Asbestos and Silica-induced Changes in Human Alveolar Macrophage Phenotype

Andrij Holian,1 Margaret O. Uthman,2 Tatiana Goltssova,2 Steven D. Brown,1 and Raymond F. Hamilton Jr.1

1Departments of Internal Medicine and 2Pathology, University of Texas Houston Health Science Center, Houston, Texas

The mechanism by which fibrogenic particulates induce inflammation that can progress to lung fibrosis is uncertain. The alveolar macrophage (AM) has been implicated in the inflammatory process because of its function and reported release of inflammatory mediators when isolated from fibrotic patients. It has been recently shown that fibrogenic, but not nonfibrogenic, particulates are highly potent in inducing apoptosis of human AM. In this study, we tested the hypothesis that fibrogenic particulates could shift the phenotypic ratio of human AM to a more inflammatory condition. The macrophage phenotypes were characterized by flow cytometry targeting the RFD1 and RFD7 epitopes. Results demonstrated that chrysotile and crocidolite asbestos, as well as crystalline silica, but not titanium dioxide or wollastonite, increased the RFD1+ phenotype (inducer or immune activator macrophages) and decreased the RFD1+RFD7+ phenotype (suppressor macrophages). These results provide a mechanistic explanation that may link apoptosis (namely, suppressor macrophages) to a shift in the ratio of macrophage phenotypes that could initiate lung inflammation. — Environ Health Perspect 105(Suppl 5):1139-1142 (1997)

Key words: RFD1, RFD7, inflammation, fibrosis, apoptosis

Introduction

Inhalation of fibrogenic particulates such as asbestos and silica results in the development of lung inflammation that can progress to lung fibrosis (1). Although these particulates have long been known to cause lung fibrosis, there is as yet no comprehensive understanding of the molecular and cellular events that lead to fibrosis. Much work in this area has focused on the alveolar macrophage (AM). The rationale behind this is 2-fold. First, AM would be expected to encounter asbestos and silica as they are the first line of lung defense against microbes and are responsible for phagocytosis of inhaled foreign material (2). Second, AM taken from fibrotic patients release a variety of proinflammatory mediators capable of stimulating fibroblast proliferation and collagen synthesis, which are hallmarks of fibrosis (3). For example, a previous study has shown that even in the absence of clinical evidence of lung fibrosis, subjects exposed heavily to asbestos for a number of years had AM that were much more active in releasing inflammatory mediators than matched subjects with lower asbestos exposures (4). Therefore, macrophage activation appears to be consistent with the development of inflammation and fibrosis.

To study the mechanism(s) of macrophage activation by fibrogenic particulates, a number of laboratories, including our own, have attempted to replicate human AM activation by these particulates. Although there is some increased release of inflammatory cytokines in response to silica and asbestos, the release is orders of magnitude less than that observed using macrophages taken from fibrotic patients (4). This suggests that some other factor or process may be involved with particulate activation of macrophages.

Several studies have demonstrated the presence of distinct macrophage phenotypes within the AM pool obtained by bronchoalveolar lavage from human subjects. The macrophage phenotypes have been described on the basis of expression of RFD1 and RFD7 epitopes found on human AM (5,6). RFD1 corresponds to a 28- to 33-kDa protein within the MHCI complex while RFD7 corresponds to a 77-kDa protein found intracellularly and on the cell surface (6). Using these markers, Poultre and colleagues have classified RFD1+ AM as those strongly stimulating T lymphocytes in allogenic-mixed lymphocyte reactions, dendriticleike, nonadherent to glass, weakly phagocytic, and containing low amounts of intracellular fluorescent material (7-9). RFD7+ AM are mature phagocytic cells (9), and RFD1+RFD7+ AM function as suppressor cells (7,9). Both RFD7+ and RFD1+ AM have significant amounts of intracellular fluorescent material, suggesting phagocytic activity. RFD1+7− cells are very poor stimulators of allogenic mixed lymphocyte reactions and were reported to actively repress the T-cell stimulatory capacity of RFD1+ AM (7,9).

Furthermore, addition of RFD1 monoclonal antibodies (MoAb) inhibits RFD1+ AM-mediated T-cell stimulatory activity (10). Studies of these AM phenotypes in patients with various inflammatory diseases strongly suggest that shifts in phenotypes could play a role in disease progression (7,11), and regulation of these phenotypes could be important in altering disease progression and/or outcome. Therefore, if fibrogenic particulates cause a shift in these populations to a higher ratio of RFD1+ to RFD1+7− cells, we propose that it could explain in vitro and in vivo findings that a shift in phenotype is required prior to a net change in AM activity. An increase in RFD1+ cells would increase T-cell activation, resulting in interferon-γ release and development of the inflammatory cascade.

A potential mechanism for change in macrophage phenotype may be apoptosis of the suppressor population. The ability of fibrogenic particulates, but not of nonfibrogenic particulates, to cause human AM apoptosis has been described recently (12,13). Apoptosis was evident at concentrations of particulates well below those causing any evidence of other forms of cell injury and below doses causing human AM release of inflammatory cytokines (4). In addition, another fibrogenic agent,
bleomycin, was reported to be a potent inducer of apoptosis in human AM (14). Whether this defines a pattern remains to be determined. Nevertheless, apoptosis of suppressor macrophages provides a mechanism that could cause a relative increase in the effector to suppressor macrophage ratio.

The present study was designed to test the hypothesis that fibrogenic particulates such as asbestos and silica could affect the phenotype ratio of human AM in vivo. Human AM were incubated with chrysotile (CHR), crocidolite (CRO), crystalline silica (SIL), wollastonite (WOL), and titanium dioxide (TIO), then examined by flow cytometry for changes in expression of RFD1 and RFD7 surface markers.

**Materials and Methods**

**Cell Cultures**

Human AM were obtained by bronchoalveolar lavage of normal, nonsmoking adult volunteers of either gender as previously described (15). Instillations of 240 to 300 ml sterile saline resulted in recoveries of 200 to 260 ml of lavage fluid that was kept at 4°C until cells were isolated from the lavage fluid by centrifugation. The saline supernatant was aspirated and discarded, and the cell pellet was resuspended in a small volume (1–5 ml) of HEPES-buffered medium 199 (GIBCO BRL, Gaithersburg, MD) with 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO) and antibiotics (50 U/ml penicillin, 50 μg/ml gentamycin, and 50 μg/ml streptomycin). The cell count was determined with a ZB1 Coulter Counter (Coulter Electronics, Hialeah, FL). Lavages yielded an average of 20 × 10⁶ cells that were greater than 92% AM, as verified by Leukostar staining (Fisher Scientific, Houston, TX). Viability was greater than 90% as determined by trypan blue exclusion.

Cells (1 × 10⁶ cells/ml) were cultured at 37°C in the media described above for 4 or 24 hr in the presence of no particulate (CNT), CHR (25 μg/ml), CRO (75 μg/ml), SIL (133 μg/ml), TIO (60 μg/ml), and WOL (200 μg/ml). Cell cultures were maintained in suspension by slow end-over-end tumbling (Labquake Shakers, Labindustries, Berkeley, CA) in sterile propylene tubes (PGC Scientific, Gaithersburg, MD) at 37°C in a water-jacketed CO₂ incubator (Queue, Parkersburg, WV).

**Particulates**

Two forms of asbestos were used in this study: CHR (Union Carbide RF144, 1–5 μm length) and CRO (UICC standard reference, 2–10 μm length). SIL (average size 5 μm) was acid-washed with min-U-sil-5 from Pennsylvania Glass Sand Corp. (Pittsburgh, PA). TIO (average particle diameter 0.45 μm) was purchased from Particle Information Services (Kingston, WA). WOL was provided by NYCO Minerals (Willsboro, NY). Particulate suspension concentrations were based on relative surface areas for the various particulates (25 μg/ml CHR is approximately the same surface area as 75 μg/ml CRO, 200 μg/ml WOL, 133 μg/ml SIL and 60 μg/ml TIO). Surface areas for asbestos were obtained from Timbrell et al. (16). WOL from information provided by the supplier, and surface areas were calculated for SIL and TIO based on particle size. Relative surface areas were selected as a measure of equivalency based on equal involvement of the AM plasma membrane upon contact with the particles. The upper limit of the suspension concentration was determined by the relative cytotoxicity of these fibers in a 24-hr cell culture. At concentrations greater than 25 μg/ml, CHR decreased cell viability, and it became the concentration-limiting factor. All particulates were dispersed in saline before use by 10 passages through a syringe fitted with a 25-gauge needle. Stock suspensions of particulates were prepared fresh just before their addition into AM cell culture.

**Immunostaining**

At the termination of cell culture (4 or 24 hr), all cultures were centrifuged in an Eppendorf Microcentrifuge 5415C (Brinkman Instruments, Engelsdorf, Germany), at maximum speed (12,000×g) for 20 sec. The culture media was aspirated and the cell pellet (5 × 10⁵ cells) was resuspended in 500 μl phosphate-buffered saline (PBS) with 3.5% bovine serum albumin (BSA). The monoclonal antibodies to RFD1* (murine IgM) and RFD7* (murine IgG1) surface antigens (Serotec, Kidlington, Oxford, England) were added concomitantly at a 1:200 dilution (2.5 μl in 500 μl). This mixture was incubated for 30 min at room temperature. The incubation was terminated as stated above with centrifugation and aspiration. The cell pellet was then washed 3 times in PBS (resuspension, centrifugation, and aspiration 3 times). The cell pellet was then again suspended in PBS/BSA buffer and the fluorescein anti-mouse IgM and the R-phycocerythrin antitumous IgG (Vector Labs, Burlingame, CA) were added concomitantly at 1:100 dilution (5 μl in 500 μl) and incubated 30 min at room temperature. This incubation was terminated and washed 3 times in PBS as described above. The cell pellet was then suspended in 1% formaldehyde (PBS buffered) and stored at 4°C before flow cytometric analysis. Flow cytometry was performed on a FACScan flow cytometer (Becton-Dickinson, Bedford, MA) using Consort 30 software. Using forward and side scatter of the total cell population, gates were drawn to include macrophages based on size and granularity of the cells. The instrument was calibrated with fluoresecin-isothiocyanate (FITC)- and phycoerythrin (PE)-coated beads to compensate for any overlap within the green and red fluorescence wavelengths. Cells stained without the inclusion of primary antibodies resulted in no significant staining.

**Statistical Analysis**

Because of variances in the RFD phenotypes of individuals, data were normalized to control values for each subject. Data are expressed, therefore, as a ratio of control, with the control value always equal to 1. For statistical analysis, particulate exposures were categorized as either fibrogenic (CHR, CRO, and SIL) or nonfibrogenic (TIO and WOL). The statistical comparison between these two categories at each time point (4 and 24 hr), and for each phenotype (RFD1*, RFD7*7) was performed by a one-tailed Mann-Whitney Test.

**Results**

**Normal Distribution of Human Alveolar Macrophage Phenotypes**

Much of the data from other laboratories characterized the distribution of the RFD1 and RFD7 epitopes on human AM using a fluorescence microscope. Since this study was done using flow cytometry to examine surface markers, the normal distribution was determined by this analysis. The results from analysis of 12 normal subjects are shown in Figure 1. The percentages of RFD1*, RFD1*7*, RFD7* were 5, 43, and 5, respectively. Previous studies have reported distributions of 11.4, 45.2, and 43.3 for RFD1*, RFD1*7*, RFD7*, respectively (17). However, those analyses were performed using immunohistochemical staining in which both surface and intracellular markers are stained. It has been reported that RFD1 is primarily surface and RFD7 is intracellular and surface (5). Therefore, the difference in the results suggest that RFD7 on double-stained cells is on
Another potent but unrelated fibrogenic agent, bleomycin, also induces apoptosis of human AM (14). This correlation between apoptosis of human AM and development of fibrosis suggests that apoptosis could be an early event in the development of lung inflammation. However, apoptosis usually does not result in the development of inflammation, especially since programmed cell death is required for normal development (18). Therefore, the potential association between human AM apoptosis and development of an inflammatory condition required further clarification. The results from the present study may provide that association.

The lung is constantly exposed to a wide variety of foreign substances that impinge on the epithelial surface of the respiratory tract. Therefore, in order to prevent constant sensitization to these non-pathogenic substances and antigens, a non-specific immune suppression mechanism must exist. This suppression of the lung mucosa can be explained in part by the presence of a relatively high fraction (40–50%) of RFD1\(^{-}\) AM, which down-regulate the immune response in the lung while providing nonspecific host defense (8). The lung also contains a low fraction (5–10%) of an immune active phenotype, characterized as being RFD1\(^{+}\) (17). Observations consistent with the role of these two cell populations are increases in the RFD1\(^{-}\)/RFD1\(^{+}\) ratio reported in lung inflammatory conditions such as asthma and sarcoidosis (11,17,19,20). Therefore, the ability of xenobiotic agents to cause a shift in macrophage phenotypes should correlate with their ability to induce lung inflammation.

The results from the present study indicate that fibrogenic, but not nonfibrogenic particulates, increase the RFD1\(^{-}\)/RFD1\(^{+}\) ratio consistent with that observed in human lung inflammatory conditions. Because there was an increase in RFD1\(^{-}\) and a decrease in RFD1\(^{+}\) cells, the net change in the ratio was approximately 4-fold. Furthermore, the lack of effect of nonfibrogenic particulates suggests that the changes observed with the fibrogenic particulates was not due to a particular induced artifact affecting flow cytometry. Therefore, there was an excellent correlation between the fibrogenic potency and a shift in macrophage phenotype to a more inflammatory ratio consistent with the disease etiology.

The effects of the fibrogenic particulates were progressive with time, especially on the RFD1\(^{+}\) population. This outcome is consistent with the time frame of apoptosis caused by these particulates which initiates within a few hours, but progresses with time (12,13). It still remains to be
established that the RFD17+ population is more sensitive to apoptosis than the RFD1+ cell population. These conclusions are based on the assumption that the macrophage phenotypes are static. However, if the macrophage phenotypes are reversible, then it is possible that fibrogenic particulates could change the phenotype without having to evoke apoptosis. Further studies will be needed to evaluate this possibility. Although work in other laboratories has demonstrated a shift in these populations during inflammatory conditions in the lung (11, 17, 19, 20), it remains to be determined whether the same also holds for lung fibrosis. Nevertheless, it appears from this study that fibrogenic particulates can cause a rapid shift in phenotypes and could precede inflammation, i.e., the shift in phenotypes is a primary rather than a secondary event. The results also indicated that fibrogenic particulates decreased the RFD7+ phenotype. Whether the presence of the RFD7 epitope could be important in apoptosis by fibrogenic particulates remains to be determined.

In summary, these results provide a mechanistic explanation to link a number of findings. The results indicate that fibrogenic particulates cause a shift in macrophage phenotypes to a more inflammatory condition without having to induce inflammation by themselves. This is consistent with the general lack of any significant release of inflammatory mediators from human AM incubated with particulates in vitro. Therefore, the altered macrophage phenotype ratio, in combination with other immune cells, would generate an inflammatory condition, which explains the highly active macrophages obtained from fibrotic patients. These findings indicate that new approaches to therapy need to be explored, with agents that could restore the normal phenotype balance and ablate the progressive inflammatory condition in fibrosis.

REFERENCES
1. Parker WR. Silicates and lung disease. In: Occupational Lung Disorders. London: Butterworths, 1983:233–332.
2. Holt PG, Schon-Hegrad MA, Oliver J. MHC class II antigen-bearing dendritic cells in pulmonary tissues of the rat. Regulation of antigen presentation activity by endogenous macrophage populations. J Exp Med 167:262–274 (1988).
3. Rom WN, Bitterman PB, Rennard SI, Cantin A, Crystal RG. Characterization of the lower respiratory tract inflammation of nonsmoking individuals with interstitial lung disease associated with chronic inhalation of inorganic dusts. Am Rev Respir Dis 136:1429–1434 (1987).
4. Perkins RC, Scheule RK, Hamilont R, Gomes G, Freidman G, Holian A. Human alveolar macrophage cytokine release in response to in vitro and in vivo asbestos exposure. Exp Lung Res 19:55–65 (1993).
5. Janossy G, Bofill M, Poulter DW, Rawlings E, Burford GD, Navarrete C, Ziegler A, Kelemen E. Separate ontogeny of two macrophage-like accessory cell populations in the human fetus. J Immunol 136:4354–4361 (1986).
6. Poulter DW, Campbell DA, Munro C, Janossy G. Discrimination of human macrophages and dendritic cells by means of monoclonal antibodies. Scand J Immunol 24:351–357 (1986).
7. van Haarst JMW, Hoogsteden HC, de Wit HJ, Werhoeven GT, Havenith CEG, Drexhage HA. Dendritic cells and their precursors isolated from human bronchoalveolar lavage: immunologic and functional properties. Am J Respir Cell Mol Biol 1:344–350 (1994).
8. Spiteri MA, Clarke SW, Poulter LW. Isolation of phenotypically and functionally distinct macrophage subpopulations from human bronchoalveolar lavage. Eur Respir J 5:717–726 (1992).
9. Spiteri MA, Poulter LW. Characterization of immune inducer and suppressor macrophages from the normal human lung. Clin Exp Immunol 83:157–162 (1991).
10. Poulter LW, Duke O. Dendritic cells of the rheumatoid synovial membrane and synovial fluid In: Quatermier Cours D’Immunorhumatologie et Seminaire Internationale d’Immunopathologie Articulaire (Clot J, Sony J, eds). Montpellier: La Societe Francaise d’Immunologie, 1983:89–99.
11. Poulter LW, Janossy G, Power C, Sreenan S, Burke C. Immunological/physiologic relationships in asthma: potential regulation by lung macrophages. Immunol Today 15:258–261 (1994).
12. Iyer R, Hamilton RF, Li L, Holian A. Silica-induced apoptosis mediated via scavenger receptor in human alveolar macrophages. Toxicol Appl Pharmacol 141:84–92 (1996).
13. Hamilton RF, Li L, Iyer R, Holian A. Asbestos induces apoptosis in human alveolar macrophages. Am J Physiol 271:L813–819 (1996).
14. Hamilton RF Jr, Li L, Felder TB, Holian A. Bleomycin induces apoptosis in human alveolar macrophages. Am J Physiol 269:L318–L325 (1995).
15. Scheule RK, Perkins RC, Hamilton R, Holian A. Bleomycin stimulation of cytokine secretion by the human alveolar macrophage. Am J Physiol 6:L386–L391 (1992).
16. Timbrell V, Gibson JC, Webster I. UICC standard reference samples of asbestos. Int J Cancer 3:406–408 (1968).
17. Hutter C, Poulter LW. The balance of macrophage subsets may be customised at mucosal surfaces. FEMS Microbiol Immunol 5:309–315 (1992).
18. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. Science 267:1456–1462 (1995).
19. Spiteri MA, Clarke SW, Poulter LW. Alveolar macrophages that suppress T-cell responses may be crucial to the pathogenetic outcome of pulmonary sarcoidosis. Eur Respir J 5:394–403 (1992).
20. Spiteri MA, Clarke SW, Poulter LW. Phenotypic and functional changes in alveolar macrophages contribute to the pathogenesis of pulmonary sarcoidosis. Clin Exp Immunol 74:359–364 (1988).