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Effects of Instilled Fibrogenic Particles on the Clonal Growth of Murine Pulmonary Alveolar Macrophages

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Murine pulmonary alveolar macrophages (PAM) form macrophage colonies *in vitro* with colony-stimulating factors, which stimulate the clonal growth of radioresistant alveolar colony-forming cells (AL-CFC). The toxic effects of fibrogenic mineral dust particles on AL-CFC were investigated after intratracheal instillation into mice. Exposure to either crocidolite asbestos or silica (Min-u-sil) induced a significant depletion of AL-CFC as well as a decrease in PAM recovery compared to either untreated or titanium dioxide-exposed animals. Such effects were also noted with different doses (50–200μg/animal) of instilled particles. The plating efficiency of AL-CFC was depleted in PAM exposed to fibrogenic particles *in vitro*, but not when exposed to nonfibrogenic titanium dioxide particles. These results indicate the toxic effects of fibrogenic dust particles on the clonal growth of PAM, cells which play a role in the clearance of inhaled particles from the lung and in subsequent pathologic processes.

Introduction

Pulmonary alveolar macrophages (PAM) have an essential role in lung clearance and in defense mechanisms against inhaled particles (*1*). Changes in the number of PAM and in their functions may therefore affect the subsequent pathologic processes in the lung. The recovery of PAM by bronchoalveolar lavage has been reported to be reduced after the inhalation of radioactive particles (*2*) and asbestos fibers (*3*). However, it is still uncertain whether this decrease in the numbers of recovered PAM is due to the direct effects of radiation or to the toxicity of particles on a stem cell population. Normally, PAM are maintained in a steady state either by self-renewal (*4,5*) or by clonal growth of a putative stem cell: alveolar colony-forming cells (AL-CFC). These cells have the capacity to replicate and form macrophage colonies *in vitro* by culturing with colony-stimulating factors (CSFs) (*6,7*).

The present study was designed to compare *in vitro* the toxic effects of instilled fibrogenic asbestos or silica particles on murine AL-CFC with those effects induced by nonfibrogenic titanium dioxide (TiO_2). We also compared the effects of *in vitro* exposures to particles with different toxicity.

Materials and Methods

Groups of C3H/He female mice (8–10 weeks old) were instilled intratracheally (IT) with 0.1 mL phosphate-buffered saline containing either TiO_2, crocidolite asbestos (CR; Uion International Centre Cancer standard reference preparation), or a microcrystalline form of silica (Min-u-sil; The Pennsylvania Glass Sand Co., Pittsburgh). Test animals, together with untreated controls, were kept under barrier conditions for 40 days after instillation. We obtained bronchoalveolar cells (BAC) from animals by repeated bronchoalveolar lavage as described previously (*8*). The total numbers of nucleated cells in BAC preparations were determined by a Coulter counter (Model ZM). We made differential counts from cytocentrifuge preparations stained with Giemsa and by indirect immunofluorescence using anti-asialo GM_1 ($AsGM_1$) antibody specific for mouse PAM. To detect AL-CFC, 1×10^4 BAC were plated in triplicate in 1 mL of 0.3% agarose medium, supplemented with 10% fetal bovine serum (FBS) and 500 U/mL rGM-CSF in 35-mm culture dishes. These were incubated for 21 days at 37°C in a humidified environment of 5% CO_2 in air. We then fixed the cultures and stained them with Giemsa to score the number of colonies containing 30 or more cells. For *in vitro* exposures, fresh PAM were incubated with 100 μg/mL of particles in suspension at 37°C for 1 hr and assayed For the clonal growth.

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Table 1. Recovery and constituents of bronchoalveolar cells after instillation of mineral dust particles.

Animals[a]	No. of bronchoalveolar cells recovered, $\times 10^{-5}$ [b]						
	Total	PAM	$AsGM_1^+M\Phi$	Dusted MΦ	Multinucleated MΦ	Lymphocyte	PMN
Control	4.1 ± 1.2[c]	3.7 ± 1.1	3.3 ± 1.6	—	0.03 ± 0.02	0.3 ± 0.2	0.05 ± 0.05
Titanium dioxide-exposed	3.6 ± 1.1	3.5 ± 1.0	3.5 ± 1.1	2.6 ± 1.0	0.04 ± 0.03	0.4 ± 0.3	0.04 ± 0.04
Min-u-sil	2.8 ± 0.7*	2.2 ± 0.3*	2.4 ± 0.2	1.6 ± 0.8	0.15 ± 0.08*	0.4 ± 0.2	0.27 ± 0.04*
Crocidolite	3.4 ± 1.4	2.8 ± 1.0*	2.9 ± 1.1	1.4 ± 0.7	0.43 ± 0.19*	0.4 ± 0.3	0.21 ± 0.06*

Abbreviations: PAM, pulmonary alveolar macrophage; $AsGM_1^+M\Phi$, asialo GM_1-positive macrophage; multinucleated MΦ, macrophage with more than two nuclei; PMN, polymorphonuclear leukocyte.

[a]Groups of mice were untreated (control) or exposed intratracheally to 200 μg of each particle preparation, and bronchoalveolar cells were harvested on day 30.

[b]Total recovery of cells was counted by a Coulter counter, and the number of each cell type was enumerated by the proportion (%).

[c]Data represent mean ± SD of six animals in each group.

*Significant difference compared to the control, $p < 0.05$.

Results

Recovery of PAM from Mice Instilled with Various Dusts

The numbers of BAC recovered from untreated control mice were in the range of 3.0–5.0 × 10^5, of which 90–95% were PAM or $AsGM_1$-positive macrophages (MΦ), and 5% or less were lymphocytes and polymorphonuclear leukocytes (PMN). The numbers of BAC and PAM recovered by lavage were reduced significantly both in the asbestos- and silica-exposed animals during the 40-day period after instillation, whereas those from TiO_2-exposed animals were not reduced significantly. Numbers of PMN and multinucleated MΦ were increased significantly both in the asbestos- and silica-exposed animals as compared to the control and TiO_2-exposed mice (Table 1). The proportions of particle-laden PAM ("dusted" MΦ) were 60–70% in TiO_2-exposed animals, 30–60% in silica-exposed animals, and 20–40% in asbestos-exposed animals.

Cloning Efficiency of AL-CFC Exposed to Dusts *in Vivo*

When normal BAC (mostly PAM) were incubated in agarose medium containing rGM-CSF for 21 days, the plating efficiency for macrophage colonies (AL-CFC) was about 1.8–2.0%. Compared to the untreated animals, the plating efficiency was considerably reduced with cells from both the asbestos- and silica-exposed animals, although the reduction was not significant in TiO_2-exposed animals (Fig. 1*A*). As shown in Figure 1*B*, the number of AL-CFC with cells recovered from TiO_2-exposed animals was not much affected by doses within the range 50–200 μg. However, the effect of dose was much more marked with cells from animals exposed to silica and asbestos.

As compared to the control, the plating efficiency of AL-CFC was much lower (about 0.3–0.4% per total BAC plated and about 0.4–0.5% per total PAM plated) both in the groups of asbestos- and silica-exposed animals (Table 2). The plating efficiency of AL-CFC was not significantly different in TiO_2-exposed animals from the control.

Cloning Efficiency of Cells Exposed to Dusts *in Vitro*

To test the effects of dust particles with a range of toxicity, normal BAC were exposed *in vitro* to 100 μg/mL of each type of dust in suspension at 37°C. After 1 hr, cells that had incorporated or bound particles were separated on a Percoll gradient solution (density 1.074), washed, resuspended, and incubated in agarose medium containing rGM-CSF. As shown in Figure 2, the number of AL-CFC after exposure to TiO_2 particles did not differ from the untreated control. Both asbestos (crocidolite) and silica (Min-u-sil and alpha quartz) depleted the plating efficiency of AL-CFC severely. Fly ash also suppressed colony formation, but not as much as either crocidolite or silica.

Discussion

In murine PAM, there is a subpopulation (AL-CFC) with proliferative capacity *in vitro*. AL-CFC have been recognized as more radioresistant (*9*) and lass sensitive to hydrocortisone acetate than hemopoietic macrophage progenitors (*10*), although their role *in vivo*, especially in relation to the renewal of PAM, has never been elucidated fully. The present results may indicate

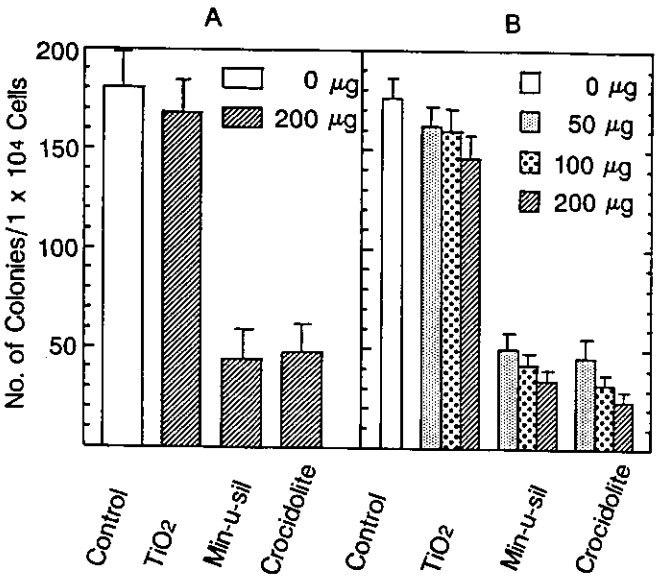

FIGURE 1. Effect of instilled mineral dust particles on the growth of alveolar macrophage colony-forming cells (AL-CFC). (*A*) Number of colonies per 1 × 10^4 bronchoalveolar cells (BAC) recovered from untreated (control) mice or from animals exposed to 200 μg of each type of dust on day 30 after instillation. (*B*) Number of colonies per 1 × 10^4 BAC recovered from untreated (control) animals or animals exposed to 50–200 μg of each type of dust on day 30 after instillation. Bars indicate the mean ± SD of triplicate experiments.

Table 2. Effect of instilled mineral dust particles on the plating efficiency of alveolar colony-forming cells.

Animals[a]	No. CFC per 1 × 10^4 cells[b]	No. PAM plated, × 10^{-3}	Plating efficiency, %	
			Per total	Per PAM
Control	181 ± 21[c]	9.6 ± 0.2	1.80 ± 0.2	2.06 ± 0.5
Titanium dioxide-exposed	158 ± 16	9.6 ± 0.1	1.58 ± 0.1	1.64 ± 0.1
Min-u-sil	37 ± 11*	8.7 ± 0.6	0.37 ± 0.1*	0.43 ± 0.1*
Crocidolite	35 ± 10*	8.7 ± 0.4†	0.33 ± 0.1*	0.45 ± 0.1*

Abbreviations: CFC, colony-forming cells; PAM, pulmonary alveolar macrophages.

[a]Groups of mice were untreated (control) or exposed intratracheally to 200 μg of each particle preparation, and bronchoalveolar cells were harvested on day 30.

[b]Indicates the number of alveolar colony-forming cells per 1 × 10^4 bronchoalveolar cells incubated for 21 days in 0.3% agarose medium containing 500 U/mL rGM-CSF.

[c]Data represent means ± SD of three separate experiments in each group.

*Significant difference compared to control, $p < 0.01$.

†Significant difference compared to control, $p < 0.05$.

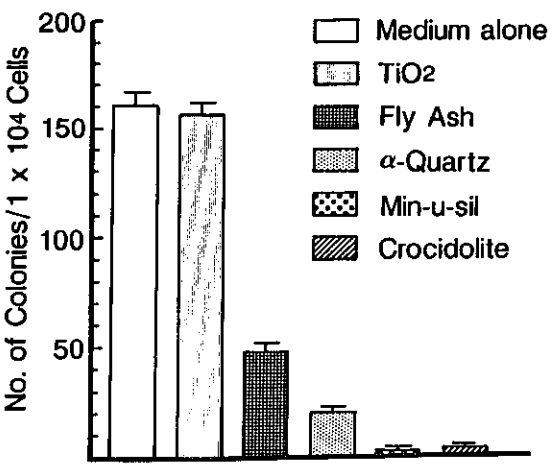

FIGURE 2. Effect of *in vitro* exposures to dust particles on the clonal growth of pulmonary alveolar macrophages (PAM). Normal PAM were either untreated (medium alone) or exposed *in vitro* to 100 μg/mL of each particle preparation in suspension at 37°C for 1 hr. The number of colonies per 1 × 10^4 viable cells plated was determined. Bars indicate the mean ± SD of triplicate experiments.

the significance of AL-CFC in the maintenance or self-renewal of PAM in the lung. The reduction of PAM recovered from asbestos- or silica-exposed animals reflects the impairment of PAM to sustain their own stem cells. After instillation of asbestos or silica, AL-CFC, a putative stem cell population, was depleted to 10% or less of the normal level, and this could account for the observed decreased in numbers of PAM. Because of the long turnover time (*11*), the population size of PAM will decline after losing the capacity for clonal growth. On the contrary, TiO_2 particles failed to induce a significant reduction in numbers of PAM and in the plating efficiency of AL-CFC, although a high proportion of PAM had phagocytized particles. After the exposure to TiO_2 particles *in vitro*, AL-CFC were detected with almost equal plating efficiency as in the untreated controls, although silica and asbestos depressed the plating efficiency of AL-CFC. These results may indicate the toxicity of fibrogenic mineral dust particles on the clonal growth of PAM, although both the specific toxic mechanism and how such acute-phase damage is related to late-developing fibrogenesis is unknown. In conclusion, toxic effects of fibrogenic mineral dusts on AL-CFC may lead to an impairment of PAM to function in the clearance of particles from the lung and in subsequent pathologic processes.

REFERENCES

1. Hocking, W. G., and Golde, D. W. The pulmonary alveolar macrophage. N. Engl. J. Med. 301: 639–645 (1979).
2. Talbot, R. J., Nicholas, L., Morgan, A., and Moores, S. R. Effect of inhaled α-emitting nuclides on mouse alveolar macrophages. Radiat. Res. 119: 271–285 (1989).
3. Kagan, E., Oghiso, Y., and Hartmann, D-P. The effects of chrysotile and crocidolite asbestos on the lower respiratory tract: analysis of bronchoalveolar lavage constituents. Environ. Res. 32: 382–397 (1983).
4. Sawyer, R. T. The significance of local resident pulmonary alveolar macrophage proliferation to population renewal. J. Leukocyte Biol. 39: 77–87 (1986).
5. Tarling, J. D., Lin, H. S., and Hsu, S. Self renewal of pulmonary alveolar macrophages; evidence from radiation chimera studies. J. Leukocyte Biol. 42: 443–446 (1987).
6. Chen, D-M., and Lin, H-S. Interaction of murine colony-stimulation factor (CSF-1) with alveolar mononuclear phagocytes. Exp. Cell Res. 140: 323–329 (1982).
7. Lin, H-S., Kuhn, C., and Kuo, T-T. Clonal growth of hamster free alveolar cells in soft agar. J. Exp. Med. 142: 877–886 (1975).
8. Oghiso, Y., Kubota, Y., Takahashi, S., and Sato, H. Effect of ^{89}Sr-induced monocytopenia on splenic and pulmonary alveolar macrophage populations in a normal steady state. J. Radiat. Res. 29: 189–202 (1988).
9. Lin, H-S., Kuhn, C., and Chen, D-M. Radiosensitivity of pulmonary alveolar macrophage colony-forming cells. Radiat. Res. 89: 283–290 (1982).
10. Lin, H. S., Kuhn, C., and Chen, D-M. Effects of hydrocortisone acetate on pulmonary alveolar macrophage colony-forming cells. Am. Rev. Respir. Dis. 125: 712–715 (1982).
11. Coggle, J. E., and Tarling, J. K. Cell kinetics of pulmonary alveolar macrophages in the mouse. Cell. Tissue Kinet. 15: 139–143 (1982).