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Cytotoxic Effect of Asbestos on Macrophages in Different Activation States

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The *in vitro* effects due to phagocytosis of asbestos by mouse peritoneal macrophages in various stages of activation have been compared. The amphiboles proved relatively inert; chrysotile, however, expressed a greater degree of cytotoxicity toward those populations of macrophages induced *in vivo* with asbestos, than toward any of the other populations of cells. These results are compared with data concerning the enzyme release from the different populations of macrophages following phagocytosis of asbestos. The results indicate that those macrophages that have been exposed to a prior stimulation of either amphibole or serpentine asbestos *in vivo* are particularly sensitive to exposure to a second dose of a toxic fiber.

Introduction

The realization that the alveolar macrophage is the first phagocytic cell in the lung to ingest inhaled asbestos fibers prompted considerable research into the direct effects of fibers on such cells *in vitro*. Early studies (*1*, *2*) have shown chrysotile to be more toxic than crocidolite or amosite, a finding confirmed by many research groups. Macrophage-activating agents, such as zymosan, are known to initiate selective release of lysosomal enzymes (*3*). A study by Davies et al. (*4*) demonstrated that mouse peritoneal macrophages, upon phagocytosis of chrysotile *in vitro*, showed a selective release of lysosomal enzymes in the absence of cell death. However, Jaurand et al. (*5*) demonstrated an additional release of lactate dehydrogenase, thus suggesting some loss of viability, for alveolar macrophages exposed to chrysotile *in vitro*. Studies by Hamilton and colleagues (*6*, *7*) showed that macrophages exposed to chrysotile *in vitro* and *in vivo* release greater quantities of the neutral protease plasminogen activator. In addition, Miller (*8*) and Donaldson et al. (*9*) have shown that asbestos-induced macrophages obtained from either lung or peritoneal cavity possess an altered surface morphology and increased number of membrane receptors consistent with cell activation.

McGee and Myrvik (*10*) demonstrated that activated macrophages are more readily injured upon phagocytosis of toxic agents than resident cells. Wright and Davis (*11*) showed that chrysotile-activated macrophages are more sensitive to the cytotoxic action of chrysotile *in vitro* than unstimulated cells; in addition, Morgan and Allison (*12*) also suggested that macrophages elicited by different biochemical means may show a different response to a further stimulus. The aim of this study was to investigate further the response of populations of macrophages in different stages of activation to the action of asbestos *in vitro*, and enzyme release from these cells was also compared.

Materials and Methods

Stimulation and Harvesting of Peritoneal Exudate Cells (PEC)

Male CBA mice, 12 weeks old, were either untreated or injected intraperitoneally with one of the following stimulating agents: 1 mL saline (Dulbecco's A); 1 mL 10% protease peptone (Difco); 1.4 mg heat-killed *Corynebacterium parvum* (Wellcome), a well-known macrophage activating agent (*13*); 1 mL of 0.1% latex beads (0.81 μ) (Difco); 2.5 mg of UICC crocidolite, UICC amosite or UICC chrysotile suspended in 1 mL saline.

Three days following injection, the mice were killed by ether overdose. The PEC were harvested by peritoneal lavage and washed.

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Spreading Assay to Assess Degree of Macrophage Activation

PECs (1×10^5) were cultured on 6×22 mm glass coverslips in Ham's F10 medium (14) + 20% fetal calf serum (FCS) at 37°C. After precisely 1 hr, the coverslips were washed vigorously to remove nonadherent cells. The remaining adherent macrophages were stained by May-Grunwald and Giemsa stains. The relative degree of activation of the population was expressed in terms of the percentage of cells completely spread. This means of assessment has been shown to correlate with other methods of activation measurement such as Fc receptor (*9*).

Culture and Treatment of PEC Populations

After harvesting, 1×10^6 PECs were cultured in 35 mm dishes in F10 + 20% FCS. After 1 hr, the cells were washed with saline to remove nonadherents. The resulting macrophage populations were cultured in F10 + 20% FCS either untreated or treated with 0.1% latex beads (0.81 μ), or 100 μg UICC crocidolite, UICC amosite or UICC chrysotile per plate.

Assessment of Phagocytic Ability of Macrophages

The phagocytic ability of the macrophage populations was assessed microscopically after 24-hr culture with latex heads. A cell was termed phagocytic if it contained more than three latex beads.

Assessment of Viability of Marcrophages

Viability of the cells was assessed, using Trypan Blue exclusion, 24 hr following treatment with latex, crocidolite, amosite or chrysotile.

Enzyme Assays

Lactate dehydrogenase (LDH) (*15*) and *N*-acetyl-β-*D*-glucosaminidase (glucosaminidase) (*16*) levels were assessed in both cells and culture medium after 24-hr culture with crocidolite or chrysotile.

In Vitro Activation of Macrophages by Lymphokine

Lymphokine, a known macrophage activating agent, was produced according to the method of Lazdins et al. (*17*) by exposing mouse splenocytes to 10 μg/mL of Concanavalin A (Con A) *in vitro* for 24 hr. Saline-induced macrophages were exposed to either the resulting lymphokine or a Con A supplemented control medium for 24 hr. The activated Con A control and untreated macrophages were then exposed to UICC chrysotile for a further 24 hr and their viabilities assessed.

Statistical Analyses

The data from the spreading assay, macrophage viability estimates and enzymes assays were examined by statistical analyses of variance, the within-experimental replication being used to provide estimates of random variation.

Results

All of the populations of PECs were found to contain 99% viable cells upon isolation from the groups of treated mice. The relative degree of activation of the adherent macrophages, according to their ability to spread on glass, is shown on Figure 1. A high degree of activation was found in those populations induced by asbestos and *C. parvum*, according to this method of activation assessment; they did not differ significantly in their ability to spread on glass. All of the remaining populations showed a much lower ability to spread on glass, the protease peptone population showing an increase over the unstimulated population ($p < 0.05$).

After 24-hr culture *in vitro* with latex heads, the macrophage groups were all found to contain 95% phagocytic and 95% viable cells following ingestion of latex heads. The effect of 24-hr incubation with chrysotile was very different from that of the other two types of asbestos (Figs. 2 and 3). Crocidolite and amosite proved noncytotoxic, and there were no significant differences observed between the macrophage populations ($p > 0.9$ overall). Chrysotile, however, while exhibiting a low degree of cytotoxicity towards the unstimulated and saline-induced populations, showed a slightly increased level of cytotoxicity towards the *C. parvum*-induced cells. All three types of asbestos-induced populations proved particularly susceptible to the cytotoxic action of chrysotile; viabilities of around 30% were obtained and no significant differences were found among these three populations. The populations of macrophages stimulated *in vitro* by Con A or lymphokine did not show an increased degree of susceptibility to the action of chrysotile (Table 1).

Ingestion of crocidolite, compared to control, stimulated an increased release of glucosaminidase in all cell populations ($p < 0.01$). An even more considerable release of this enzyme in all populations of cells followed chrysotile ingestion ($p < 0.01$) (Figs. 4 and 5). The level of release of LDH was lower than that observed for the glucosaminidase, although the asbestos-induced populations released a greater quantity of LDH than the unstimulated, saline and *C. parvum*-induced populations ($p < 0.025$).

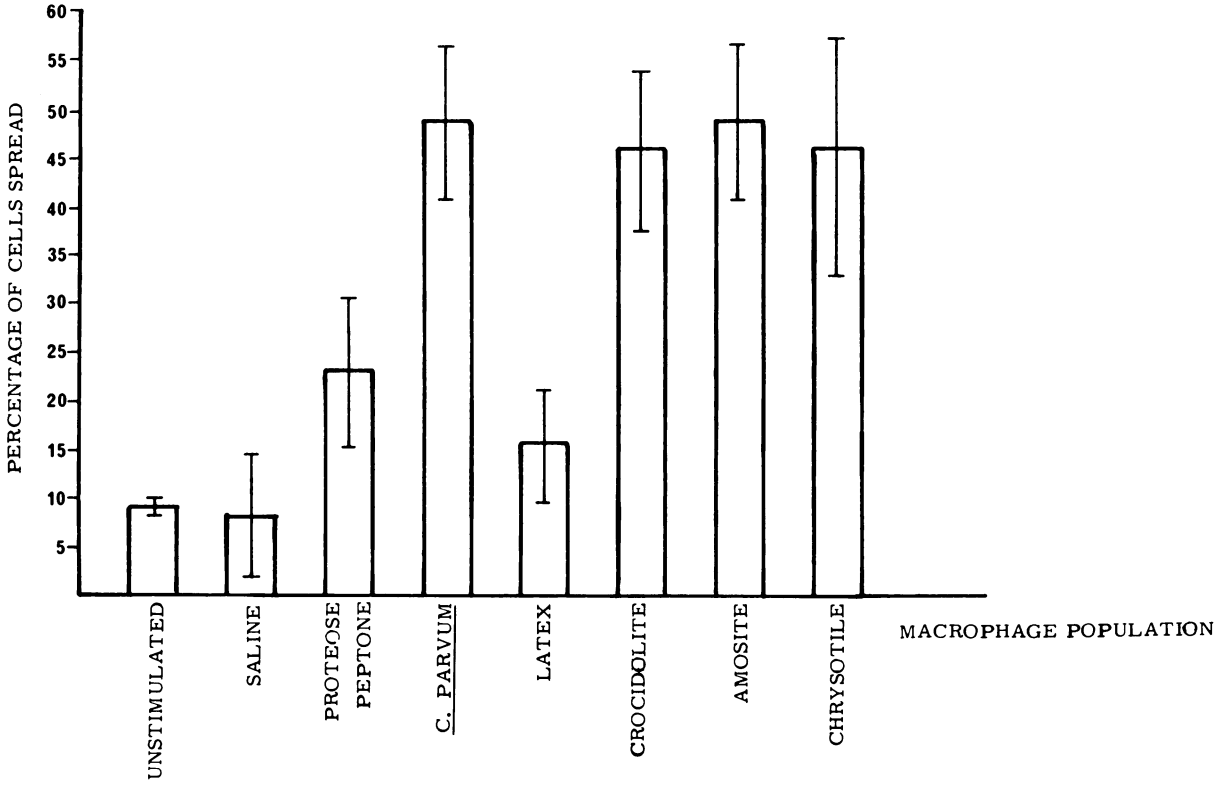

FIGURE 1. Relative degree of activation of macrophage populations according to spreading assay. Calculated as % cells spread = (number of cells spread/total number of cells counted) × 100. Results are means of at least three experiments ± SD.

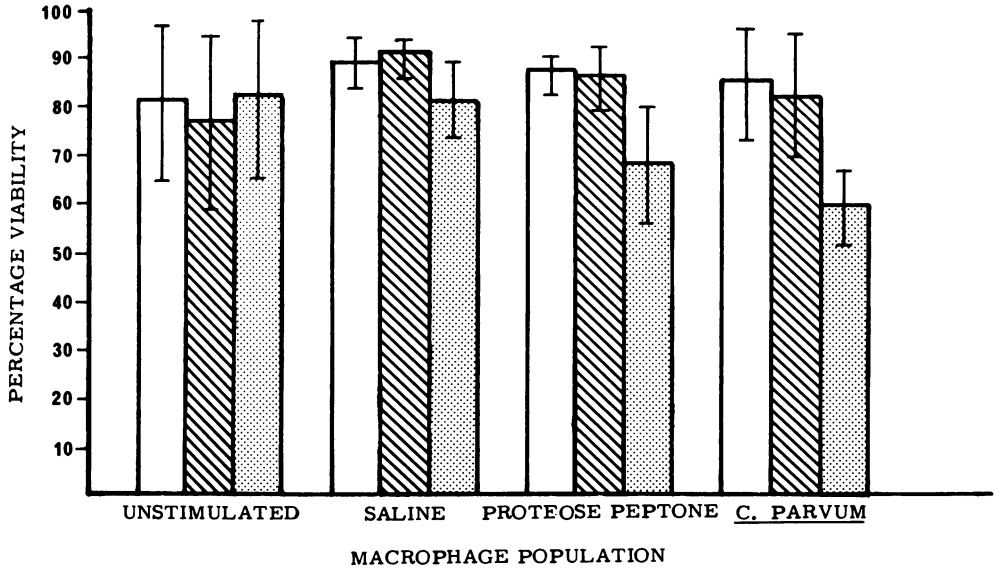

FIGURE 2. Percentage viability of macrophage populations after 24-hr exposure to asbestos *in vitro*. Treatment *in vitro*: □ crocidolite; ▨ amosite; ⊠ chrysotile. Macrophages unstimulated or stimulated with saline, protease peptone and *C. parvum*. Viability calculated as % viability = (number of viable cells on treated plate/number of viable cells on control plate) × 100. Results are means of at least three experiments ± SD.

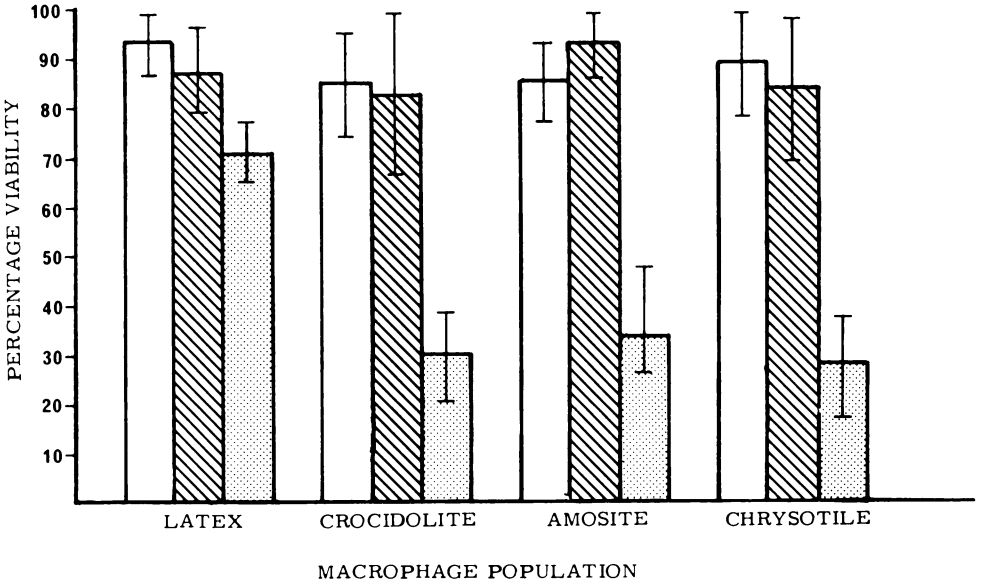

FIGURE 3. Percentage viability of macrophage populations after 24-hr exposure to asbestos *in vitro*. Treatment *in vitro*: □ crocidolite; ▨ amosite; ▒ chrysotile. Macrophages stimulated with latex and asbestos. Results are means of at least three experiments ± SD.

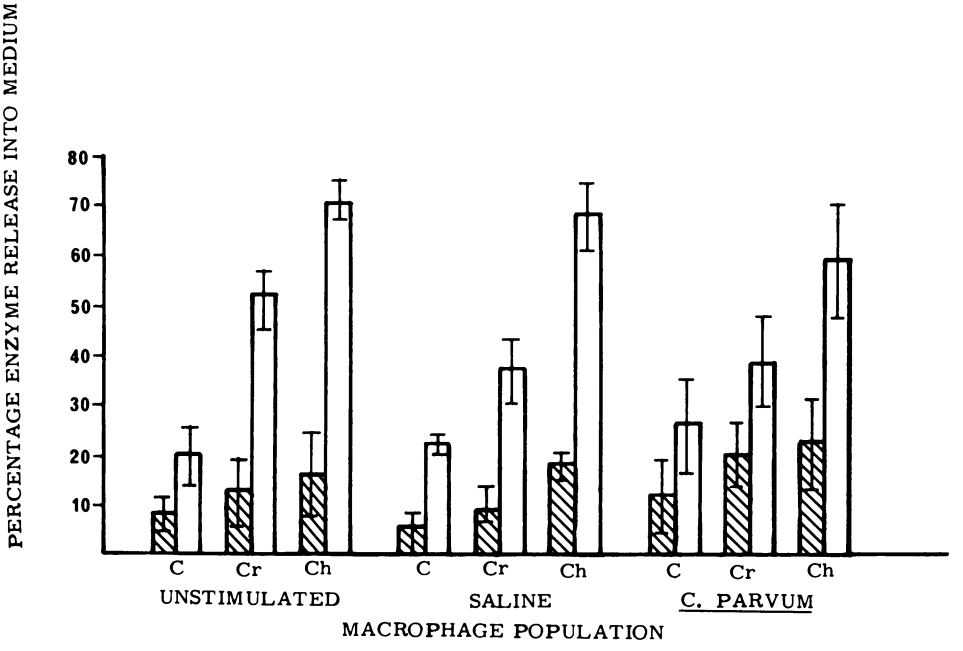

FIGURE 4. Enzyme release into medium after 24-hr exposure to asbestos. Enzyme: ▨ LDH; glucosaminidase. Treated with: C = control; Cr = crocidolite; Ch = chrysotile. Results are means of three experiments ± SE.

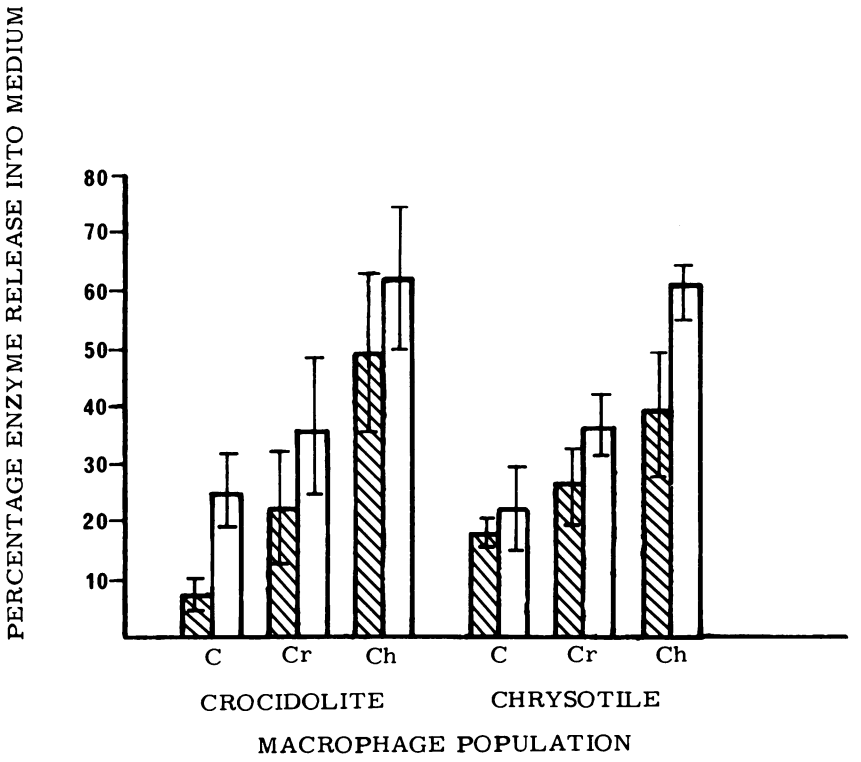

FIGURE 5. Enzyme release into medium after 24-hr exposure to asbestos. Enzyme: ▨ LDH; □ glucosaminidase. Treated with: C = control; crocidolite; Ch = chrysotile. Results are means of three experiments.

Table 1. Percentage viability of *in vitro* activated macrophages following 24-hr treatment with chrysotile.

In vitro treatment of saline-induced macrophages	% viability following ingestion of chrysotile[a]
Untreated control	67.2 ± 7.0
Con A supplemented medium	69.5 ± 8.7
Lymphokine	69.8 ± 9.2

[a] % viability = (number of viable cells on chrysotile treated plate/number of viable cells on control plate) × 100. Results are means $\bar{X}$ of three experiments ± SD.

Discussion

Peritoneal macrophages can be obtained in a variety of states of activation possessing a variety of altered properties (*18*). In general, activated macrophages are larger, have more granules, spread to a greater extent on glass and have a greater capacity to kill microorganisms and tumor cells than unstimulated, resident cells (*19*). The degree of activation of the macrophage can vary considerably, depending on the nature of the stimulating agent; and this is illustrated in Figure 1 by using a single parameter for activation assessment. Studies (*6, 9*) have shown that intraperitoneal injection of asbestos can produce a population of viable macrophages with characteristics consistent with cellular activation. In the present study, crocidolite, amosite and chrysotile have all induced intraperitoneal populations of cells both viable and apparently activated to a degree similar to *C. parvum*-induced macrophages.

All the populations of cells showed a similarly high rate of phagocytosis, regardless of the activation state, and no cell death was observed because of ingestion of nontoxic latex beads alone. The amphiboles displayed a similar level of low cytotoxicity toward all types of macrophages (Figs. 2 and 3). These cells, however, showed a diverse response to chrysotile. The nonstimulated and saline-induced macrophages appeared resistant to the cytotoxic action of the dust, whereas the more activated populations showed an increased susceptibility, the asbestos-induced cells proving the most sensitive. These results agree with those of McGee and Myrvik (*10*), in that activated macrophages tend to lose viability more rapidly than nonstimulated cells upon phagocytosis of a toxic agent. The cells activated by lymphokine *in vitro* (Fig. 4) did not display an increased sensitivity to the action of chrysotile, thus suggesting that macrophages activated *in vivo* probably possess differing properties to those activated *in vitro*. It is of interest to note that, while the amphiboles—crocidolite and amosite—appeared relatively inert *in*

vitro, both types of fiber have the capacity *in vivo* to induce macrophages that show a high sensitivity to the action of a cytotoxic dust. This is not due simply to an *in vivo* stimulating activity of particulate alone, as latex-induced macrophages did not display a high sensitivity to chrysotile.

The data regarding enzyme release from the macrophages (Figs. 4 and 5) agree with the finding of Hamilton (*6*), in that asbestos-induced macrophages secreted a similar quantity of lysosomal enzyme into the culture medium to that seen for the nonstimulated cells. Phagocytosis of crocidolite induced a slight release of glucosaminidase by all populations; however, chrysotile stimulated a large release of enzyme similar to that seen in other reports (*20*). This large release of lysosomal enzyme was not accompanied by a corresponding release of cytoplasmic LDH for unstimulated, saline and *C. parvum*-induced macrophages. However, an increased release of LDH was observed for the asbestos activated populations, corresponding to the increased loss of viability illustrated in Figure 3.

In conclusion, this report illustrates that asbestos-induced macrophages, upon phagocytosis of a second dose of dust, do not respond in a manner similar to that observed for other types of macrophage populations. This must be taken into consideration when investigating the effect of inhaled particles on macrophages in the lung. Such cells may already have received prior stimulation by other toxic agents or pathogens and also persistent exposure to different dusts, rather than the single dose often used in the *in vitro* situation.

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