The Effects of Asbestos Inhalation on the Distribution and Enhancement of Immunoassociated Antigen Expression of Alveolar Macrophage Subpopulation

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ABSTRACT. We have studied the effects of *in vivo* asbestos exposure on the surface immune-associated (Ia) antigen expression and distribution of alveolar macrophage subpopulations defined by continuous iso-osmotic Percoll gradients (density range: 1.006 to 1.123 g/ml) using a rat model of asbestos inhalation. Two groups of rats were exposed by intermittent inhalation (6 hr/day for 5 days/week over 4 weeks) to either amphibole (crocidolite) or serpentine (chrysotile) asbestos. A group of control rats was sham-exposed to clean air only. Alveolar macrophages from rats of three groups were obtained by bronchoalveolar lavage. During exposure, distinct differences appeared within 7 days of asbestos exposure, and some of these findings persisted in the crocidolite-exposed group for as long as 2 to 5 months after the cessation of exposure. Furthermore, relatively greater proportions of Ia-antigen positive cells were detected in several density fractions obtained from both asbestos-exposed groups (especially the crocidolite-exposed group). Multinucleated alveolar macrophages were seen frequently in all Percoll fractions after both types of asbestos inhalation. A significant proportion of multinucleated alveolar macrophages in these fractions expressed surface Ia-antigen positivity. The finding of enriched numbers of higher-density phagocytes in bronchoalveolar lavage cell subpopulations from asbestos-exposed rats may reflect the presence of newly recruited-immature monocytes and/or macrophages at sites of intrapulmonary asbestos deposition. Also, increased proportions of Ia-antigen positive cells suggest that a part of them were functionally activated.—**KEY WORDS:** alveolar macrophage subpopulation, amphibole, asbestos inhalation, Ia-antigen, serpentine asbestos.

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Asbestosis is a disease of diffuse interstitial pulmonary fibrosis caused by inhalation of asbestos fibers. The mechanisms whereby asbestos dusts exert their fibrogenic effects in the lungs are not satisfactorily understood, although a number of factors are clearly operative. There is considerable evidence that alveolar macrophages play a pivotal role in the pathogenesis of asbestosis [12, 13], because these phagocytic cells are a notable feature of histologic lesions at sites of asbestos deposition in the lungs [2, 4, 18]. Multinucleated giant cells are also a prominent component of the macrophage response to inhaled asbestos [10]. It is conceivable that the accumulation of alveolar macrophages in evolving asbestotic lesions may reflect a localized tissue response to the release of asbestos-induced chemotactic stimuli within the lungs. Support for this concept is provided by experimental studies that have demonstrated that asbestos inhalation can evoke the release of chemoattractants for alveolar macrophages [11, 21].

Asbestos exposure is also associated with enhanced expression of immuno-associated (Ia) surface antigens on alveolar macrophages [8, 9]. Since

the surface expression of Ia antigens on macrophages is a useful morphologic correlate of macrophage activation [7, 15], these findings collectively suggest that contact with asbestos may induce alveolar macrophages to become functionally activated.

Studies in several species, using continuous or discontinuous density gradients, have shown that pulmonary alveolar macrophage populations are heterogeneous in nature, comprising subsets of both young and functionally more mature macrophages [3, 6, 16, 20]. It is possible that alveolar macrophages that aggregate at sites of intrapulmonary asbestos deposition may reflect increased proportions of newly recruited-immature cells, as well as increased percentages of activated phagocytic cells. Our study was designed to test this hypothesis by examining the effects of chrysotile (i.e., serpentine) and crocidolite (i.e., amphibole) asbestos inhalation on the distribution of alveolar macrophage subpopulations in the rat, as an experimental model of the human asbestos exposure situation, defined by continuous iso-osmotic Percoll gradients.

MATERIALS AND METHODS

Experimental animals and exposure protocol: The study entailed a comparison of three matched groups of adult male, inbred Fischer-344 rats. One group of rats was exposed to a time-weighted average concentration of 10.52 mg/m^3 of crocidolite asbestos. A second group of rats received a time-weighted concentration of 10.23 mg/m^3 of chrysotile asbestos. A control group of rats was sham-exposed only to filtered clean air. The exposures were performed under contract at the University of Vermont. The rats were exposed by intermittent inhalation for 6 hr/day, 5 days/week, over 4 weeks. Some animals were sacrificed on days 7 or 17, respectively, of the exposure regimen, whereas others were sacrificed at 2 to 3 weeks or 2 to 5 months, respectively, after the cessation of exposure. Histologically mild interstitial pulmonary fibrosis were seen in the lungs from asbestos-exposed group of rats sacrificed at 2–5 months, whereas were not detectable in the lungs from animals sacrificed at 2–3 weeks.

Bronchoalveolar lavage studies: Animals from the 3 exposure groups were sacrificed at various times during exposure and after the terminating of exposure. Prior to lavage, the rats were exsanguinated after anesthesia with ketamine hydrochloride (Parke-Davis, NJ) and Innovar-Vet (Pitman-Moore, Washington Crossing, NJ), 50 mg/kg and 0.1 ml/kg given intramuscularly respectively [11]. After bleeding from both side of Subclavian artery, the lung was removed from chest cavity. The trachea was cannulated with polypropylene tubing. To obtain bronchoalveolar lavage (BAL) cells, the lung was lavaged with a 150 ml warmed (37°C) calcium and magnesium free phosphate-buffered saline (PBS) supplemented with 0.6 mM EDTA (Sigma, St. Louis, MO) [20] accompanied by gentle massage of the lung. Cells were washed 3 times in PBS to remove EDTA and resuspend in RPMI-1640 medium (GIBCO, Grand Island, N.Y.) supplemented with 10% of heat-inactivated fetal calf serum and $60 \mu\text{g/ml}$ of gentamicin (RPMI-FCS). Cell number and viability were assessed by trypan blue exclusion in a hemocytometer. BAL cells as alveolar macrophages were determined by morphological features on Giemsa stained cytocentrifuge preparation and confirmed by peroxidase [14] and nonspecific esterase [22] staining.

Generation of iso-osmotic continuous density gra-

dient: A continuous iso-osmotic Percoll density gradient was generated by using a modification of the method of Chandler *et al.* [3]. Briefly, an iso-osmotic working solution of Percoll (Pharmacia) was prepared by mixing 9 part of Percoll stock solution and 1 part of 10X medium 199 (GIBCO, Grand Island, N.Y.). At room temperature, a two-chamber gradient mixer was used in which 40 ml of sterile iso-osmotic Percoll working solution was placed in the mixing chamber and equal volume of sterile RPMI-1640 was placed in second chamber. The linear gradient was formed on ice with a 6 ml of Percoll cushion in polycarbonate centrifuge tube (IEC 2806 Needham Hts, MA) at rate of 1–2 ml/min controlled by gravity. The gradient were stored overnight in the refrigerator to remove microbubbles [19]. The density of the gradient varied from 1,006 to $1,123 \text{ g/ml}$ as determined by refractive index (Bousch and Lomb refractometer, at 23°C) and density marker beads (Pharmacia).

Fractionation of alveolar macrophages: Unfractionated bronchoalveolar cells (containing at least 1×10^7 cells) suspended in 5 ml of RPMI-FCS were layered on to the gradient. The linear gradients were centrifuged at $800 \times g$ and 4°C for 30 min with no brake. The density-defined alveolar macrophages were collected with the aid of a tapping cap (Halpro Inc., Rockville, MD) using a dense sucrose solution (2.5 M solution) to displace the gradient. Serial fractions of gradient (Table 1) were collected in 10 sterile polypropylene centrifuge tubes (Corning 25319) on an ice bath at a rate of 3 ml/min using a peristaltic pump (LKB 2115-002). The fractionated density-defined alveolar macrophages were diluted with 1.5 volumes of sterile cold PBS and centrifuged at $400 \times g$ for 10 min. After 3rd washing with RPMI-1640, cell number and viability were assessed in the same manner as BAL cells.

Table 1. Density range of continuous iso-osmotic Percoll gradients

Fraction No.	Density range (g/ml)
1	<1.018
2	1.018–1.028
3	1.028–1.037
4	1.037–1.046
5	1.046–1.055
6	1.055–1.064
7	1.064–1.072
8	1.072–1.081
9	1.081–1.090
10	>1.090

Macrophage morphologic assessment: The composition of unfractionated and density-defined cell fractions (including the proportion of multinucleated macrophages) was enumerated by proportional analysis on Giemsa-stained cytocentrifuge preparations. The various fractions comprised approximately 90 to 99% macrophages in virtually all instances, as defined by nonspecific esterase positivity and by morphologic criteria. Determinations were also made of the macrophage yield (i.e., the proportion of unfractionated macrophages recoverable) in each density-defined fraction.

Assessment of Ia surface antigen expression on macrophages: The surface expression of Ia antigen on rat alveolar macrophages was determined by an immunoperoxidase technique on cytospin preparations, as described previously [8]. For this purpose, a monoclonal mouse IgG anti-rat Ia antiserum (Accurate Chemicals, Westbury, CT) was used as the primary antibody, and a biotin-labeled horse anti-mouse IgG antiserum (Vector Laboratories, Burlingame, CA) as the secondary antibody.

Interpretation of data: Results were expressed as the mean $\pm$ standard error of the mean of three to five experiments. Statistical comparisons were made using Student's *t*-test.

RESULTS

Density-defined distribution of alveolar macrophages: When unfractionated alveolar macrophages from the three groups of exposed rats were layered on continuous iso-osmotic Percoll gradients, the majority (i.e., more than 70%) of the cells were recoverable from density fractions 6 and 7 (Fig. 1). There were, nevertheless, distinct differences between sham-exposed and asbestos-exposed groups of rats with respect to the density-defined distribution of alveolar macrophage subpopulations. Thus, when rats were sacrificed on days 7 and 17 during exposure, significantly greater proportions of alveolar macrophages were recoverable in the highest density fractions (i.e., fractions 8 to 10) after crocidolite exposure than after sham-exposure

Fig. 1. Density-defined distribution of alveolar macrophage subpopulations. *: Significantly different from Sham-exposed group ($P<0.05$). **: Significantly different from Sham-exposed group ($P<0.01$). Upper and lower row of asterisks indicate differences of Crocidolite- and Chrysotile-exposed group respectively.

($P < 0.01$). Similar albeit less striking, changes were detectable after chrysotile inhalation ($P < 0.05$). The crocidolite-related effect persisted and was detectable in rats sacrificed at 2 to 3 weeks and 2 to 5 months, respectively, after the cessation of exposure ($P < 0.05$).

Proportion of multinucleated alveolar macrophages: Both crocidolite and chrysotile exposures were associated with significantly increased numbers of multinucleated alveolar macrophages recoverable by bronchoalveolar lavage, when compared with those in sham-exposed control (Fig. 2). It is noteworthy that the proportion of multinucleated macrophages was significantly increased in virtually all density-defined fractions after both types of asbestos exposure (Table 2). This effect occurred within 7 days of the onset of asbestos exposure and persisted throughout the entire period of the study.

Ia surface antigen expression on alveolar macrophages: The proportion of Ia antigen-bearing alveolar macrophages was also significantly increased after asbestos exposure in several density-defined fractions (Table 3). Although this finding was noted in the context of both types of asbestos exposure, it was more prominent after crocidolite than chrysotile exposure. Once again, the effect could be detected within 7 days of beginning asbestos inhalation and, in the case of crocidolite, persisted throughout the entire course of the study. Furthermore, when consideration was given to the presence of Ia-antigen positive multinucleated

alveolar macrophages in the various density-defined fractions, both types of asbestos inhalation were associated with relatively large proportion of such cells (Table 4). Thus, approximately 2 to 32% of multinucleated macrophages expressed the Ia surface marker in different density-defined fractions. By contrast, Ia-positive multinucleated cells were almost never detected in any Percoll fraction after sham exposure.

DISCUSSION

The study has demonstrated that the deposition of asbestos fibers in the lungs produces striking changes in the composition and distribution of alveolar macrophage subpopulations defined by continuous iso-osmotic Percoll gradients. These alterations are characterized by increased proportions of multinucleated and higher-density macrophages after asbestos exposure. Alveolar macrophages in the higher-density fractions have been shown to be developmentally immature [5, 20]. Thus, the finding of enriched numbers of higher-density phagocytes in bronchoalveolar lavage cell subpopulations from asbestos-exposed rats may reflect the presence of newly recruited-immature monocytes and/or macrophages at sites of intrapulmonary asbestos deposition. Studies demonstrating the presence of asbestos-related chemoattractants for alveolar macrophages give credence to this notion [11, 21].

Because alveolar macrophage populations are heterogeneous in nature, their overall function is dependent on mixtures of cells with variable functional capabilities. A disproportionate increase in the number of functionally activated phagocytic cells can result in macrophage-mediated lung injury, as has been shown for sarcoidosis and other granulomatous pulmonary diseases [7, 15]. A similar mechanism may pertain to asbestosis, because this study has demonstrated enhancement of Ia antigen expression in most density-defined fractions after asbestos exposure. These enhancement of Ia antigen expression provides morphologic correlate of functional macrophage activation induced by asbestos. This phenomenon was particularly prominent among multinucleated alveolar macrophage subpopulations. Although the significance of these multinucleated cells in asbestosis has not been established, they may represent a distinct subset of functionally activated phagocytes. Their appearance

Fig. 2. Micrograph of Ia-antigen positive multinucleated alveolar macrophage (MAM) from crocidolite-exposed rat at 5 months after the cessation of exposure. Some of the alveolar macrophages were pierced with asbestos fibers (arrow). $\times 2,160$.

Table 2. Proportions of multinucleated alveolar macrophages (%)

	Fraction No.	Rat exposure group		
		Sham-exposed	Chrysotile-exposed	Crocidolite-exposed
During exposure (day 7)	Unfractionated	0.99±0.30	4.03±1.24*	9.50±0.87**
	1	—	2.00±2.00	8.89±6.48
	2	0.74±0.53	3.86±1.64	5.46±1.23**
	3	0.78±0.45	6.77±1.27**	5.11±1.27**
	4	1.34±0.47	5.36±1.36*	6.64±1.64**
	5	1.23±0.51	4.99±1.28*	5.91±1.19**
	6	0.96±0.42	4.55±1.04**	5.76±0.57**
	7	1.01±0.65	4.56±1.10*	6.07±1.26**
	8	0.60±0.38	7.14±1.32**	8.47±0.89**
	9	0.54±0.54	7.55±1.39**	11.64±2.00**
	10	0.20±0.20	9.55±2.31**	12.15±1.55**
During exposure (day 17)	Unfractionated	1.63±0.63	8.05±0.96**	6.55±0.83**
	1	—	1.33±1.33	—
	2	—	3.24±1.52	0.72±0.72
	3	1.29±0.54	6.96±1.77**	5.08±1.57*
	4	0.83±0.26	6.61±0.76**	5.94±1.01**
	5	0.81±0.30	5.68±0.62**	5.62±1.60**
	6	0.75±0.24	5.24±0.81**	5.91±0.65**
	7	0.81±0.15	7.24±0.86**	5.86±1.48**
	8	0.94±0.14	8.61±1.44**	8.54±1.32**
	9	1.36±0.59	6.34±0.82**	9.64±0.69**
	10	0.26±0.26	5.34±0.91**	13.53±1.26**
2-3 weeks Post-exposure	Unfractionated	1.59±0.18	5.76±1.64*	5.86±1.19*
	1	—	6.25±6.25	1.95±1.95
	2	1.13±0.57	5.38±1.31*	1.10±0.80**
	3	0.73±0.55	7.53±1.16**	3.78±0.95*
	4	0.87±0.29	5.33±0.83**	5.15±0.71**
	5	2.00±0.55	6.80±0.49**	4.83±0.58**
	6	0.90±0.29	5.21±0.49**	4.85±0.49**
	7	0.87±0.49	5.28±0.77**	4.98±0.58**
	8	1.73±0.70	7.25±3.35**	5.73±0.76**
	9	1.13±0.36	8.98±2.62*	12.20±1.88**
	10	0.50±0.50	8.20±0.52**	12.57±1.29**
2-5 months Post-exposure	Unfractionated	1.82±0.64	4.85±1.56	4.68±1.36*
	1	1.00±1.00	1.62±1.14	1.82±1.82
	2	2.53±2.07	1.08±0.80	1.27±0.60
	3	0.74±0.25	6.89±0.62**	4.08±1.08**
	4	1.02±0.33	5.07±1.32**	4.15±0.89**
	5	1.12±0.31	5.48±1.10*	4.57±1.59*
	6	1.14±0.33	4.62±0.91**	3.16±0.57**
	7	0.58±0.29	5.23±1.28**	5.06±1.28**
	8	1.72±0.71	8.04±1.02**	7.43±1.87*
	9	1.13±0.52	7.80±2.27*	11.33±2.24**
	10	0.52±0.39	5.35±1.27**	13.98±1.10**

* P<0.05 (sham-exposed vs. asbestos-exposed)

** P<0.01 (sham-exposed vs. asbestos-exposed)

mean±S.E.M. (n=5)

Table 3. Proportions of Ia-antigen positive alveolar macrophages (%)

		Rat exposure group		
	Fraction No.	Sham-exposed	Chrysotile-exposed	Crocidolite-exposed
During exposure (day 7)	Unfractionated	3.43±1.60	4.67±1.01	7.95±1.40*
	1	—	—	—
	2	0.58±0.58	—	0.15±0.15
	3	0.49±0.27	1.72±0.56*	2.01±0.47*
	4	1.53±0.84	2.55±0.67	2.55±0.90
	5	2.93±1.67	4.04±1.82	3.01±0.44
	6	1.74±0.49	3.38±1.53	4.69±1.27*
	7	1.04±0.35	3.43±1.10*	5.84±1.90*
	8	1.72±0.40	1.03±0.62	4.13±1.50*
	9	0.49±0.49	1.14±0.48	5.10±1.38**
	10	0.20±0.20	3.77±2.14	3.24±0.97**
During exposure (day 17)	Unfractionated	2.81±1.57	9.89±1.48**	11.84±2.62**
	1	—	—	—
	2	—	0.80±0.51	0.27±0.27
	3	1.46±1.02	5.16±1.81	2.28±0.80
	4	1.25±0.65	5.82±1.83*	3.52±1.73
	5	1.44±0.70	8.89±2.80*	9.10±3.58*
	6	1.27±1.06	6.58±2.70	9.38±2.20**
	7	2.06±1.42	6.21±1.57*	6.19±2.15
	8	1.59±0.62	3.91±0.79*	4.38±1.51
	9	2.13±2.13	3.13±1.27	7.92±4.93
	10	2.64±2.33	2.14±1.24	4.78±1.36
2-3 weeks Post-exposure	Unfractionated	1.54±0.55	9.86±3.23*	19.53±2.29**
	1	—	—	—
	2	1.03±1.03	1.02±0.49	2.97±1.43
	3	1.63±0.67	4.53±3.43	4.95±1.34
	4	2.54±1.21	6.83±2.94	10.15±0.92**
	5	1.34±0.81	7.52±5.02	11.95±2.35**
	6	0.91±0.52	6.80±5.06	9.08±2.63*
	7	1.21±0.66	5.98±4.58	10.14±2.96*
	8	5.04±0.86	6.47±3.07	9.27±2.20
	9	3.42±1.40	6.23±3.65	11.04±3.20
	10	5.12±2.87	12.15±7.56	15.75±2.82*
2-5 months Post-exposure	Unfractionated	4.12±1.86	9.89±2.39*	13.82±4.95
	1	0.68±0.68	1.16±1.16	2.50±2.50
	2	0.88±0.88	1.99±1.78	5.44±5.44
	3	3.22±1.38	1.20±0.60	4.39±2.65
	4	2.80±0.98	6.37±2.83	6.61±3.57
	5	3.94±1.42	4.95±1.52	9.64±1.78*
	6	1.70±0.47	5.97±2.97	10.31±4.98
	7	1.99±0.20	8.69±3.85	7.80±1.63**
	8	2.30±0.49	2.80±1.79	5.64±1.03**
	9	4.01±2.26	6.19±3.16	8.57±3.85
	10	5.63±0.66	8.73±1.79	12.43±6.66

* P<0.05 (sham-exposed vs. asbestos-exposed)

** P<0.01 (sham-exposed vs. asbestos-exposed)

mean±S.E.M.(n=5)

Table 4. Proportions of Ia-antigen positive multinucleated alveolar macrophages (%)

	Fraction No.	Rat exposure group		
		Sham-exposed	Chrysotile-exposed	Crocidolite-exposed
During exposure (day 7)	Unfractionated	—	5.22±3.23	7.89±1.70
	1	—	—	—
	2	—	—	—
	3	—	—	2.57±2.57
	4	—	2.22±2.22	1.97±1.97
	5	—	—	1.97±1.97
	6	—	1.96±1.96	2.08±2.08
	7	—	12.50±12.50	6.08±3.34
	8	—	—	1.67±1.67
	9	—	—	6.36±4.43
	10	—	—	4.00±2.68
During exposure (day 17)	Unfractionated	—	18.91±4.77	16.02±6.28
	1	—	—	—
	2	—	—	4.17±4.17
	3	—	31.50±16.04	—
	4	—	7.41±7.41	1.87±1.86
	5	—	10.85±2.07	7.78±4.01
	6	—	17.05±13.12	9.56±2.38
	7	—	11.87±1.63	10.53±1.33
	8	—	4.34±2.23	4.73±2.47
	9	—	2.38±2.38	8.51±2.19
	10	—	7.04±3.53	11.21±4.02
2–3 weeks Post-exposure	Unfractionated	—	14.87±2.55	32.20±6.54
	1	—	—	3.70±3.70
	2	—	7.00±7.00	6.07±6.07
	3	—	18.50±13.59	5.87±5.87
	4	—	16.70±6.39	11.80±3.39
	5	—	12.87±3.96	15.10±5.61
	6	—	11.40±4.30	11.93±0.99
	7	—	18.33±13.64	14.17±8.70
	8	—	2.93±2.93	15.43±7.93
	9	11.10±11.10	9.87±2.60	25.40±2.81
	10	—	11.40±2.95	16.57±5.67
2–5 months Post-exposure	Unfractionated	4.76±4.76	19.37±1.41	27.24±5.93
	1	—	—	—
	2	—	11.00±11.00	1.67±1.67
	3	—	1.73±1.73	5.88±5.88
	4	—	—	9.58±5.42
	5	—	8.67±8.67	11.79±7.85
	6	—	16.42±11.07	22.22±11.75
	7	—	24.44±12.37	18.73±7.30
	8	3.33±3.33	13.88±10.02	13.06±1.80
	9	—	11.37±6.64	11.88±7.99
	10	—	3.51±3.51	15.10±13.66

mean±S.E.M. (n=3)

may result from the fusion of macrophage membranes induced by interferon [17]. Further studies are needed to establish whether enhancement of Ia antigen expression reflect the secretion of specific alveolar macrophage secretory product (e.g. interleukin 1 and fibronectin) and lymphokines (e.g. γ -interferon and BCDF) from "activated" alveolar macrophages.

Both amphibole and serpentine exposure are capable of inducing the changes seen in this study, and these effects develop shortly (i.e., by day 7) after the start of asbestos exposure. However, the effects of crocidolite exposure tend to be more prominent and to persist for longer period after the cessation of asbestos inhalation. These variations in the biological effects of the two asbestos fiber types may relate to the relatively more efficient clearance and greater solubility of chrysotile, compared with that of crocidolite [1].

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