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Interactions of Asbestos-Activated Macrophages with an Experimental Fibrosarcoma

by K. Donaldson,* J. M. G. Davis,* A. Ewing†
and K. James†

Supernatants from *in vivo* asbestos-activated macrophages failed to show any cytostatic activity against a syngeneic fibrosarcoma cell line *in vitro*. UICC chrysotile-induced peritoneal exudate cells also failed to demonstrate any growth inhibitory effect on the same cells in Winn assays of tumor growth. Mixing UICC crocidolite with inoculated tumor cells resulted in a dose-dependent inhibition of tumor growth; this could, however, be explained by a direct cytostatic effect on the tumor cells of high doses of crocidolite, which was observed *in vitro*.

Introduction

Recently there has been an accumulation of experimental evidence suggesting that asbestos can have a stimulatory or activating effect on macrophages *in vivo* and *in vitro*. Davies et al. (1) reported that peritoneal macrophages treated with asbestos *in vitro* released lysosomal hydrolases without evidence of cell death, and Hamilton and co-workers (2, 3) demonstrated that asbestos *in vivo* and *in vitro* induces selective release of the neutral protease plasminogen activator from peritoneal macrophages. Humes et al. (4) and Sirois (5) have reported the induction of prostaglandin release by peritoneal and alveolar macrophages treated with asbestos *in vitro*. Selective release of lysosomal hydrolases and neutral proteases from macrophages can be induced by a range of macrophage activating agents, both immunological and nonimmunological (6).

Miller and Kagan (7) have reported that the alveolar macrophages of rats inhaling crocidolite show evidence of activation by morphology, Fc receptor avidity and the ability to stimulate T-lymphocytes to take up thymidine. We have studied the macrophages induced in the peritoneal cavity by intraperitoneal asbestos injection and have found macrophages to be activated by several criteria (8, 9) but

full activation to the tumor cell cytotoxic state was not found (8). Small significant reductions in the size of experimental subcutaneous fibrosarcomas were, however, found in mice which had received UICC chrysotile intraperitoneally compared with mice which had received saline intraperitoneally.

In this paper we present further studies into the interactions of asbestos, macrophages and an experimental fibrosarcoma.

Materials and Methods

Macrophage Plasma Membrane 5'-Nucleotidase and Lysosomal Acid Phosphatase

These assays were carried out according to the method of Raz et al. (10) using glycerophosphate (Sigma) and 5'-adenosine monophosphate (Sigma) as substrates, and assaying for phosphate release by the method of Ames and Dubin (11). Protein was assayed by the method of Lowry (12).

Experimental Fibrosarcoma

The experimental tumor line used was the CCH₁ fibrosarcoma derived from a subcutaneous methylcholanthrene-induced fibrosarcoma in CBA/Ca mice. It has been maintained in long-term culture and passaged through mice at intervals (13).

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Macrophage Supernatants, Leachate and Asbestos

Peritoneal exudate cells (PEC) were harvested 3 days after intraperitoneal injection of UICC crocidolite, chrysotile, latex spheres (0.81 μm diameter, Difco) or saline. The macrophages were maintained in culture over 24 hr in complete RPMI (RPMI 1640, Gibco) containing 10% fetal calf serum (Gibco) and antibiotics (cRPMI). The supernatant was centrifuged, aliquoted and stored frozen at -20°C until use. Leachate was prepared by incubating crocidolite or chrysotile (5 mg/mL) in cRPMI for 3 days at 37°C then spinning out the fiber by centrifuging at $3500g$ for 1 hr. Asbestos was serum-coated to reduce direct cytotoxic effects by preincubating for 1 hr at 37°C in cRPMI.

CCH₁ Tumor Cell Proliferation Assay

The same assay system was used to measure the effect of macrophage supernatants, asbestos or asbestos leachate on CCH₁ cell proliferation *in vitro*. 5×10^4 CCH₁ cells were inoculated into microtiter plates (Sterilin) in 100 μL of cRPMI. Various volumes of supernatant, leachate or asbestos (preincubated in cRPMI for 1 hr at 37°C) were then added, and the final volume was adjusted to 200 μL with cRPMI. After 24 hr, 0.25 μCi of ^3H -thymidine were added to each well and after a further 24 hr the cells were harvested in a cell harvester (Skatron); cell-bound ^3H -thymidine counts were measured by liquid scintillometry.

Winn Assay

The assay used was essentially the same as that described by Gabizon and Trainin (14). PECs obtained 3 days after IP injection of saline or chrysotile (5 mg) were harvested, counted and mixed with CCH₁ tumor cells in the following ratios: (1) 10:1, i.e., 10^5 CCH₁ cells plus 10^6 PEC in 0.1 mL Dulbecco's phosphate buffered saline (Dul. A) and (2) 100:1, i.e., 5×10^2 CCH₁ cells plus 5×10^4 PEC in 0.1 mL Dul. A. Groups of 10 mice received 0.1 mL subcutaneously in the right hind limb. Tumor growth was monitored by taking the mean of the two major diameters of the tumor.

Table 1. Plasma membrane 5'-nucleotidase and lysosomal acid phosphatase activity in 3-day saline- and 3-day chrysotile-induced macrophages.

Macrophage source	5'-Nucleotidase, $\mu\text{mole phosphate}/$ mg protein/hr	Acid phosphatase $\mu\text{mole phosphate}/$ mg protein/hr
Saline-induced (3-day)	3.7	0.4
Chrysotile-induced (3-day)	0.5	4.7

Chrysotile was arbitrarily chosen for this assay since previous studies (8) had shown crocidolite and chrysotile to be similar in their ability to activate macrophages.

Inclusion of Crocidolite in Tumor Cell Inoculum

UICC crocidolite was incubated in normal mouse serum for 1 hr at 37°C and then mixed with CCH₁ cells so that 0.1 mL of inoculum contained 10 μL of normal mouse serum; 0, 5, 50, or 500 μg of crocidolite; and 5×10^2 CCH₁ cells. Groups of 10 mice received 0.1 mL subcutaneously in the right hind limb and tumor growth was monitored.

Statistical Analysis

All differences were examined for statistical significance using Student's *t*-test.

Results

Table 1 shows that chrysotile-induced macrophages have decreased plasma membrane 5'-nucleotidase and increased acid phosphatase content, compared to saline-induced macrophages.

The effect of 24-hr supernatants of macrophages induced by various agents on tumor cell proliferation is shown in Figure 1. Statistical analysis re-

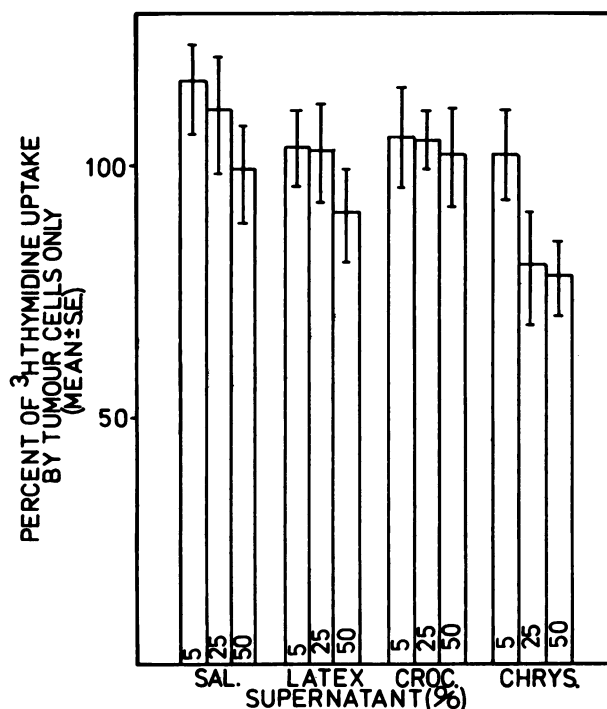


FIGURE 1. Effect of supernatants from 3-day saline-, latex-, crocidolite- or chrysotile-induced peritoneal exudate macrophages on proliferation of tumor cells *in vitro* as measured by ^3H -thymidine uptake.

vealed no significant effects of macrophage supernatants from any source, on CCH₁ cell proliferation, compared to CCH₁ cells alone. There was considerable variation in the response of CCH₁ cells to these supernatants between experiments, and this is reflected in the large amount of variation evident in Figure 1, which represents the mean and standard errors of five separate experiments.

Although there were differences in the mean tumor diameter produced by tumor cells alone, tumor cells mixed with saline-induced PEC and tumor cells mixed with chrysotile-induced PEC, at both 10:1 and 100:1 (Figs. 2 and 3), these differences were not statistically significant.

Figure 4 shows a dose-dependent inhibition of tu-

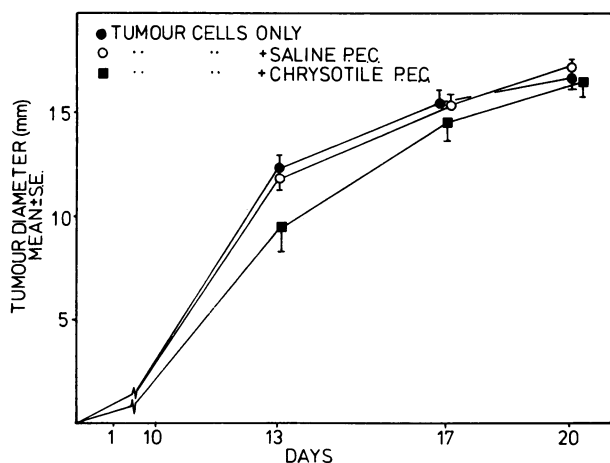


FIGURE 2. Effect of 3-day saline- and 3-day chrysotile-induced peritoneal exudate cells on subcutaneous tumor growth in a Winn assay; peritoneal exudate cell: tumor cell ratio of 10:1 at subcutaneous inoculation. Although no positively cytostatic Winn assay control was included in these experiments, intraperitoneal *C. parvum* is known to inhibit this tumor model by inducing tumor cell cytotoxic macrophages (8).

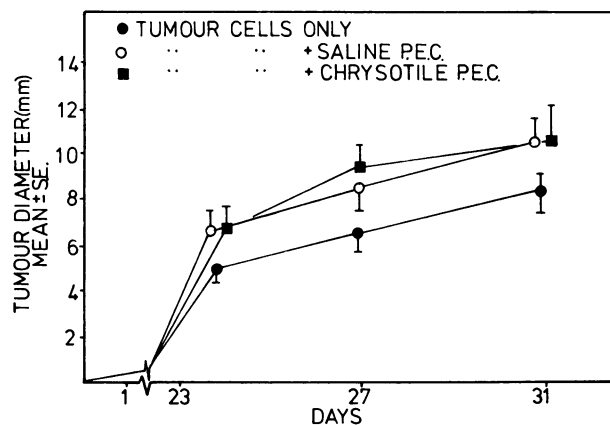


FIGURE 3. Effect of 3-day saline- and 3-day chrysotile-induced peritoneal exudate cells on tumor growth in a Winn assay; peritoneal exudate cell:tumor cell ratio of 100:1 at subcutaneous injection.

mor growth produced by mixing crocidolite that had been preincubated with normal mouse serum with the CCH₁ tumor cell inoculum.

The effects of crocidolite and chrysotile, at doses which extend over four orders of magnitude, on proliferation of CCH₁ tumor cells *in vitro* are shown in Figure 5; remarkably little effect is seen except at the very highest doses of asbestos, where crocidolite caused marked inhibition of ³H-thymidine uptake by tumor cells. Figure 5 also shows that crocidolite leachate, at the highest concentration, produced inhibition of ³H-thymidine uptake.

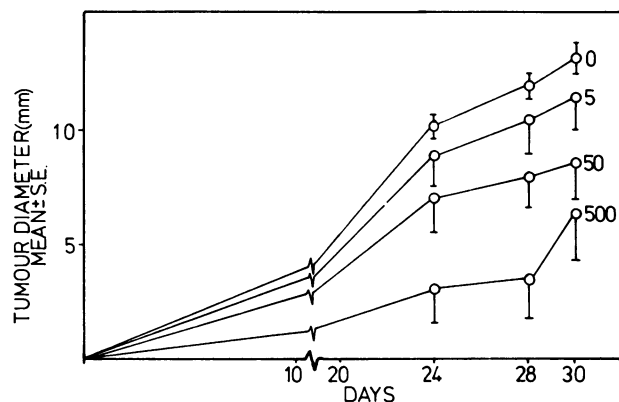


FIGURE 4. Effect of tumor growth of 0, 5, 50 or 500 µg of crocidolite percent along with tumor cells at subcutaneous inoculation.

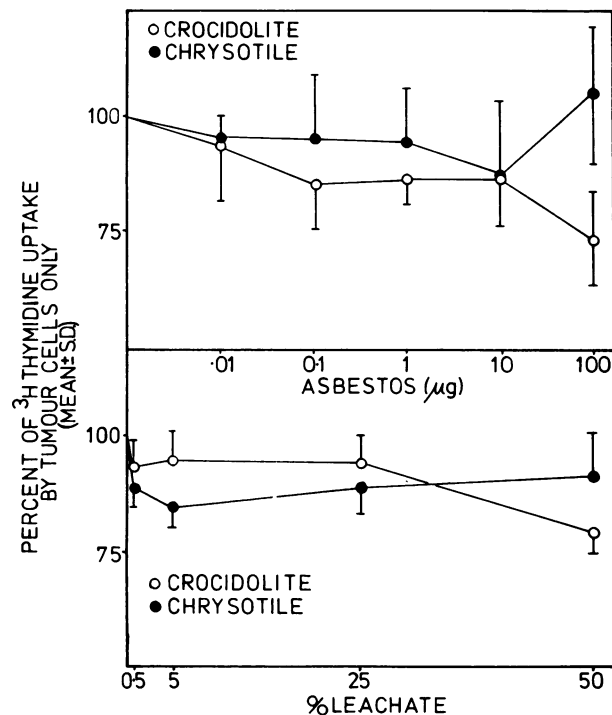


FIGURE 5. Effect of various doses of asbestos and asbestos leachate on tumor cell proliferation *in vitro*.

Discussion

In this paper we describe experiments which were aimed at further elucidating the degree of activation of macrophages induced in the murine peritoneal cavity by injection of asbestos.

Macrophage activation apparently involves the sequential adoption of properties which can culminate in the fully tumoricidal state (15). In keeping with other reports, we have found that asbestos can lead to activation of macrophages *in vivo* but, using an *in vitro* assay, we failed to detect any evidence of tumor cell cytotoxicity by asbestos-activated macrophages although *Corynebacterium parvum*-activated macrophages were tumoricidal in this system (8). However, in the same study, there was the contradictory finding *in vivo* that the administration of asbestos by intraperitoneal injection to mice caused a small but significant reduction in the growth of an experimental subcutaneous tumor. The present study follows on from this work and examines whether the tumor-retarding effect that was noted *in vivo* could be associated with macrophage activation.

Macrophage cytostatic activity, as well as cytotoxic activity, can play a role in retarding tumor growth, and this can be mediated by soluble factors (16). The supernatants from asbestos-activated macrophages were therefore tested in an assay of CCH₁ tumor cell proliferation *in vitro*. No significant inhibitory activity against tumor cell proliferation was detected in crocidolite, chrysotile, latex or saline-induced macrophage supernatants using Student's *t*-test.

In a further attempt to detect cytostatic activity in asbestos-activated macrophages, and in an effort to circumvent the problem produced by difference between the site of the tumor (leg) and the site of primary macrophage activation (the peritoneal cavity), two approaches were tried. In the first approach, Winn assays were used to bring 3-day asbestos- or saline-induced PEC into close contact with the target tumor cells by mixing them together at various effector : target ratios; this mixture was then inoculated and tumor growth compared to that of tumors produced with the appropriate number of tumor cells alone and with similar effector : target ratios of saline-induced PEC and tumor cells. No significant differences in tumor size were obtained with chrysotile-activated macrophages at ratios of either 10:1 or 100:1 compared to controls, and therefore no evidence of tumor cell cytostatic activity in asbestos-activated macrophages was detected.

In a second approach various doses of crocidolite asbestos that had been preincubated in serum-con-

taining medium for 1 hr were mixed with inoculating tumor cells and tumor growth monitored. The dose-dependent decrease in tumor size which was obtained could have been due to local macrophage activation to the cytostatic state or could have been due to a direct toxic effect of crocidolite on the tumor cells. It was specifically to avoid such a direct toxic effect that crocidolite was chosen rather than chrysotile, since the amphiboles have a less active surface than chrysotile, and this is also why the crocidolite was precoated by incubation in serum-containing medium. However, in order to test for such a direct toxic effect of crocidolite on tumor cells, crocidolite was used in the *in vitro* CCH₁ cell proliferation assay. It was clear that both chrysotile and crocidolite and a fiber-free leachate of both asbestos types produced very little effect at all doses, except for inhibition at the highest doses with crocidolite. Since the doses of crocidolite present in inocula compared closely with, or exceeded, the doses used in the *in vitro* assay, it is evident that the reduction of tumor size produced could be accounted for by direct toxic effects of crocidolite on CCH₁ tumor cells. The induction of local tumor cell cytostatic macrophages does not therefore have to be evoked as a mechanism.

It seems likely that the small but significant reduction in the size of experimental tumors in mice injected with chrysotile which we reported previously (8) was not macrophage-mediated and that the overall adjuvant effect of asbestos could result in a nonspecific mobilization of some of the other well-documented antitumor immune responses.

The tumoricidal potential of macrophages has been used in these studies in the context of assessing the degree of macrophage activation and the results suggest that asbestos-activated macrophages have not attained the fully activated tumoricidal state. Tumor cell cytotoxic macrophages have been considered to be part of the surveillance system which acts to eliminate transformed cells before tumors can develop, and so the potential of asbestos-activated macrophages to attain full tumoricidal status has relevance for asbestos carcinogenesis. Previous studies have produced evidence that asbestos can act as a tumor promoter in classical two-stage carcinogenesis after initiation by polycyclic hydrocarbons (17, 18). A recent report has shown that a tumor promoter can block lymphokine-mediated activation of macrophages to the tumoricidal state (19). If this is shown to be a general property of tumor promoters, then it could be of particular relevance to asbestos carcinogenesis.

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