION

Many agents injected parenterally elicit chronic inflammatory reactions. Carrageenan, a mixture of sulphated D-galactose and 3·6-dehydro-D-galactose, induces a chronic granulomatous response in experimental animals such as guinea-pigs (Williams, 1957) and rats (Morris, Weinberg & Spector 1968). This model is widely used by experimental pharmacologists and pathologists. Recent experiments in our laboratory indicate that certain types of carrageenan also induce intense chronic inflammatory lesions after i.m. injection in mice (Davies, Allison, Dym & Cardella, 1976a). Because of the heterogeneous nature of carrageenan we prefer dextran sulphate which has a uniform polysaccharide structure and offers the chance of varying independently the degree of substitution with sulphate groups as well as the molecular size of the carrier. When we inject experimental animals with different amounts of dextran sulphate and after appropriate time intervals quantify the macrophage infiltrate by measurement of specific marker enzymes at the injection site, the dose-response curves show that the capacity of dextran sulphate to elicit inflammatory responses depends on the mol. wt and degree of sulphation (Schorlemmer, H.U., Gugig, M., Burger, R. and Allison, A.C. manuscript in preparation) in a manner which closely parallels the capacity of these compounds to activate complement by the alternative pathway (Burger *et al.*, 1975) and to induce secretion of hydrolases from macrophage cultures (Schorlemmer *et al.*, 1977b). A variety of other agents injected s.c. or i.m. elicit similar chronic inflammatory responses. Some of these are listed in Table 3.

The chronic inflammatory lesion characteristic of rheumatoid arthritis includes infiltration of the synovial connective tissues by mononuclear phago-

Table 3. *In vivo* and *in vitro* responses of macrophages to some activators of the alternative pathway of the complement system

Stimulus for activation or release	Response	Reference
A. viscosus	Enzyme secretion from macrophages	Page *et al.*, 1974
	Activation of the complement system	Schorlemmer *et al.*, 1977
	Chronic inflammatory periodontal disease in monoinfected germ-free rodents	Guggenheim & Schroeder, 1974
BCG	Enzyme secretion from macrophages	Schorlemmer *et al.*, 1977
	Activation of the complement system	
	Splenomegaly, increased immunity to parasites, virus and tumours	Clark, Allison & Cox, 1976 Juy & Chedid, 1975
Carrageenan	Enzyme secretion from macrophages	Davies & Allison, 1976 Schorlemmer *et al.* 1977
	Activation of the complement system	Davies, 1963 Schorlemmer *et al.* 1977
	Capacity to induce acute (oedema) and chronic inflammation (granuloma) in rats and guinea-pigs	Di Rosa, 1972
C parvum	Activation of the complement system	McBride, Weir, Kay, Pearce & Caldwell, 1975
	Enzyme secretion from macrophages	Schorlemmer *et al.*, 1977
	Splenomegaly, increased immunity to parasites, viruses, and tumours	Clark *et al.*, 1977 Kirchner, Holden & Herberman, 1975
Dental plaque	Enzyme secretion from macrophages	Page *et al.*, 1973
	Activation of the complement system	Schorlemmer *et al.*, 1977
	Chronic inflammatory periodontal disease	Page & Schroeder, 1976
Endotoxin	Enzyme secretion from macrophages	Allison, Davies & Page, 1973 Wahl, Wahl, Mergenhagen & Martin, 1974
	Activation of the complement system	Bitter-Suermann *et al.*, 1975 Dierich, Bitter-Suermann, König, Hadding, Galanos & Rietschel, 1973 Gerwurz, Mergenhagen, Nowotny & Phillips, 1968
	Macrophage stimulation, increased immunity against tumours	Evans & Alexander, 1972
Streptococcal cell walls	Enzyme secretion from macrophages	Davies *et al.*, 1974 Page *et al.*, 1974
	Activation of the complement system	Schorlemmer *et al.*, 1977
	Rheumatic fever, chronic inflammatory lesions	Ginsburg 1972 Page *et al.*, 1974
Mouldy hay dust	Activation of the complement system	Edwards, 1976
	Enzyme secretion from macrophages	Schorlemmer *et al.*, 1977b
	Pulmonary inflammation	Edwards *et al.*, 1976
Immune complexes	Enzyme secretion from macrophages	Cardella *et al.*, 1974
	Activation of the complement system	König *et al.*, 1973 Osler, Oliveira, Shin & Sandberg, 1969
	Rheumatoid arthritis, nephritis chronic inflammatory lesions	Bonamo, Tursi, Trizio, Gillardi & Damacco, 1970 Pincus, Haberkern & Christian, 1968 Spector & Heesom, 1969
Micropolyspora faeni	Activation of the complement system	Edwards, 1976
	Enzyme secretion from macrophages	Schorlemmer *et al.*, 1977
	Pulmonary inflammation	Wenzel *et al.*, 1965 Edwards *et al.*, 1976
Polyanions (Dextran sulphate; Polyanethol sulphonate)	Enzyme secretion from macrophages	Schorlemmer *et al.*, 1977 Schorlemmer *et al.*, 1977
	Activation of the complement system	Hadding *et al.*, 1973 Burger *et al.*, 1975
	Splenomegaly, chronic inflammation e.g. arthritis	Page Thomas, Cook & Ricketts, 1961
Zymosan	Enzyme secretion from macrophages	Schorlemmer *et al.*, 1977 Weismann *et al.*, 1971a
	Activation of the complement system	Pillemer, Schoenberg, Blum & Wurz, 1955 Brade, Lee, Nicholson, Shin & Mayer, 1973
	Chronic inflammatory reactions in mice and guinea-pigs e.g. arthritis	Keystone *et al.*, unpublished

cytes. The presence of immune complexes in these lesions is well documented (Ziff, 1973) and mononuclear phagocytes interact with certain kinds of immune complexes since they have receptors for the Fc region of antibody molecules (Hay, Torrigiani & Roitt, 1972). It is well known that immune complexes can induce the selective release of hydrolases from polymorphonuclear leucocytes (Henson, 1971; Weissmann, Zurier, Spieler & Goldstein, 1971) and mononuclear phagocytes (Cardella *et al.*, 1974). Group A streptococcal infections are associated with rheumatic fever which is still an important disease in some countries. Lesions like those in rheumatic fever in the heart, joints and other sites can be produced in experimental animals by, purified cell walls of Group A streptococci (PPG) (Page, Davies & Allison, 1974; Ohanion, Schwab & Cromartie, 1969; Ginsburg, 1972). These lesions do not appear to have an immunopathological origin, (Page *et al.*, 1974). Addition of small quantities of group A streptococcal cell wall preparations to cultures of macrophages induces marked hydrolase secretion (Davies *et al.*, 1974). The observations now presented show that group A streptococcal cell walls efficiently activates complement by the alternative pathway. This is consistent with a recent report that group A streptococcal cell walls and membranes activate complement by the alternative pathway (Tauber, Polley & Zabriskie, 1976). The activation required factor D but not properdin.

Periodontal disease is the commonest cause of tooth loss in human adults. The disease is caused by dental plaque, consisting largely of bacterial cell wall residues which accumulate between the teeth and the gingiva. Pure cultures of *A. viscosus* can reproduce the disease when used as single contaminants of germ-free rats (Guggenheim & Schroeder, 1974). Dental plaque in doses of 5–50 μg/ml causes a rapid and massive redistribution of acid hydrolases from mouse peritoneal macrophages into the culture medium (Page *et al.*, 1973). The experiments here reported show that dental plaque and *A. viscosus* efficiently activate complement by the alternative pathway.

Inhalation of mouldy hay dust produces the disease farmer's lung. The main causative organism is the thermophilic fungus *Micropolyspora faeni* (Wenzel, Emanuel, Lawton & Magnin, 1965). This disease can be reproduced by injecting *M. faeni* into the tracheas of experimental animals not previously sensitized to the antigens (Edwards *et al.*, 1974). Mouldy hay dust, *M. faeni* and fractions derived from this organism all induce the release of hydrolytic enzymes from macrophages in culture (Schorlemmer *et al.*, 1977a) and they are known as efficient activators of the complement system via the alternative pathway (Edwards, 1976). All the inducers of chronic inflammation listed in Table 3 were found to stimulate the secretion from cultured mouse peritoneal macrophages of acid hydrolases into the culture medium; the release is time- and dose-dependent. This is not a non-specific loss of enzymes from dying macrophages: the cells remain healthy and show no loss of the cytoplasmic enzyme lactate dehydrogenase.

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, as was in part suggested previously by Dukor, Dietrich, Gisler, Schumann & Bitter-Suermann (1974). 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). Of special interest is the finding that cathepsin G which is abundant in stimulated macrophages efficiently cleaves factor B of the alternative pathway at physiological pH (Bitter-Suermann, D., Schorlemmer, H.U., Hadding, U., Barrett & Allison, A.C. manuscript in preparation). It is remarkable that Factor B, C3 and cathepsins are all synthesized by macrophages (Bentley, Bitter-Suermann, Hadding & Brade, 1976; Lai Fat & van Furth, 1975) and together comprise a self-activating system, (Fig. 7 and Bitter-Suermann, Burger, Brade & Hadding, 1977). Hence the system for activating macrophages by agents inducing chronic inflammation is produced by the macrophages themselves and does not require serum constitutents. This could be highly relevant to the pathogensis of chronic inflammation in lesions containing large numbers of macrophages. These could form a self-activating system generating chemotactic factors, which recruit more mononuclear phagocytes in the lesions and stimulate the secretion of enzymes which are themselves involved in the activating process. The secreted enzymes could also be important in the degradation of articular cartilage, proteogylcan, collagen and other connective tissue constituents.

Another feature of chronic inflammatory lesions

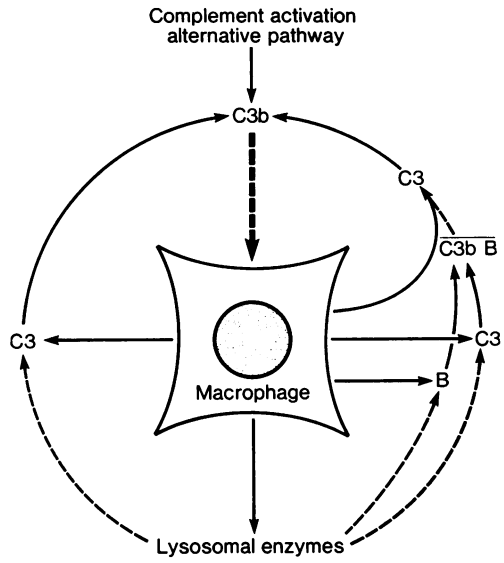

Figure 7. Possible amplification loops in the activation process of mononuclear phagocytes.

is fibrogenesis. Evidence has been presented that this occurs by a two-stage mechanism: activated macrophages secrete a factor or factors which stimulate proliferation and/or collagen synthesis by macrophages (Allison, Clark & Davies, 1977). Since C3b induces enzyme secretion from macrophages it may also induce secretion of fibrogenic factors, but this has not yet been demonstrated.

All of the agents which have been discussed induce enzyme secretion from cultures of macrophages, and it seems likely that the inflammatory responses which they elicit *in vivo* can occur without the participation of acquired immune responses. However, immune responses mediated by T and B lymphocytes appear to play a role in the pathogenesis of other chronic inflammatory reactions, e.g. that occurring around a schistosome egg (Warren, 1972) and where immune complexes in antibody excess are generated (Spector & Heesom, 1969).

ACKNOWLEDGMENTS

This work was supported by a grant (Scho 215/1) of the Deutsche Forschungsgemeinschaft, Bad Godesberg, Germany and by grants of the SFB 107 Mainz, Germany.

REFERENCES

Allison A.C., Clark I.A. & Davies P. (1977) Cellular interactions in fibrogenesis. *Ann. rheum. Dis.* (In press.)

Allison A.C., Davies P. & Page R.C. (1973) Effects of endotoxin on macrophages and other lymphoreticular cells. *J. infect. Dis.* **128**, 2125.

Bentley C., Bitter-Suermann D., Hadding U. & Brade V. (1977) *In vitro* synthesis of factor B of the alternative pathway of complement activation by mouse peritoneal macrophages. *Europ. J. Immunol.* **6**, 393.

Bitter-Suermann D., Burger R., Brade V. & Hadding U. (1977) Mouse factor B of the alternative pathway of complement activation *J. Immunol.* (In press.)

Bitter-Suermann D., Hadding U., Schorlemmer H.U., Limbert M. & Dukor P. (1975) Activation by some T-dependent antigens and B-cell mitogens of the alternative pathway of the complement system. *J. Immunol.* **115**, 425.

Bitter-Suermann D., Hadding U., Melchert F. & Wellensieck H.J. (1970) Independent and consecutive action of the complement components C5, C6 and C7 in immune hemolysis. I. Preparation of EAC 1–5 with purified guinea pig C3 and C5. *Immunochemistry*, **7**, 955.

Bonamo L., Tursi A., Trizio D., Gillardi U. & Damacco F. (1970) Immune complexes in rheumatoid synovitis: a mixed staining immunofluorescence study. *Immunology*, **18**, 557.

Brade V., Lee G.D., Nicholson A., Shin H.S. & Mayer M.M. (1973) The reaction of zymosan with properdin system in normal and C4-deficient guinea pig serum. *J. Immunol.* **111**, 1389.

Burger R., Hadding U., Schorlemmer H.U., Brade V. & Bitter-Suermann D. (1975). Dextran sulphate a synthetic activator of C3 via the alternative pathway I. Influence of molecular size and degree of sulphation on the activation potency. *Immunology*, **29**, 549.

Cardella C.J., Davies P. & Allison A.C. (1974) Immune complexes induce selective release of lysosomal hydrolases from macrophages. *Nature* (*Lond.*), **247**, 46.

Clark I.A., Cox F.E. & Allison A.C. (1977) Protection of mice against Babesia and Plasmodium with killed Corynebacterium parvum. *Parasitology*, (In press.)

Clark I.A., Allison A.C. & Cox F.E. (1976a) Protection of mice against Babesia and Plasmodium with BCG. *Nature* (*Lond.*), **259**, 309.

Conchie J., Findlay J. & Levy G.A. (1959) Mammalian glycosidases. Distribution in the body. *Biochem. J.* **71**, 318.

Davies G.E. (1963) Inhibition of guinea-pig complement *in vitro* and *in vivo* by carrageenan. *Immunology*, **6**, 561.

Davies P. & Allison A.C. (1976) Secretion of macrophage enzymes in relation to the pathogenesis of chronic inflammation. *Immunobiology of the Macrophage*, (ed. by D.S. Nelson), p. 427, Academic Press Incorporated, New York, San Francisco, London.

Davies P., Allison A.C., Dym M. & Cardella C. (1976a) The selective release of lysosomal enzymes from mononuclear phagocytes by immune complexes and other materials causing chronic inflammation. *Infection and Immunology in the Rheumatic Diseases* (ed. by D.C.

Dumonde) p. 365, Blackwell Scientific Publications, Oxford.

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.

DIERICH M.P., BITTER-SUERMANN D., KÖNIG W., HADDING U., GALANOS C. & RIETSCHEL E.T. (1973) Analysis of bypass activation of C3 by endotoxic LPS and loss of this potency. *Immunology*, **24,** 721.

DIROSA M. (1972) Biological properties of carrageenan. *J. pharm. Pharmac.* **24,** 89.

DUKOR P., DIETRICH F.M., GISLER R.H., SCHUMANN G. & BITTER-SUERMANN D. (1974) Possible targets of complement action in B-cell triggering. *Progress in Immunology II*, volume 3, (ed. by L. Brent and J. Holborow) p. 99. North-Holland Publishing Company, Amsterdam.

EDWARDS J.H. (1976) A quantitative study on the activation of the alternative pathway of complement by mouldy hay dust and thermophilic actinomycetes. *Clin. Allergy*, **6,** 19.

EDWARDS J.H., WAGNER J.C. & SEAL R.M.E. (1976) Pulmonary responses to particulate materials capable of activating the alternative pathway of complement. *Clin. Allergy* **6,** 155.

EDWARDS J.H. (1974) Experimental immunopathology with specific antigen fractions. *Aspergillosis and farmer's lung in man and animals* (ed. by R. de Haller and F. Suter), Hans Huber, Vienna.

EDWARDS J.H., BAKER J.T. & DAVIES B.H. (1974) Precipitin test negative farmer's lung-activation of the alternative pathway of complement by mouldy hay dusts. *Clin. Allergy*, **4,** 379.

EVANS R. & ALEXANDER P. (1972) Mechanism of immunologically specific killing of tumor cells by macrophages. *Nature (Lond.)*, **236,** 168.

GERWURZ H., MERGENHAGEN S.E., NOWOTNY A. & PHILLIPS J.K. (1968) Interaction of the complement system with native and chemically modified endotoxins. *J. Bact.* **95,** 397.

GINSBURG J. (1972) Mechanisms of cell and tissue injury induced by group A streptocci: relation of poststreptococcal sequelae. *J. infect. Dis.* **126,** 294.

GOLDSTEIN I.M. & WEISSMANN G. (1974) Generation of C5-derived lysosomal enzyme-releasing activity (C5a) by lysates of leukocyte lysosomes. *J. Immunol.* **113,** 1583.

GUGGENHEIM B. & SCHROEDER H.E. (1974) Reactions in the periodonteum to continuous antigenic stimulation in gnotobiotic rats. *Infect. Immunol.* **10,** 565.

HADDING U., DIERICH M., KÖNIG W., LIMBERT M., SCHORLEMMER H.U. & BITTER-SUERMANN D. (1973) Ability of the T-cell replacing polyanion Dextran to trigger the alternate pathway of complement activation. *Europ. J. Immunol.* **3,** 527.

HAY F.C., TORRIGIANI G. & ROITT I.M. (1972) The binding of human IgG subclasses to human monocytes. *Europ. J. Immunol.* **2,** 257.

HENSON P.M. (1971) Interaction of cells with immune complexes, adherence release of constituents and tissue injury. *J. exp. Med.* **134,** 1145.

JUY D. & CHEDID L. (1975) Comparison between macrophage activation and enhancement of nonspecific resistance to tumors by mycobacterial immunoadjuvants. *Proc. nat. Acad. Sci. (Wash.)*, **72,** 4105.

KIRCHNER H., HOLDEN H.T. & HERBERMAN R.B. (1975) Inhibition of *in vitro* growth of lymphoma cells by macrophages from tumour-bearing mice. *J. nat. Cancer Inst.* **55,** 971.

KÖNIG W., BITTER-SUERMANN D., DIERICH M. & HADDING U. (1973) Bypass-activation of the complement system starting with C3. II. C3 activation by gamma-1-immune aggregates in guinea pig serum. *Immunochemistry*, **10,** 431.

LAI A. FAT R.F.M. & VAN FURTH R. (1975) *In vitro* synthesis of some complement components (C1q, C3 and C4) by lymphoid tissues and circulating leucocytes in man. *Immunology*, **28,** 359.

MCBRIDE W.H., WEIR D.M., KAY A.B., PEARCE D. & CALDWELL J.R. (1975) Activation of the classical and alternate pathway of complement by *Corynebacterium parvum. Clin. exp. Immunol.* **19,** 143.

MORRIS B., WEINBERG T. & SPECTOR G.J. (1968) The carrageenan granulomas in the rat. A model for the study of the structure and function of macrophages. *Brit. J. exp. Pathology*, **49,** 302.

OHANIAN S.H., SCHWAB J.H. & CROMARTIE W.J. (1969) Relation of rheumatic like cardiac lesions of the mouse to localization of group A streptococcal cell walls. *J. exp. Med.* **129,** 37.

OSLER A.G., OLIVEIRA B., SHIN H.S. & SANDBERG A.L. (1969) The fixation of guinea pig complement by γ 1 and γ 2 immunoglobulins. *J. Immunol.* **102,** 269.

PAGE R.C. & SCHROEDER H.E. (1976) Pathogenesis of inflammatory periodontal disease. *Lab. Invest.* **33,** 236.

PAGE R.C., DAVIES P. & ALLISON A.C. (1974) Pathogenesis of the chronic inflammatory lesions induced by group A streptococcal cell walls. *Lab. Invest.* **39,** 568.

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.

PAGE THOMAS D.P., COOK F.R. & RICKETTS C. (1961) Polysaccharide induced proliferative synovitis. *Communicazione al X. Congresso della Lega Internationale control il Rheumatismo* Volume II, p. 948. Turin.

PILLEMER L., SCHOENBERG M.D., BLUM L. & WURZ L. (1955) Properdin system and immunity. II. Interaction of the properdin system with polysaccharides. *Science*, **122,** 545.

PINCUS T., HABERKERN R. & CHRISTIAN C.L. (1968) Experimental chronic glomerulitis. *J. exp. Med.* **127,** 819.

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., EDWARDS J.H., DAVIES P. & ALLISON A.C. (1977a) Macrophage responses to mouldy hay dust, Micropolyspora faeni and zymosan, activators of complement by the alternative pathway. *Clin. exp. Immunol.* (In press.)

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.* (In press.)

SPECTOR W.G. & HEESOM N. (1969) The production of granulomata by antigen-antibody complexes. *J. Path.* **98,** 31.

TALALAY P., FISHMAN W.M. & HUGGINS C. (1946) Chromiogenic substrates II. Phenolphthalein glucuronic acid as substrate for the assay of glucuronidase activity. *J. biol. Chem.* **166,** 757.

TAUBER J.W., POLLEY M.J. & ZABRISKIE J.B. (1976) Non-specific complement activation by streptococcal structures II. Properdin-independent initiation of the alternative pathway. *J. exp. Med.* **143,** 1352.

WAHL L.M., WAHL S.M., MERGENHAGEN S.E. & MARTIN G.R. (1974) Collagenase production by endotoxin-activated macrophages. *Proc. nat. Acad. Sci. (Wash.),* **71,** 3598.

WARREN K.S. (1972) The immunopathogenesis of schistosomiasis: a multidisciplinary approach. *Trans. roy. Soc. trop. Med. Hyg.* **66,** 417.

WEISSMANN G., ZURIER R.B., SPIELER P.T. & GOLDSTEIN I.M. (1971) Mechanism of lysosomal enzyme release from leukocytes exposed to immune complexes and other particles. *J. exp. Med.* **134,** 1495.

WEISSMANN G., DUKOR P. & ZURIER R.B. (1971a) Effect of cyclic AMP on release of lysosomal enzymes from phagocytes. *Nature: New Biology,* **231,** 131.

WENZEL F., EMANUEL D.A., LAWTON B.R. & MAGNIN E.G. (1965) Isolation of the causative agent of farmer's lung. *Annals of Allergy,* **22,** 533.

WILLIAMS G. (1957) A histological study of the connective tissue reaction to carrageenan. *J. Path. Bact.* **73,** 557.

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

ZABRISKIE J.B. (1976) Rheumatic fever: a streptococcal induced autoimmune disease? *Infection and Immunology in the Rheumatic Diseases* (ed. by D.C. Dumonde) p. 97, Blackwell Scientific Publications, Oxford.

ZIFF M. (1973) Pathophysiology of rheumatoid arthritis. *Fed. Proc.* **32,** 131.