Br. J. exp. Path. (1977) **58**, 315

THE SELECTIVE RELEASE OF LYSOSOMAL ACID HYDROLASES FROM MOUSE PERITONEAL MACROPHAGES BY STIMULI OF CHRONIC INFLAMMATION

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Received for publication February 3, 1976

Summary.—The parallelism between the capacity of various agents to elicit chronic inflammatory responses *in vivo* and to induce the selective release of lysosomal enzymes from cultures of mouse peritoneal macrophages *in vitro* is discussed.

Zymosan elicits an intense inflammatory response when injected i.m. in mice. Chrysotile asbestos produces a response of a similar nature and intensity as is seen with zymosan, while injections of acid-leached asbestos and polystyrene latex are not followed by inflammation.

It is also shown that zymosan and asbestos induce a dose-dependent increase in the total enzyme activity of an inflamed muscle. On the other hand latex and acid-leached asbestos caused no significant increases in lysosomal enzyme levels.

Agents eliciting inflammatory responses, such as zymosan and chrysotile asbestos induce a selective release of acid hydrolases from cultured macrophages; in contrast agents lacking the capacity to induce inflammation, such as latex and acid-leached chrysotile asbestos, do not induce the release of lysosomal enzymes from cultured macrophages.

STUDIES in this and other laboratories over the last few years have established that mouse peritoneal macrophages maintained in culture release a large proportion of their lysosomal hydrolases when exposed to a variety of stimuli known to elicit chronic inflammatory responses (Page, Davies and Allison, 1973; Cardella, Davies and Allison, 1974; Davies, Page and Allison, 1974*a*; Davies *et al.*, 1974*b*; Page, Davies and Allison, 1974; Pantalone and Page, 1975; Schorlemmer and Allison, 1976; Schorlemmer, Davies and Allison, 1976*a*; Schorlemmer *et al.*, 1977*b*; Schorlemmer *et al.*, 1977).

The release is selective, the cells remaining viable as judged by a number of criteria. Indeed under certain conditons cells exposed to inflammatory stimuli show higher levels of nonlysosomal enzymes such as lactate dehydrogenase and leucine-2-naphthylamidase, suggesting an enhanced protein synthetic activity. It is also clear that selective release of lysosomal enzymes does not occur when macrophages are exposed to stimuli which are not inflammatory (Axline and Cohn, 1970; Davies *et al.*, 1974*a*). In this paper we illustrate by means of *in vitro* and *in vivo* studies a close correlation between the capacity of stimuli to elicit chronic inflammatory lesions when injected i.m. in mice and to induce the selective release of lysosomal enzymes from mouse peritoneal macrophages maintained in culture. For this purpose we have used zymosan, known to be inflammatory *in vivo* (Edwards, Wagner and Seal, 1976) and

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latex, which produces little or no inflammation *in vivo*. It is known that removal of the surface magnesium groups from chrysotile asbestos diminishes its haemolytic potency (Morgan, 1975). We now show that this treatment also diminishes greatly the ability of this type of asbestos to elicit inflammation and selective release of lysosomal enzymes.

MATERIAL 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, U.K. and M199 from Burroughs Wellcome, Beckenham, Kent, U.K., and swine serum from Bio Cult Laboratories Ltd, Glasgow, Scotland.

Biochemical reagents.—Bovine serum albumin, penicillin, streptomycin, phenolphthalein glucuronic acid 0·01M, pH 7·0, and zymosan prepared from *S. cerevisiae* yeast were from Sigma Chemical Co., Surbiton, Surrey, U.K.; polystyrene latex particles (0·81 μm diameter) were from Difco, U.S.A., *p*-nitrophenyl-β-D-galactopyranoside and *p*-nitrophenyl-2-acetamido-β-D-deoxyglucopyranoside from Koch-Light Laboratories, Colnbrook, Bucks, U.K., heparin, preservative-free, from Boots, Nottingham, U.K., pyruvate and nicotinamide adenine dinucleotide from Boehringer Mannheim Gmbh, Germany, and Triton X-100 from British Drug Houses Ltd, Poole, Dorset, U.K.

Macrophage collection and culture.—Swiss mice were inoculated i.p. with 2 ml proteose-peptone 3 days previously and macrophages were obtained by peritoneal lavage with 5 ml M199 containing 100 u/ml of penicillin and streptomycin and 10 i.u./ml heparin. Samples 5-ml 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 air at 37° for 1–2 h to allow attachment of adherent cells. Non-adherent cells were removed by washing × 4 with phosphate-buffered saline. After washing, the cells were cultured in M199 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 washed once with phosphate-buffered saline. The cells were then 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.

β-glucuronidase was assayed by the method of Talalay, Fishman and Huggins (1946).

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

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

Preparation of inflammatory stimuli.—Zymosan was suspended in phosphate-buffered saline, boiled 3× for 10 min in a water-bath and resuspended in a volume of vehicle required to give appropriate concentrations of the particle. Polystyrene latex was washed 4× in phosphate-buffered saline before addition to the medium.

Chrysotile asbestos (UICC standard reference sample of Rhodesian chrysotile) was used as a starting material in all experiments. We are grateful to Mr A. Morgan and Mr A. Holmes (Health Physics and Medical Division, Atomic Energy Research Establishment, Harwell, Didcot, Berks) for supplying us with acid-treated chrysotile asbestos and a water-treated control sample prepared by methods described by Morgan, Lally and Holmes (1973). A full account of the chemical composition and some of the biological properties of these samples will be published elsewhere. The acid-treated chrysotile asbestos was leached with 1M hydrochloric acid for 432 h while another sample serving as a non-leached control was suspended in distilled water for 625 h. Calculations based on an initial magnesium content of 25% showed that acid treatment removed 92·8% of magnesium, while water treatment removed only 0·53% of magnesium. After treatment both asbestos samples were washed thoroughly with phosphate-buffered saline and then with distilled water; the washed samples were then lyophilized. All weights of asbestos mentioned in the results section refer to the lyophilized samples of water and acid-treated asbestos.

Induction of chronic inflammation in vivo.—The material for injection was suspended at

the appropriate concentration in phosphate-buffered saline and 0·1 ml injected into the right hamstring muscle of mice. An equal volume of the vehicle was injected into the contralateral muscle. After 7 days animals were killed and whole muscle removed for homogenization or fixation for microscopical examination. Homogenization was carried out by dispersion with a MSE homogenizer at top speed for 2 min in a total added volume of 5 ml phosphate-buffered saline containing 0·1% Triton X-100. Samples were centrifuged at 1000 rev/min for 10 min and supernatants retained for enzyme assays. This procedure was adopted after showing that the combined effects of the detergent and the disruptive procedure were effective in releasing completely the acid hydrolases of the muscle tissue.

RESULTS

The inflammatory capacity of latex, zymosan, water and acid-treated asbestos in vivo

Both histological examination and biochemical measurement on excised tissue were performed 7 days after injection of stimuli, after establishing that this time was close to optimal for demonstrating a chronic inflammatory response by morphological and biochemical criteria. Fig. 1 compares the response to latex and zymosan in the hamstring muscle of mice. It is clear (Fig. 1a) that latex does not elicit inflammation; the muscle tissue appears normal with no convincing evidence of the presence of infiltrating inflammatory cells. In contrast zymosan causes an intense inflammatory response in the muscle. This is evident from Fig. 1b which reveals a dense infiltrate of inflammatory cells displacing muscle fibres that show some degeneration. The inflammatory cells which infiltrate between muscle fibres are seen to be large cells with abundant vacuolated cytoplasm (Fig. 1c) characteristic of macrophages. A similar contrast in morphological response was observed in muscle tissue injected with acid-leached and water-treated chrysotile asbestos (Fig. 2). The unleached asbestos elicits an inflammatory response of a similar nature and intensity (Fig. 2a) as is seen with zymosan, while the acid-leached material induces only a low grade response consisting of a few inflammatory cells (Fig. 2b). Little or no muscle damage is observed.

Biochemical estimation of inflammatory responses to zymosan, latex, acid-leached and water-treated chrysotile asbestos

Lysosomal enzyme activity in muscle is very low (Canonico and Bird, 1970), the specific activity of various acid hydrolases being several orders of magnitude less than that seen in macrophages. We have taken advantage of this large quantitative difference between macrophages and muscle tissue and used detectable increases in lysosomal enzyme activity as an indicator of infiltration by a small number of macrophages. This allows measurement of total infiltration when the overall enzymic content of an inflamed muscle is determined.

Fig. 3 compares the activity of N-acetyl-β-G-glucosaminidase in control muscle injected with vehicle only with a series of muscle samples from animals injected with increasing amounts of latex or zymosan. It is clear that injection of zymosan results in a dose-dependent increase in total lysosomal enzyme activity, a maximum being observed at 400 μg/muscle. On the other hand, injections of latex particles did not result in any significant increase in the activity of N-acetyl-β-D-glucosaminidase at any of several doses up to 400 μg/muscle (Fig. 3). Similar differences were observed with acid-leached and water-treated asbestos (Fig. 4). The water-treated asbestos gave a dose-dependent increase in lysosomal enzyme content, β-glucuronidase in this instance, while injection of acid-leached asbestos did not result in significant increases of lysosomal enzyme levels in treated muscles at any of the dose levels used (50–400 μg). These biochemical measurements (Figs. 3 and 4) provide quantitative confirmation of the histological findings illustrated in Figs 1 and 2.

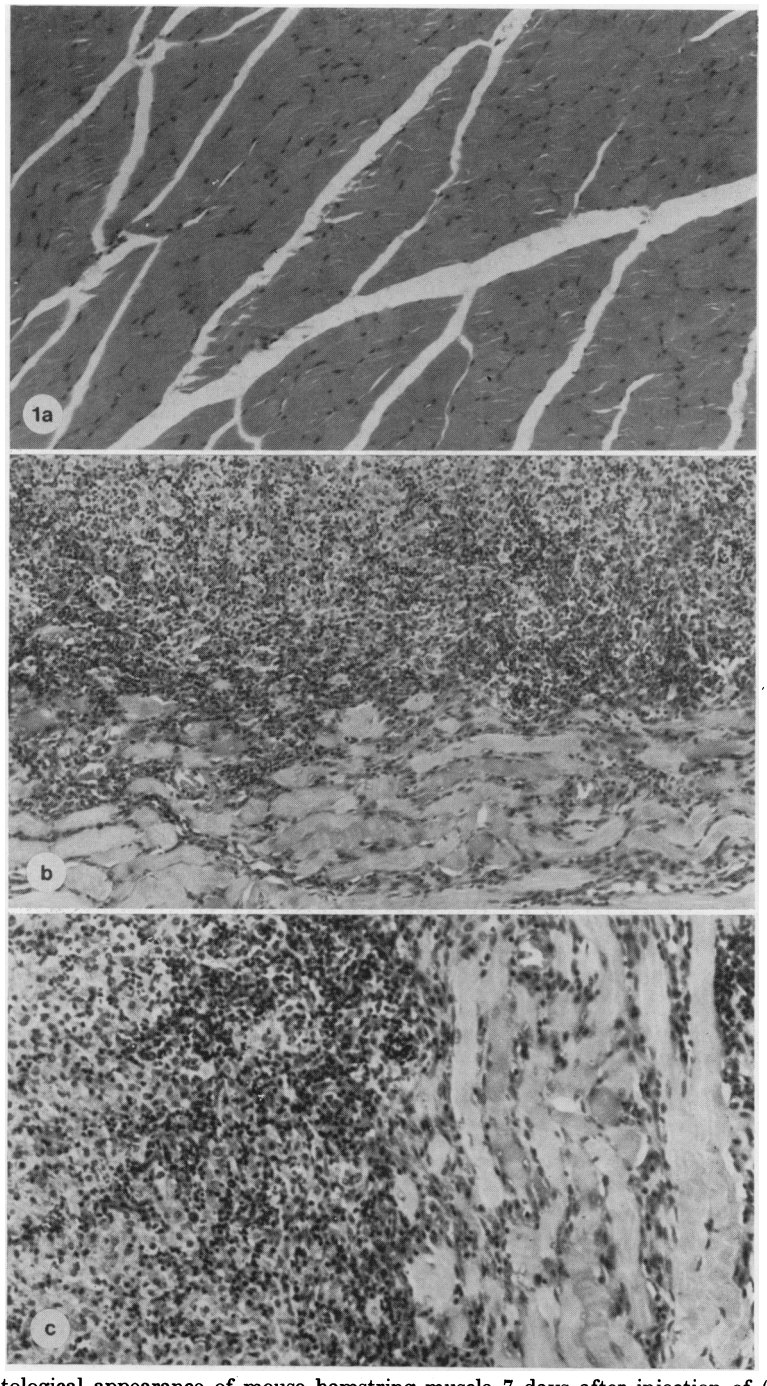

FIG. 1.—Histological appearance of mouse hamstring muscle 7 days after injection of (a) 200 μg latex, (b) 200 μg zymosan ($\times$ 90). (c) Shows details of the morphology of infiltrating macrophages in the zymosan lesion ($\times$ 145).

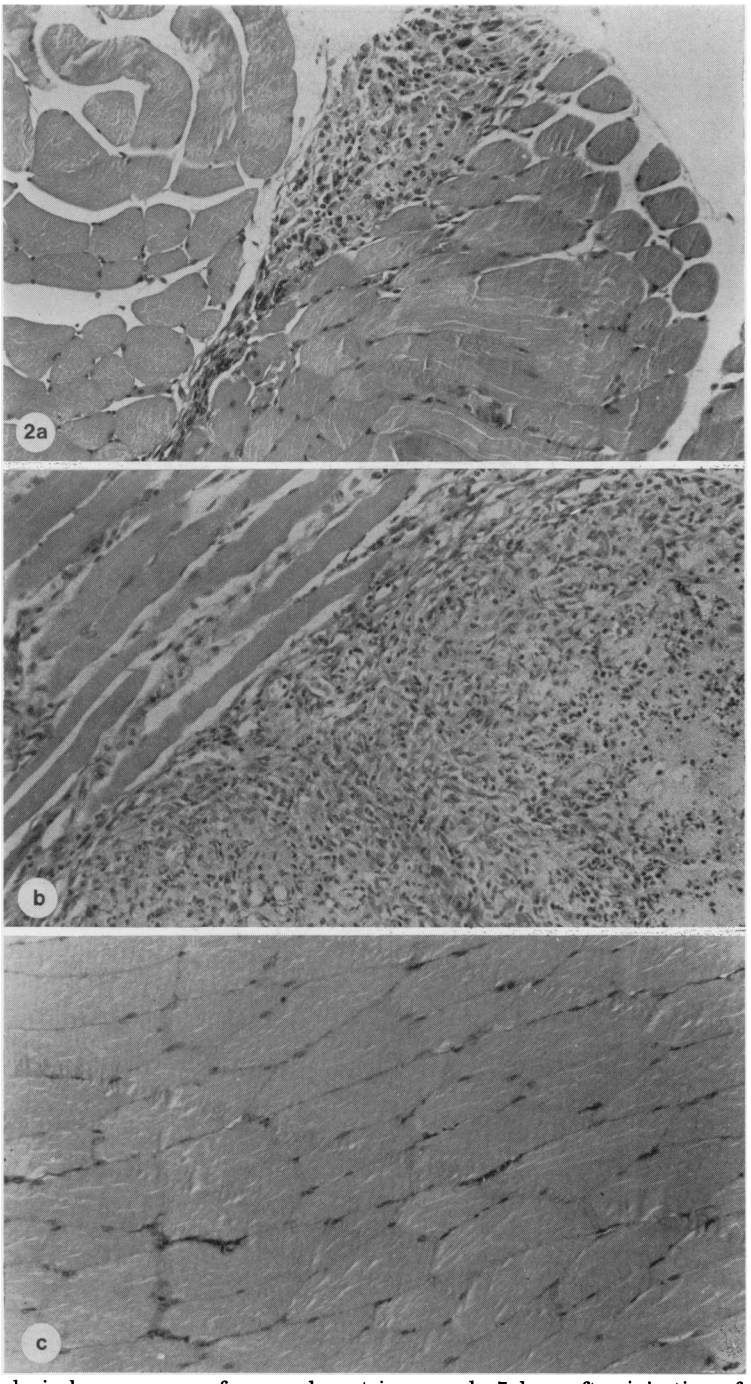

FIG. 2.—Histological appearance of mouse hamstring muscle 7 days after injection of 400 μg (a) acid-leached chrysotile asbestos and (b) water-treated chrysotile asbestos ($\times$ 145). (c) Into the control muscle 0·1 ml of phosphate-buffered saline was injected ($\times$ 145).

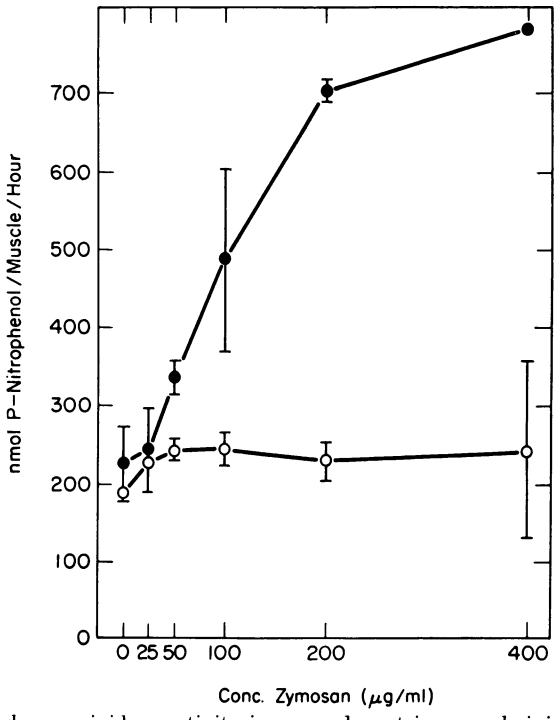

FIG. 3.—N-acetyl-β-D-glucosaminidase activity in mouse hamstring muscle injected with increasing amounts of latex (○——○) or zymosan (●——●) in 0·1 ml phosphate-buffered saline. Animals not receiving any latex or zymosan were injected with 0·1 ml of vehicle.

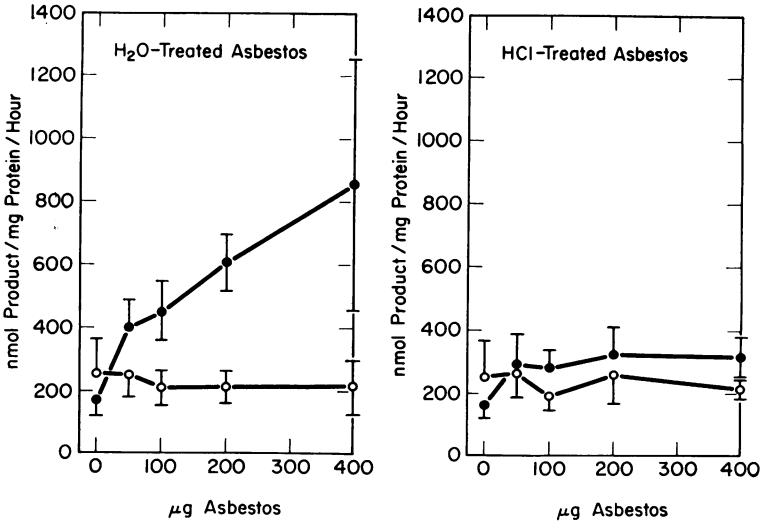

FIG. 4.—N-acetyl-β-D-glucosaminidase activity in mouse hamstring muscle injected with increasing amounts of acid-leached chrysotile asbestos (●——●) or water-treated asbestos (●——●) in 0·1 ml phosphate-buffered saline. Animals were injected with 0·1 ml of vehicle (○——○) in the contralateral hamstring muscle.

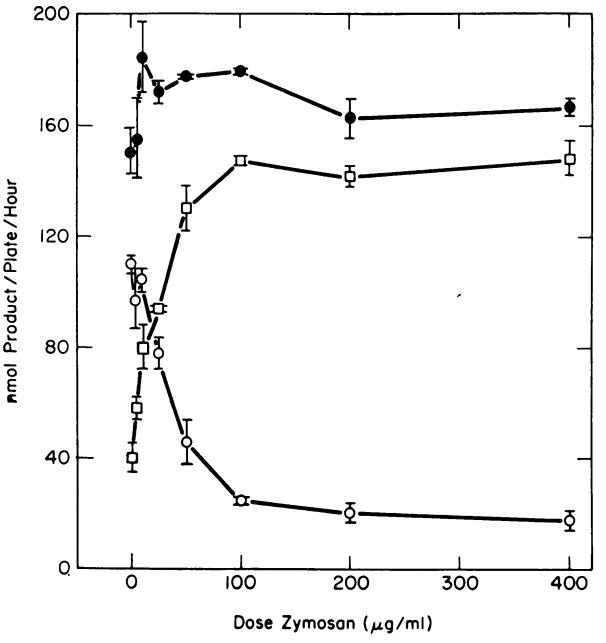

FIG. 5.—The release of β-glucuronidase from mouse peritoneal macrophages exposed to increasing amounts of zymosan for 24 h. (●——●) indicates total enzyme activity in the culture, (○——○) cellular levels of enzyme and (□——□) enzyme activity in the culture medium.

The capacity of latex, zymosan, water and acid-treated chrysotile asbestos to induce selective release of acid hydrolases from mouse peritoneal macrophages in culture

In vitro studies with cultures of mouse peritoneal macrophages were designed to examine the capacity of stimuli with inflammatory properties, such as zymosan and chrysotile asbestos, to induce selective release of acid hydrolases. These responses were then compared to those obtained with stimuli lacking inflammatory properties, such as latex and acid-leached chrysotile asbestos.

Fig. 5 shows that in the presence of zymosan there is a major redistribution of lysosomal enzymes from cells into their culture medium over a 24-h period. The release is seen to be dose-dependent, maximal release being observed with 100 μg/ml zymosan. In separate experiments, it was shown that the selective release of acid hydrolases was also time-dependent, with the majority of enzyme release occurring within 12 h of adding zymosan to the cultures. In contrast latex does not cause any release of acid hydrolyases from macrophages over a 24-h period at concentrations up to 1000 μg/ml (Fig. 6).

That the release of lysosomal enzymes from macrophages induced by zymosan is shown in Fig. 7. It is clear that none of the doses of zymosan used result in increases in the activity of the cytoplasmic enzyme lactate dehydrogenase in the culture medium (Fig. 7). In fact, in the presence of zymosan slight increases in cellular levels of lactate dehydrogenase are observed, a further indication of their viability despite the selective release of a large proportion of their lysosomal enzymes. Fig. 7 also shows that latex does not induce any detectable release of cellular lactate dehydrogenase at concentrations up to 1000 μg/ml.

Fig. 8 compares the extent of the selective release of a representative lysosomal enzyme, β-galactosidase, from

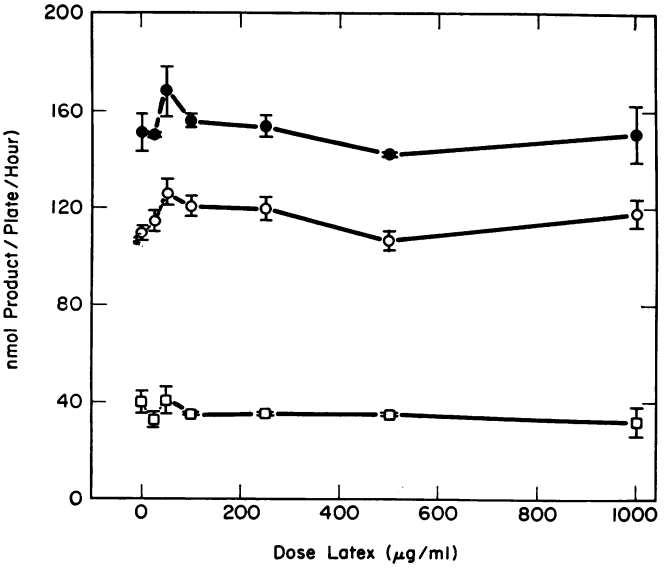

FIG. 6.—β-glucuronidase activity in cultures of mouse peritoneal macrophages exposed to latex in increasing amounts up to 1000 μg/ml. (●——●) indicates total activity in the culture, (○——○) cellular levels of enzyme and (□——□) enzyme activity in the culture medium.

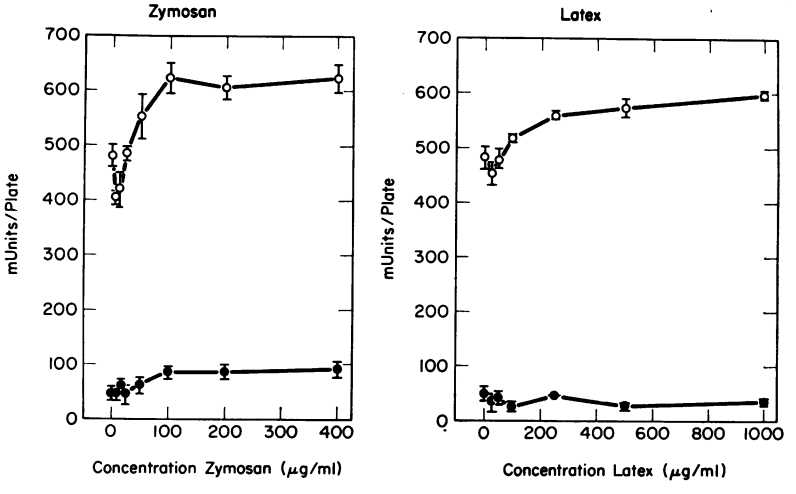

FIG. 7.—Lactate dehydrogenase in cultures of mouse peritoneal macrophages exposed to increasing amounts of latex or zymosan for 24 h. (○——○) indicates enzyme activity in cells and (●——●) enzyme activity in culture medium.

macrophages exposed to acid-leached and water-treated chrysotile asbestos. The water-treated asbestos induces a dose-dependent increase in the amount of lysosomal enzyme in the culture medium. This level increases from a baseline amount of approximately 10% of total culture activity to nearly 50% in the presence of 50 μg/ml of water-treated chrysotile asbestos. In contrast the acid-leached asbestos increased lysosomal enzyme release into the culture medium from the baseline amount of approximately 10% to only 20% of the total

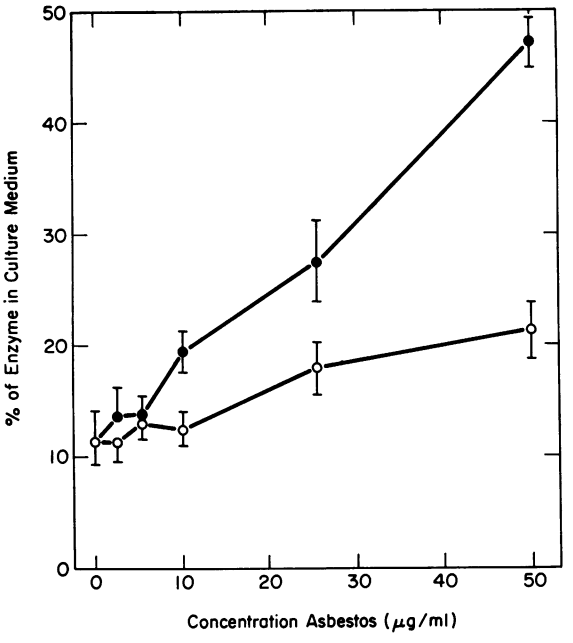

FIG. 8.—The release of β-galactosidase into the culture medium of mouse peritoneal macrophages after 24 h of exposure to increasing amounts of acid-leached chrysotile asbestos (○——○) or water-treated chrysotile asbestos (●——●).

activity in the culture. Similar findings were made when two other representative lysosomal enzymes, namely N-acetyl-β-D-glucosaminidase and β-glucuronidase were assayed. There was no evidence of any cytotoxic effect of either type of asbestos as indicated by measurement of lactate dehydrogenase levels in cells and culture media (Table).

TABLE.—*Lactate Dehydrogenase Activity in Mouse Peritoneal Macrophage Cultures Exposed to Increasing Concentrations of Acid-leached or Water-treated Asbestos for 24 h*

Concentration asbestos (μg/ml)	Enzyme activity (m units/plate)			
	Acid-leached asbestos		Water-treated asbestos	
	Cells	Medium	Cells	Medium
0	444±81	39±29		
2	487±145	28±17	401±18	81±28
5	381±89	40±30	424±14	55±15
10	454±50	52±57	441±3	95±29
25	452±79	64±19	494±84	35±14
50	441±81	33±48	513±46	85±60

DISCUSSION

The studies described in this paper show that agents eliciting inflammatory responses induce the selective release of lysosomal hydrolases from mouse peritoneal macrophages maintained in culture. On the other hand stimuli of a similar physical nature but lacking inflammatory activity do not release lysosomal enzymes from macrophages *in vitro*. The possibility that the differences in activities of the stimuli is due to differences in particle used requires consideration. Zymosan particles have a diameter of approximately 3 μm while the latex particles used for most experiments had a diameter of 0·81 μm. However, similar findings were made with latex particles of diameters up to 15·8 μm. Acid-leached chrysotile asbestos has been examined by electron microscopy (Morgan *et al.*, 1973) and shown to retain the fibrous morphology of the undepleted material. These results show similar correlations to those described

in other studies where inflammatory and non-inflammatory stimuli have been examined for their effect on lysosomal enzyme release from macrophages. Antigen–antibody complexes formed at equivalence elicit chronic inflammatory responses (Spector and Heesom, 1969) and also induces selective release of lysosomal enzymes from macrophages *in vitro* (Cardella *et al.*, 1974). Antigen or antibody alone are not inflammatory nor do they result in lysosomal enzyme release from macrophages. Products of lymphocytes stimulated with mitogens or antigens cause chronic inflammatory changes when injected into various tissue sites (Andreis, Stasny and Ziff, 1974; Dumonde *et al.*, 1975) and also elicit the selective release of lysosomal enzymes from macrophages (Pantalone and Page, 1975). Supernatants from unstimulated lymphocytes show no activity either *in vivo* or *in vitro*. Similar correlations of *in vivo* inflammatory capacity have also been reported for different types of carrageenan (Davies *et al.*, 1976).

The essential role played by macrophages in chronic inflammation is well recognized and has been discussed elsewhere (van Furth, 1970, 1975; Davies and Allison, 1976a). It has also become clear that macrophages secrete a number of products under various conditions *in vitro* and that several of these may be directly involved and play a major role in the participation of macrophages in chronic inflammatory responses. These include hydrolytic enzymes derived from lysosomes (Davies and Allison, 1976b) and other cellular sources (Werb and Dingle, 1976), complement components (Bentley *et al.*, 1976; McClelland and van Furth, 1976; Einstein, Schneeberger and Colten, 1976), prostaglandins (Gordon, Bray and Morley, 1976; Bonney *et al.*, 1977), factors affecting lymphocyte function (Waksman and Namba, 1976) and a factor promoting fibroblast growth (Leibovich and Ross, 1976). The secretion of these products in response to inflammatory stimuli has only been studied to a limited extent. For example, neutral proteinase secretion occurs in an optimal fashion when cells have been collected from animals previously given an intraperitoneal injection of an inflammatory stimulus such as thioglycollate broth (for review see Werb and Dingle, 1976). In some instances secretion *in vitro* can be increased further by addition of a phagocytic stimulus such as latex, not necessarily possessing inflammatory properties (Gordon, Unkeless and Cohn, 1974; Werb and Gordon, 1975*b*). Hamilton, Vassalli and Reich (1976) have shown that macrophages from mice given intraperitoneal injections of chrysotile asbestos secrete considerable amounts of plasminogen activator when cultured *in vitro*. In contrast, mice injected with latex particles did not yield macrophages which secreted amounts of plasminogen activator above control levels.

Further investigations are required to elucidate the mechanism by which the selective release of lysosomal enzymes by inflammatory stimuli is related to the establishment of chronic inflammatory lesions.

We have recently drawn attention to the interaction of complement components with macrophages. Enzymes released from macrophages stimulated by chrysotile asbestos are able to cleave C3 (Schorlemmer and Allison, 1976). Agents able to activate the alternative pathway of complement (as zymosan does) induce macrophages cultured in a serum-free medium to secrete the complement cleavage products C3a and C3b. Activation of complement, generation of kinins and other mediators could increase vascular permeability. Enzymes released from stimulated macrophages can cleave C5 to generate a product chemotactic for macrophages (Snyderman, Shin and Dannenberg, 1972). Thus hydrolase release induced from macrophages may be related to the generation of factors that recruit more mononuclear phagocytes from the circulation and, possibly, immobilize them in the sites of the lesions.

Dr H. U. Schorlemmer was supported by a grant (Scho 215/1) from the Deutsche Forschungsgemeinschaft, Bad Godesberg, Germany.

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