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Selective Differences in Macrophage Populations and Monokine Production in Resolving Pulmonary Granuloma and Fibrosis

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Alveolar macrophages (AM) and their production of interleukin-1–like activity (IL-1) and macrophage-derived growth factor for fibroblasts (MDGF) were examined during chronic inflammatory reactions leading to either granuloma formation or fibrosis. Groups of five rats each received, respectively, a single transtracheal injection of xonotlite, attapulgite, short chrysotile 4T30, UICC chrysotile B asbestos, or saline. One month later, such treatments induced either no change (xonotlite), granuloma formation (attapulgite and short chrysotile 4T30), or fibrosis (UICC chrysotile B). By 8 months, however, the granulomatous reactions had resolved or greatly diminished, whereas the fibrosis persisted irreversibly. Parallel examination of cell populations obtained by bronchoalveolar lavage revealed that multinucleated giant macrophages (MGC) were present in lavage fluids of animals with resolving granulomatous reactions but absent in those obtained from animals with lung fibrosis. Evaluation of monokine production by inflammatory macrophages also revealed significant differences. Enhanced production of IL-1–like activity was seen in both types of lung injury, although especially during the early stage (1 month) and decreased thereafter (8 months). By contrast, augmentation of MDGF production was observed in animals with lung fibrosis only and persisted up to 9 months. Taken together, these data indicate that production of selected cytokines, as well as AM differentiation along a given pathway, may modulate the outcome of a chronic inflammatory response. (Am J Pathol 1991, 138:487–495)

Inflammation is a protective response of the host to invading microorganisms or injury. However chronic inflammation, when it takes place, is often a destructive process associated with many human diseases.[1] It is characterized by granuloma formation, which, under certain circumstances, may evolve toward irreversible fibrosis.[2] The inflammatory response is a complex phenomenon involving many cell types and mediators. Among these macrophages and their derivatives, epithelioid and giant cells are a characteristic feature of chronic inflammation. However the precise role and function of various macrophage populations in the maintenance, progression, and outcome of inflammatory reactions is unknown. It is believed that these cells are an important source of molecular mediators that dictate the progression of the inflammatory response. In this regard, previous studies showed that interleukin-1 (IL-1)[3–5] and macrophage-derived growth factor(s) for fibroblasts (MDGF)[6–10] may play a role. However the particular significance of these mediators in inflammation is not clearly understood. Therefore, to elucidate the potential role of these mediators and of selected cell populations in the progression of chronic inflammatory reactions, it is necessary to relate cell populations and mediator production to observed morphologic and physiologic changes.

In recent years, our laboratory developed a rat model of lung inflammation and fibrosis induced by various silicate fibers.[11,12] In the present study, we isolated bronchoalveolar cells from animals with different types of pulmonary lesions (granuloma *versus* fibrosis) and examined their morphologic characteristics as well as their capacity to produce IL-1–like activity and MDGF. Our findings indicate that the nature of both the cell populations involved and the mediators produced may play a determining role in the outcome of the inflammatory response.

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Materials and Methods

Animals

Male Wistar rats weighing 250 to 300 g were purchased from Charles River Canada, Inc. (St. Constant, Quebec). These animals were derived from a pathogen-free colony, shipped behind filter barriers, and housed in a horizontal laminar flow isolator (Johns Scientific Inc., Toronto). They were given Purina Rat Chow (Ottawa, Canada) and water *ad libitum* and were used 1 week later.

Induction of Lung Granuloma and Fibrosis

UICC standard sample of chrysotile B (58% were less than 5 μm) was obtained from the National Research Institute for Occupational Diseases, Johannesburg, South Africa. Very short chrysotile 4T30 fibers (100% were less than 5 μm) were isolated as described[13] from Johns-Manville grade 4T30 chrysotile fibers according to the sedimentation procedure of Jolicoeur et al.[14] The calcium silicate xonotlite (100% were less than 3 μm) was a commercially available product (Promatek) and the clay mineral attapulgite (100% were less than 1 μm) was obtained from the Quincy deposits (Florida). All these fiber preparations were provided by the Laboratoire de Caracterisation de l'Amiante (SNA), University of Sterbrooke, Sterbrooke. The preparation and the physicochemical properties and characteristics of the fibers used in this study were described in detail earlier.[12,13] Each sample was autoclaved for 45 minutes and suspended in sterile phosphate-buffered saline (PBS; pH 7.4) with a Dounce glass homogenizer (Fisher Scientific, Ottawa, Canada) before instillation into the animals. Rats received a single transtracheal injection, as described previously,[9] of either saline, UICC chrysotile B, short chrysotile 4T30, attapulgite, or xonotlite. Groups of five animals each received various doses (1, 5, or 10 mg) of these materials and a total of 130 animals were examined by bronchoalveolar analyses 1 and 8 months after treatment. Animals also were examined for histologic changes as described previously.[11,12] After lavage, the lungs were removed and a median longitudinal section of the upper left lobe (1-mm thick) was fixed by immersion in phosphate-buffered 4% formaldehyde. Such sampling allows adequate discrimination between normal (negative) and positive findings. After fixation, lung tissue was embedded in paraffin and representative sections measuring 5-μm thick were cut and stained with hematoxylin and eosin.

Obtainment and Culture of Bronchoalveolar Cells

Bronchoalveolar cells were obtained by bronchoalveolar lavage as described.[15] Briefly, after the animals were killed, the abdominal aorta was severed and the trachea cannulated. A total volume of 48 ml of PBS (pH 7.4) in 8-ml aliquots was infused in each animal, 93% (45 ml) of which was recovered in control rats. The bronchoalveolar cells were obtained by centrifugation at 200*g* at 4°C for 10 minutes, washed in PBS, and finally resuspended in Dulbecco's modified Eagle medium (DMEM) (Grand Island Biological Co., Grand Island, NY). Cells were counted in a hemacytometer chamber and viability was determined by trypan blue exclusion. For comparison, values of total cell counts in lavage fluid at each time studied were corrected for the corresponding fluid recovery at that particular interval, and all values were calculated as cell counts per 45 ml of lavage fluid recovered. Bronchoalveolar cells (0.5×10^6) from control and treated rats were incubated in 1 ml of DMEM supplemented with 0.5% dialyzed fetal bovine serum (FBS) (Grand Island Biological Co.) for 4 hours at 37°C in a humidified 95% air–5% CO_2 atmosphere. The culture supernatants were collected, centrifuged, and frozen at −80°C until assayed for MDGF activity. For evaluation of their production of IL-1–like activity, bronchoalveolar cells (0.5×10^6) were incubated in 1 ml of Roswell Park Memorial Institute medium (RPMI) 1640 containing 0.5% FBS for 24 hours at 37°C in the presence and absence of muramyl dipeptide (MDP) (20 μg/ml) (Calbiochem, La Jolla, CA).

Analysis of Bronchoalveolar Cells and Multinucleated Giant Macrophages

Differential counts of lavage cells were made from cytocentrifuge smears prepared with 2.5×10^4 cells and stained with Wright-Giemsa stain. The proportion of multinucleated giant macrophages (MGC) defined as alveolar macrophages (AM) with three or more nuclei in bronchoalveolar preparations was quantitated by counting 10^3 cells and calculating the percentage of cells that contained three or more nuclei. The occurrence of MGCs also was evaluated by dividing the number of nuclei within MGCs by the total number of nuclei in 10^3 cells, as described by Crawford and coworkers,[16] to obtain a fusion index.

Thymocyte Proliferation Assay

C3H/HeJ thymocytes (1.5×10^6/well) were cultured[17] in 96-well round-bottomed microculture plates in a final volume of 200 μl RPMI 1640 containing 7.5% FBS with dilutions of samples in the presence and absence of phytohemagglutinin PHA (1 μg). Appropriate controls con-

tained medium with PHA and MDP. Cultures in triplicate were incubated for 66 hours at 37°C, pulsed with 1 μCi/well [^{3}H]TdR, and harvested at 72 hours onto glass filters for measurement of [^{3}H]TdR incorporation by scintillation spectrometry. Bioassay data were calculated as net cpm by the following formula: net cpm = (cpm of thymocytes + PHA + sample) − (cpm of thymocytes + PHA). Thymocytes incubated with PHA alone incorporated 300 to 600 cpm of [^{3}H] TdR. Incorporation of [^{3}H] TdR in the presence of sample dilutions was compared with that in the presence of dilutions of a standard Il-1 preparation obtained from P388D$_1$ cells stimulated with MDP (20 μg/ml). Interleukin-1–like activity was quantified by calculation of half-maximal units.[18] As shown in Table 2, goat anti-mouse IL-1 alpha antibody (provided by Dr. R. Chizzonite, Hoffmann-LaRoche Inc., Nutley, NJ) completely neutralized the activity found in standard P388D$_1$ supernatants.

MDGF Assay

The human embryonic lung fibroblasts IMR-90 were used to measure levels of MDGF in culture supernates using a modification of the procedure described previously.[9] Briefly IMR-90 lung fibroblasts (2×10^4) were cultured in the presence or absence of culture supernates from bronchoalveolar cells of control and treated rats for 48 hours in a final volume of 200 μl DMEM supplemented with 0.5% FBS. Fibroblast cultures were pulsed with I μCi/ml tritiated thymidine (^{3}H-TdR, spc. act. 2.0 Ci/mM, New England Nuclear, Mississauger, Ontario, Canada) for the last 16 hours and thymidine incorporation was measured by counting the radiolabeled TCA-precipitable material. Bioassay data were calculated as net cpm by the following formula: net cpm = (cpm of fibroblasts + sample) − (cpm of fibroblasts alone). Fibroblasts incubated alone incorporated 400 cpm of [^{3}H]TdR. Incorporation of [^{3}H]TdR in the presence of sample dilutions was compared with that in the presence of dilutions of a standard preparation of MDGF obtained from AMs incubated for 4 hours. MDGF activity was quantified by calculation of half-maximal units.[18]

Statistical Analysis

Results are expressed as mean values ± standard error of the mean (SEM). The statistical significance between treated and control groups was determined using a one-way ANOVA and Tukey test (Statsgraphics) ($P < 0.05$).

Results

Lung Lesions Induced by Various Silicate Fibers

Table 1 summarizes the lung histologic changes observed 1 and 8 months after exposure to various doses of xonotlite, UICC chrysotile B, short 4T30 chrysotile, or attapulgite. One month after exposure, the histopathologic changes in the lung were essentially the same as we described previously.[12] While xonotlite at all the doses

Table 1 *Lung Histologic Changes 1 and 8 Months After Instillation of Various Silicate Fibers*

Treatment	Time after exposure (months)	Dose (mg)	Number of animals examined	Lung histology		
				Normal	Granuloma	Fibrosis
Saline	1	—	25	25	—	—
Xonotlite	1	1	5	5	—	—
		5	10	10	—	—
		10	5	5	—	—
UICC Chrysotile B	1	1	5	—	—	5(+)
		5	5	—	—	5(+)
4T30	1	1	3	—	3(+)	—
		5	7	—	7(+)	—
		10	5	—	5(+)	—
Attapulgite	1	1	5	—	5(+)	—
		5	10	—	10(+)	—
		10	5	—	5(+)	—
Saline	8	—	5	5	—	—
Xonotlite	8	5	5	5	—	—
	8	10	5	5	—	—
4T30	8	5	5	5	—	—
	8	10	5	3	2(±)*	—
Attapulgite	8	5	5	—	5(±)*	
	8	10	5	—	5(±)	

* Lesions are fewer and smaller and some appear to resolve.

tested caused no significant alteration of rat lung morphology, UICC chrysotile B (1 and 5 mg) caused severe fibrotic lesions located primarily in peribronchiolar tissues. In contrast, treatment with 1, 5, and 10 mg of short 4T30 chrysotile and attapulgite caused granulomatous reactions characterized by accumulation of macrophages, epithelioid cells, and giant cells in the alveolar structures. By 8 months, some differences in the lesions observed were seen. The granulomatous reactions originally found with short 4T30 chrysotile fibers (5 and 10 mg) resolved or decreased, whereas those observed with attapulgite (5 and 10 mg) decreased significantly in number and size. In contrast, the fibrosis observed with UICC chrysotile B persisted up to 6 months[9] and 9 months (unpublished data) after exposure. No significant alteration of lung morphology was seen with xonotlite (5 and 10 mg) even at 8 months.

Changes in Bronchoalveolar Cell Populations

Differential analysis of bronchoalveolar cells obtained from rats treated with various fibers was performed in correlation with lung histology. Figure 1 shows that 1 month after exposure, this was a significant increase in the number of macrophages from attapulgite- and chrysotile B-treated rats as well as significant increases in neutrophils (6% and 1.6% of total cells, respectively [5 mg], compared to 0.1% for controls), and lymphocytes (3.6% and 1.2% of total cells, respectively, [5 mg] compared to 0% in controls). In contrast, cell populations from 4T30- and xonotlite-treated animals were not significantly different from controls. On the other hand, 8 months after treatment the increases in macrophage seen in animals exposed to attapulgite were no longer apparent, although the accumulation of neutrophils and lymphocytes was still present (1.6% and 1% of total cells, respectively [5 mg] compared to 0.2% and 0% for controls) (Figure 1). Furthermore no significant change in cell populations was noticed, even 8 months after exposure to 4T30 chrysotile or xonotlite. As reported earlier,[9] no significant change in bronchoalveolar cell population was found in animals exposed to chrysotile B 6 months after exposure.

Subsequent analysis of the morphology of AMs recovered from the lungs of these animals revealed striking morphologic differences and the number of MGCs was monitored. By 1 month (Figure 2), a significant number of multinucleated giant macrophages were found in lavage fluids of all the animals exposed to attapulgite and 4T30 chrysotile fibers (5 mg). By contrast, the number of MGCs found in animals exposed to chrysotile B and xonotlite

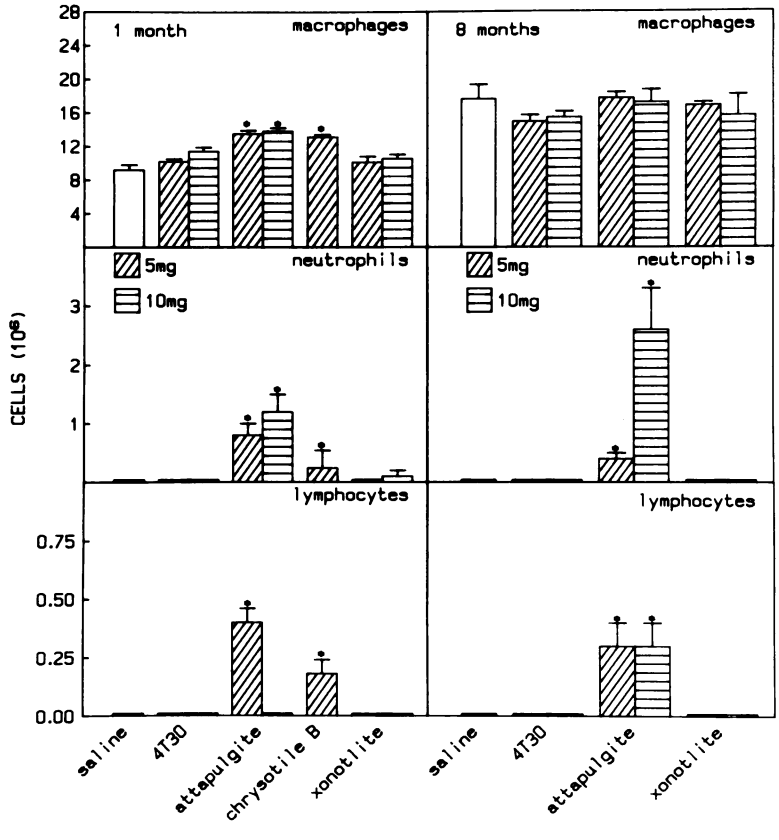

Figure 1. *Differential analysis of bronchoalveolar cells obtained from rats treated with various fibers. Bronchoalveolar cells were obtained by bronchoalveolar lavage and differential counts were made from cytocentrifuge smears stained with Wright-Giemsa, as described in Materials and Methods. Absolute numbers of cells were derived by multiplying the percentage of total cells for each category by the total cell counts. Values are mean ± SEM of at least five animals per group. *Significantly different from control at* $P < 0.05$.

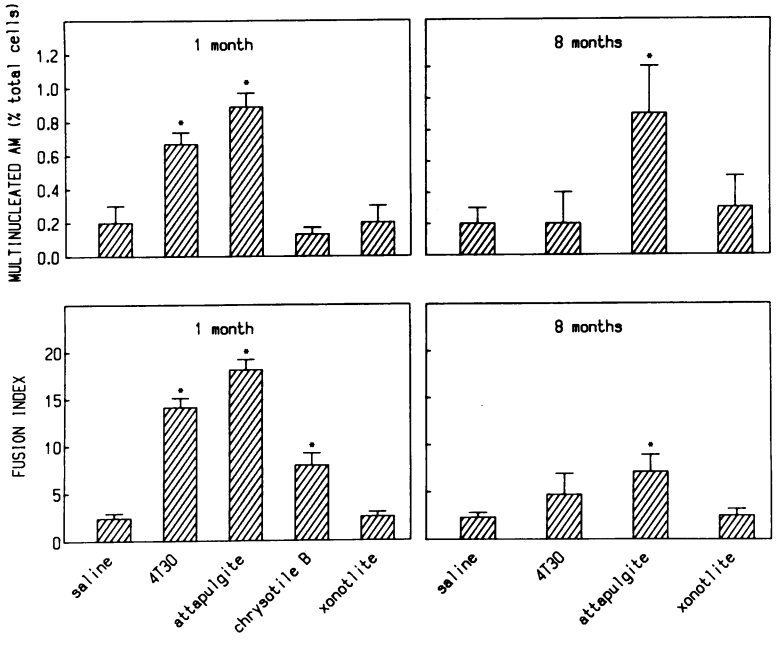

Figure 2. *Analysis of multinucleated giant AM present in bronchoalveolar lavage of control and treated animals (5 mg). One thousand cells were counted by two different observers. Values are expressed as percentage of AM with three or more nuclei or fusion index (fi) and represent mean ± SEM of five animals. *Significantly different from control at* $P < 0.05$.

was not significantly different from controls. Giant macrophages were still elevated 8 months after treatment in animals exposed to attapulgite (Figure 2).

Production of Macrophage-derived Growth Factor

Accumulating evidence indicates that AMs produce growth factor(s) for fibroblasts and previously we showed that lung lavage cells (99% AM) from animals exposed to chrysotile asbestos produced enhanced levels of these mediators for periods up to 6 months[9] and 9 months (unpublished). Therefore it was of interest to investigate the production of MDGF by AMs from animals with pulmonary granuloma. As shown in Figure 3, AMs from animals exposed to 4T30 chrysotile, attapulgite, and xonotlite did not produce higher levels of fibroblast-stimulating activity than controls 1 month after exposure. Only AMs from animals exposed to UICC chrysotile B asbestos exhibited higher production of MDGF. Furthermore MDGF production could not be stimulated further *in vitro* by MDP, a macrophage activator,[19] both in control and treated animals.

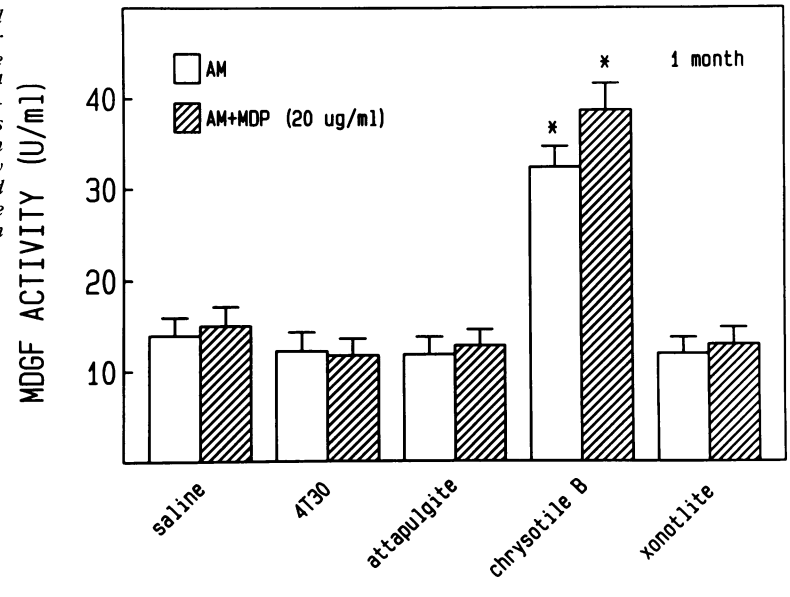

Figure 3. *MDGF production by AM of control and treated animals (5 mg). Bronchoalveolar cells were obtained by lung lavage and were incubated at a density of* 0.5×10^6*/ml for 4 hours. The culture media were collected, centrifuged, and incubated at various dilutions with* 2×10^4 *fibroblasts.* ^{3}*H-TdR incorporation was measured at 48 hours and MDGF activity was calculated as described in Materials and Methods. Values are mean ± SEM of at least five animals per group. *Significantly different from control at* $P < 0.05$.

Production of IL-1–like Activity

Alveolar macrophages released significant levels of thymocyte-activating factor (6.7 U/ml) with stimulation with MDP. When AM supernatants from control and chrysotile B-treated rats were reacted with goat anti-mouse IL-1–alpha antibody, thymocyte activation was inhibited, indicating that the bulk of the activity could be attributed to IL-1 alpha (Table 2). Figure 4 shows the pattern of IL-1–like activity produced by unstimulated and MDP-stimulated AMs from control and treated animals. One month after instillation, AMs from short 4T30 chrysotile-, attapulgite-, and chrysotile B-treated rats all produced higher levels of IL-1–like activity in response to MDP. The MDP-dependent increases seen with attapulgite (47 times) and chrysotile B (57 times) were higher than those found with 4T30 (3.9 times). Unstimulated AMs from treated rats did not produce higher levels of IL-1–like activity, except in the case of attapulgite where low levels (4.8 times more than control) of IL-1–like activity were released spontaneously. By 8 months, however, the small increases seen with 4T30 fibers were no longer apparent and unstimulated AMs from attapulgite-treated animals produced amounts comparable to those of controls. While MDP-induced IL-1–like activity was still significantly higher in attapulgite-treated rats (two times greater than control), the amount produced was much lower than that observed at 1 month.

Discussion

Accumulating evidence suggests that macrophages and monokines play a critical role in granuloma formation.[3,5] Alveolar macrophages produce a plethora of molecular mediators[20] with potential role in dictating the progression and development of inflammatory responses, including IL-1, TNF-α, and fibroblast growth factors. However the precise function, relative contribution, and orchestration of these mediators during chronic inflammatory reactions is unknown. Also the role of macrophage 'subpopulations' or differentiation in regulating the progression and outcome of inflammatory reactions is unclear. In this study, we used an animal model to study AM populations and selected monokine production in two types of lung injury: resolving granuloma and irreversible fibrosis. Our findings indicate that, depending on the outcome of chronic inflammatory reactions, selective change in AM populations and monokine production occurs (Table 3). Pulmonary granuloma and fibrosis were associated with significant changes in AM morphology that parallel those seen in lung section.[11,12] Animals with lung fibrosis had an increase in the number of large mature AMs and epithelioid-like cells (manuscript submitted for publication), whereas those with granuloma and resolving granuloma exhibited a significant increase in the proportion of MGCs. These findings raise interesting questions concerning the physiologic role of these cell populations. Available evidence indicates that AMs are markedly heterogeneous, having different characteristics and functions.[21–23] With respect to this, AMs separated on Percoll density gradients, which fractionate cells on the basis of cell maturity, are functionally different for IL-1 production, expression of Ia antigen, phagocytosis, and prostaglandins. Similarly AMs obtained by a two-step lavage procedure are markedly different in their chemotactic responsiveness and their potential for aggregation.[24] Furthermore epithelioid cells, which reportedly originate from macrophages,[25] have important biosynthetic properties and share, as secretory cells, features in common with activated macrophages. These cells have been found to be poorly phagocytic, to have reduced expression of Fc and C3b receptors compared with macrophages, but to express Ia antigen.[1,26] Also MGCs, which arise from epithelioid cells,[27] were shown to be as metabolically active as macrophages. They express Ia antigen[28] and produce a variety of lysosomal enzymes[29] as well as high levels of superoxide anion.[30] It was suggested that MGCs may represent highly stimulated cells of macrophages lineage at a terminal stage of maturation.[30] Collectively all these observations support the assumption that changes in the differentiation status of AMs alter their functions and thus the progression and outcome of inflammatory reactions. However establishment of a causal role of AM-related cells in the development of granuloma and fibrosis must await characterization of the mediators produced by isolated subpopulations of AMs.

Our study also demonstrates selective changes in monokine production by AMs in both types of lung injury. While enhanced levels of IL-1–like activity were found in animals with lung granuloma and fibrosis, MDGF production was higher only in animals with irreversible fibrosis. This suggests that AM supernatants from the latter contain additional growth factors other than IL-1 and that in-

Table 2. *Inhibition of Thymocyte-stimulating Activity by Anti-Mouse IL-1 Alpha Antibody*

Supernatants	IL-1 alpha antibody	Thymocyte proliferation (% of control)
$P388D_1$	1/1000	3.4 ± 0.9
	1/2000	3.0 ± 0.8
	1/4000	3.6 ± 0.9
	1/10000	2.9 ± 0.7
Control AM + MDP*	1/4000	0
Chrysotile B AM + MDP	1/4000	3.4 ± 0.8

* MDP (20μg/ml).

Supernatants were incubated with thymocytes as described in Materials and Methods in the presence and absence of appropriate dilutions of goat anti-mouse IL-1 alpha antibody. Data represent mean ± SEM of three experiments.

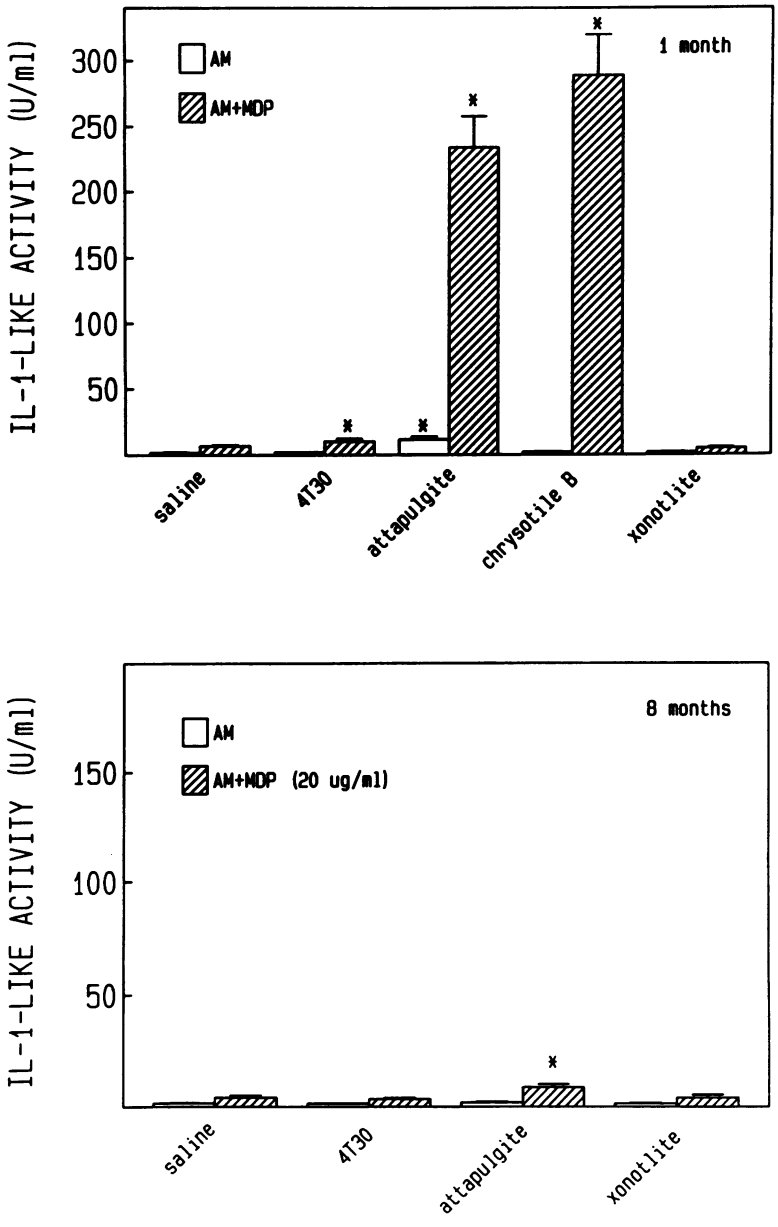

Figure 4. *IL-1 production by AM of control and treated animals (5 mg). AM (0.5×10^6) were cultured in the presence or absence of MDP (20 μg/ml) for 24 hours and their supernatants were tested at various dilutions in a standard thymocyte assay as described in Materials and Methods. Values are mean ± SEM of at least five animals per group. *Significantly different from control at* $P < 0.05$.

duction of fibroblast proliferation may result from the interactions between a combination of growth factors. In support of this, purified IL-1 was found to be unable to stimulate DNA synthesis of fibroblasts at concentrations that were mitogenic for thymocytes but could amplify the effects of suboptimal concentrations of other growth factors such as platelet-derived growth factor.[31]

Interestingly the profile of production of IL-1–like ac-

Table 3. *Summary of Findings*

	MGC		MDGF		IL-1-like activity		Histologic changes	
Months	1	8	1	6*	1	8	1	8
Saline	no	no	normal	normal (+)	normal	normal (−)	normal	normal
Xonotlite	no	no	normal	−	normal	normal (−)	normal	normal
UICC Chrysotile	no	−	++	++	++	−	fibrosis	fibrosis
Short 4T30 Chrysotile	+	no	normal	−	+	normal	granuloma	normal/resolving granuloma
Attapulgite	+	±	normal	−	++	±	granuloma	granuloma (−)

* As described by Lemaire et al.[9]

tivity and MDGF by inflammatory AMs was also quite different. Although levels of IL-1–like activity were greatly increased 1 month after treatment, they declined thereafter and returned to control levels by 8 months. This is consistent with a role of IL-1 in the early stages of inflammation possibly associated with macrophage recruitment and activation. In support of this, IL-1 has been shown to be chemotactic for monocytes[32] and AM[33] and to induce its own production[34] as well as the production of other mediators of inflammation.[34,35] Furthermore IL-1 could induce changes compatible with monocyte/macrophage differentiation[36] and in doing so may regulate the extent of its own secretion. In this regard, macrophages produce less IL-1 than monocytes and their capacity for IL-1 production decreases as they differentiate into more mature cells.[21,22,37] Thus the decreased release of IL-1–like activity seen with AMs from older animals as well as with inflammatory AMs 8 months after treatment may be related to a shift in macrophage maturation. However other mechanisms, including regulation by other cytokines or presence of inhibitory factors, cannot be ruled out. By contrast, in earlier studies[9] (and also unpublished data) MDGF production was still significantly higher at later stages of the inflammatory response (6 and 9 months), suggesting that AMs did not loose their ability to produce this mediator(s). In fact, AMs from older animals also produced higher basal levels of MDGF,[9] suggesting that mature AMs may have a greater ability to elaborate this mediator(s). Whether enhanced production of MDGF is due to an increase in the proportion of mature AMs during the inflammatory response or to induction by other cytokines remains to be elucidated. Preliminary experiments from our laboratory indicate that MDGF production cannot be induced by lipopolysaccharide LPS or MDP. The participation of other cytokines such as colony-stimulating factor and interferon gamma is being investigated.

In conclusion, these data provide evidence that the nature of AM populations and monokine production may influence the outcome of inflammatory reactions. The complexity of the cytokine network in macrophage may find its physiologic meaning during inflammatory reactions in the type of macrophages present at sites of inflammation.

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