Exposure to ambient particles accelerates monocyte release from bone marrow in atherosclerotic rabbits

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Goto, Yukinobu, James C. Hogg, Chih-Horng Shih, Hiroshi Ishii, Renaud Vincent, and Stephan F. van Eeden. Exposure to ambient particles accelerates monocyte release from bone marrow in atherosclerotic rabbits. Am J Physiol Lung Cell Mol Physiol 287: L79–L85, 2004. First published March 5, 2004; 10.1152/ajplung.00425.2003.—Exposure to air pollution [particulate matter, particles <10 μm (PM|0)] causes a systemic inflammatory response that includes stimulation of the bone marrow (BM) and progression of atherosclerosis. Monocytes are known to play a key role in atherogenesis by migration into subendothelial lesions where they appear as foam cells. The present study was designed to quantify the BM monocyte response in Watanabe heritable hyperlipidemic (WHHL) rabbits after PM10 exposure. WHHL rabbits were given twice weekly intrapharyngeal instillations of 5 mg of PM10 for 4 wk to a total of 40 mg and compared with control WHHL or New Zealand White (NZW) rabbits. The thymidine analog 5′-bromo-2′-deoxyuridine was used to label dividing cells in the BM and a monoclonal antibody to identify monocytes in peripheral blood. The transit time of monocytes through the BM was faster in WHHL than in NZW rabbits (30.4 ± 1.9 h vs. 35.2 ± 0.9 h, WHHL vs. NZW; P < 0.05). PM10 instillation exposure increased circulating band cell counts, caused rapid release of monocytes from the BM, and further shortened their transit time through the BM to 23.2 ± 1.6 h (P < 0.05). The percentage of alveolar macrophages containing particles in the lung correlated with the BM transit time of monocytes (r2 = 0.45, P <0.05). We conclude that atherosclerosis increases the release of monocytes from the BM, and PM10 exposure accelerates this process in relation to the amount of particles phagocytosed by alveolar macrophages.

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estimated units/ml or <3.0 ng/ml) that has been shown not to stimulate AM or bronchial epithelial cells in vitro or cause either a local or systemic effect when instilled into the lung of rabbits (3, 9, 17).

Experimental animals. Female WHHL rabbits (38) (n = 10; weight, 2.9 ± 0.3 kg; Covance Research Products, Denver, PA) were used in this study. All animals were 42 wk old at the start of the experimental protocol and were fed standard rabbit chow. We also used female New Zealand White (NZW) rabbits (n = 6; weight, 2.5 ± 0.9 kg) without atherosclerosis as additional controls. The protocol was approved by the Animal Experimentation Committee of University of British Columbia.

Experimental design. The animals were challenged with intrapharyngeal instillation (18) of either the particles suspended in saline (WHHL, n = 5) or just saline (WHHL, n = 5; NZW, n = 6). Briefly, the rabbits were anesthetized with 4% isoflurane, and 1 ml of normal saline or PM10 (5 mg of EHC-93 mixed with 1 ml of saline) was instilled twice a week for 4 wk, as previously described in detail (18, 30). The dividing cells in the bone marrow were labeled by infusing 100 mg/kg of BrdU (Sigma Chemical, St. Louis, MO) intravenously intravenously 24 h before the sixth instillation (30). Blood samples obtained from the central ear artery just before (baseline) and at intervals 7 days after the initial instillation of PM10 were used to measure total leukocyte counts and were also taken at intervals from 4 to 168 h for wk or 24–168 h after BrdU injection to determine the number of BrdU-labeled monocytos (MOBrdU) or PMN (PMNBrdU), respectively (12, 34). Differential white blood cell (WBC) counts were determined by counting 200 leukocytes in randomly selected fields of view on Wright-Giemsa-stained blood smears. Sedation [fentanyl (20 μg/kg) and droperidol (1 mg/kg)] was administered by subcutaneous injection to facilitate blood collection. The rabbits were killed 4 days after the last (8th) instillation with an overdose of pentobarbital sodium, and the lungs and aorta were removed for histological evaluation using methods previously described in detail (18, 30).

Immunohistochemical detection of BrdU-labeled leukocytes in the circulation. Monocytes were identified using RbM2 (ICN Biomedicals, Aurora, OH), a monoclonal antibody specific for rabbit monocyte cytoplasmic lysosomal antigen (28). To determine the fraction of MOBrdU in the circulation, cells on cytospin preparations (12) were stained for the presence of both monocyte cytoplasm RbM2 antigen (red) and nuclear BrdU (blue) (12) using the alkaline phosphatase and antialkaline phosphatase method (6).

Cytospin preparations were also stained by the APAAP method using anti-BrdU monoclonal antibody Bu20a (Dako Laboratories, Copenhagen, Denmark) to determine the fraction of PMNBrdU (4). MOBrdU and PMNBrdU or the different subpopulations of PMNBrdU (G3, G2, and G1 cells) were evaluated, and their transit through the bone marrow was calculated as previously described in detail (12, 34). All slides were coded and evaluated by investigators without knowledge of their origin.

Histology of the lungs. Random histological sections of formalin-fixed, paraffin-embedded lung tissue stained with hematoxylin-eosin were examined for AM-containing particles. AM were divided into three categories: macrophages containing no particles in their cytoplasm (negative), <5% of the AM cytoplasm containing PM10, or >5% of the AM cytoplasm containing PM10 (18).

Statistical analysis. The results are expressed as means ± SE and analyzed using a repeated-measure ANOVA over time where the effect of multiple comparisons was corrected using the Bonferroni method. The transit times of MOBrdU or PMNBrdU were compared by one-way ANOVA, followed by Fisher’s protected least significant differences test as the post hoc test among the groups. The correlation between parameters was examined by Spearman’s rank correlation test. A corrected P < 0.05 was considered significant throughout the study.

RESULTS

Distribution of PM10 in the lung. AM-containing particles in the PM10 group of WHHL rabbits were distributed diffusely in all lung regions and had a higher percentage of particle-positive AM (17.1 ± 5.4% vs. 3.5 ± 0.7%, PM10 vs. control, P = 0.026; Fig. 1). Most positive AM had <5% of the AM cytoplasm occupied by particles (14.4 ± 4.3% vs. 3.3 ± 0.6%, PM10 vs. control, P = 0.021). There was no statistical difference in the percentage of particle-positive AM between WHHL and NZW controls.

Atherosclerosis in rabbits. The volume fraction (Vv) of atherosclerotic lesions of the aorta was estimated as previously described (30). The values tended to be higher and more variable in the PM10 compared with the control group, but this was not statistically significant (29.5 ± 12.9% vs. 20.9 ± 3.5%, PM10 vs. control, P = not significant). However, there was a correlation between the percentage of AM positive for particles and the Vv of atherosclerotic lesions in the vessels (r² = 0.694, P = 0.011). No atherosclerotic lesions were observed in the NZW rabbits.

Leukocytes in the circulation. The repeated instillation exposure to PM10 did not change WBC, red blood cell, and platelet counts (data not shown) or monocyte and PMN counts compared with the control group (Fig. 2A). However, the percentage of circulating nonsegmented PMN (band cells) increased from the third week of exposure (Fig. 2B). The number of band cells in the circulation followed the same pattern (15; 17.4 ± 3.9 vs. 5.2 ± 2.7 × 10⁸/liter, PM10 vs. control, P < 0.05; day 20: 22.1 ± 2.2 vs. 4.9 ± 0.6 × 10⁸/liter, PM10 vs. control, P < 0.05). No significant difference was seen between the circulating leukocyte counts in WHHL and NZW controls.

MOBrdU in the circulation. Figure 3 shows the release of MOBrdU and PMNBrdU in the circulation from the marrow with

![Fig. 1. Distribution of particulate matter, particles <10 μm (PM10), in the cytoplasmic surface area of alveolar macrophages exposed to PM10 (filled bars) for 4 wk or saline (control, open bars). A higher percentage of alveolar macrophages that phagocyted particles was seen in PM10-exposed group, especially in which <5% of the cytoplasmic surface area was occupied by particles. Each value represents mean ± SE from 5 Watanabe heritable hyperlipidemic (WHHL) rabbits. *P < 0.05 vs. control group. Please refer to the histology images in Refs. 18 and 30.](http://ajplung.physiology.org/)

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Fig. 2. Circulating polymorphonuclear leukocyte (PMN; squares) and monocyte (circles) counts (A) and the percentage of band cells (triangles; B) in the circulation of rabbits exposed to PM10 (filled symbols) for 4 wk or saline (control, open symbols). Arrows show the time points of PM10 instillation. The percentage of the circulating band cells increased from the third week of exposure (day 15). Values at each time point represent means ± SE from 5 WHHL rabbits. *P < 0.05 vs. control group.

Fig. 3. Release of 5'-bromo-2'-deoxyuridine (BrdU)-labeled monocytes (MOBrdU; circles) and BrdU-labeled PMN (PMN-BrdU; squares) into the circulation after instillation exposure of WHHL rabbits to PM10 (filled symbols; n = 5) or saline (control, open symbols; n = 5) for 4 wk. The first MOBrdU and PMN-BrdU appear in the circulation at 4 and 24 h after BrdU labeling, respectively. The data show that the percentage of MOBrdU increased more rapidly in the PM10-exposed group (a peak at 16 h) compared with the control group (a peak at 24 h). *P < 0.05 vs. controls. Each value represents means ± SE.
or without PM$_{10}$ instillation exposure in WHHL rabbits. The first MO BrdU appeared in the circulation 4 h after labeling, which was earlier than PMN BrdU (at 24 h). The fraction of MO BrdU reached a peak at 16 h (PM$_{10}$ group) and 24 h (control), with a more rapid increase of MO BrdU in the circulation in the PM$_{10}$ group (at 12–16 h, $P < 0.05$). The curves for the percentage of MO BrdU in the circulation were similar to the curves for the numbers of MO BrdU. In contrast, there was no significant difference in the fraction and the number of PMN BrdU in the circulation between groups.

To determine the size of the bone marrow pool, the cumulative number of MO BrdU or PMN BrdU in the circulation was calculated as previously described (33). Figure 4 shows the cumulative frequency distribution of MO BrdU, all PMN BrdU, and G1 cells (weakly stained PMN BrdU). The repeated PM$_{10}$ instillation exposure did not change the overall size of the bone marrow pool of monocytes (Fig. 4A), but there was an increase in the size of the bone marrow mitotic pool of PMN (G1 cells, $P < 0.05$, Fig. 4C) in the PM$_{10}$ group. Moreover, there was a correlation between the percentage of AM that have phagocytosed particles in the lung and the size of the bone marrow mitotic pool of PMN ($r^2 = 0.404$, $P = 0.029$; Fig. 5B).

**Transit time of monocytes through the marrow.** Table 1 shows the calculated mean transit time of MO BrdU, all PMN BrdU, and the different subpopulations of PMN BrdU (G3 and G1 cells) through the bone marrow. The monocyte transit times of the control WHHL rabbits was shorter than the control NZW rabbits (30.4 ± 1.9 h vs. 35.2 ± 0.9 h, $P = 0.041$). Moreover, the instillation exposure of the WHHL rabbits to PM$_{10}$ further shortened monocyte marrow transit time (23.2 ± 1.6 h vs. 30.4 ± 1.9 h, $P = 0.021$). The transit time of MO BrdU through the marrow also correlated with the percentage of AM that had phagocytosed particles in the lung ($r^2 = 0.456$, $P = 0.019$; Fig. 5A). The PM$_{10}$ exposure did not change the transit time of all PMN BrdU through the marrow, but there was a trend toward a reduction of their transit time through the postmitotic pool of PMN (G3 cells, $P = 0.054$).

**DISCUSSION**

Recent work from our laboratory (30) has shown that repeated exposure to PM$_{10}$ causes the progression of atherosclerosis in WHHL rabbits. These studies also showed an increase in circulating immature PMN counts with an increase in the size of the bone marrow mitotic pool of PMN. The present study extends these observations by showing that PM$_{10}$ exposure accelerates the release of monocytes from the bone marrow as a part of the systemic response to the deposition of PM$_{10}$ in the lung. This was associated with an increase in the bone marrow turnover of monocytes and a shortening of their transit time through the marrow without increasing the monocyte marrow pool size. The extent of this stimulation of the bone marrow by PM$_{10}$ exposure was related to the extent of PM$_{10}$ phagocytosed by AM in the lung. These monocytes released from the bone marrow appear into the circulation earlier than PMN. Together, these results show that PM$_{10}$ exposure stimulates the bone marrow to accelerate the release of monocytes with a different pattern from PMN.

Controlled inhalation of ambient particles by animals induces both pulmonary and cardiovascular changes (2, 5, 37). Several reports, including our own (1, 13, 18), have used...
instillation of particles and documented similar cardiopulmonary change. Instillation of particles allows more accurate control of the amount of particles that reaches the airways and lung cells that process these particles. Experimental animals have different patterns of breathing and filtering ambient particles; instillation, therefore, provides a convenient experimental tool to quantify the local and the systemic inflammatory response elicited by exposure to particles. We used intrapharyngeal instillation in our study to promote diffuse deposition and avoid localized deposition of particles in the lung. This method is well described in our previous studies (18, 30). These studies showed that ~20% (1 mg) of the dose delivered by intrapharyngeal instillation was aspirated into the lung, and <4% reached the alveolar surface. This dose of particles is comparable with other animal experiments and is relevant to human exposure (2, 18, 31). We calculated an alveolar exposure of 3.1 ng/cm² for each dose or 24.8 ng/cm² throughout the study period. Assuming a 6.5-m² alveolar surface area for a 2.9-kg rabbit, the magnitude of exposure is smaller than an estimated human exposed to 35.1 ng/cm² for 90 days at the average concentration in six U.S. cities (7).

The WHHL rabbits are hyperlipidemic as a result of consistent inbreeding from mutant rabbits (38). Their clinical features include spontaneous development of aortic atherosclerosis that bears a marked resemblance to human atherosclerosis (38). Because subjects with preexisting vascular disease and the elderly are at particular risk for these air pollution-induced adverse health effects (21, 26, 39), we have used these rabbits that naturally develop atherosclerosis. The present results confirmed that repeated exposure to PM₁₀ increased the size of the bone marrow mitotic pool of PMN and released more immature PMN (band cells) without a clearly measurable effect on PMN bone marrow transit times (Figs. 2B and 4C and Table 1). Because of lower circulating PMN counts in PM₁₀-exposed rabbits in our current study (Fig. 2A), the

### Table 1. Transit times of monocytes and PMN through bone marrow

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Monocytes</th>
<th>All PMN</th>
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<th>G1</th>
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<tr>
<td>PM₁₀ 5</td>
<td>5</td>
<td>23.2±1.6*</td>
<td>100.9±2.6</td>
<td>68.0±1.6</td>
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<td>123.2±2.0</td>
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<tr>
<td>Controls 6</td>
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<td>35.2±0.9</td>
<td>101.6±1.2†</td>
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Values are hours, means ± SE. n, Number of subjects. All polymorphonuclear leukocytes (PMN), total transit time of 5′-bromo-2′-deoxyuridine-labeled PMN; G3, transit time of PMN through the postmitotic pool; G1, transit time of PMN through the mitotic pool; NZW, New Zealand White rabbits, PM₁₀, particle matter <10 μm; WHHL, Watanabe heritable hyperlipidemia rabbits. *P < 0.05 vs. control group. †P < 0.05 vs. NZW group. ‡Data from Ref. 18.

Fig. 5. Correlation between the percentage of alveolar macrophages that have phagocytosed PM₁₀ and the transit time of monocytes through the bone marrow (A) and the bone marrow mitotic pool size of PMN (G1 cells; B) in WHHL rabbits.
calculated PMN mitotic pool size was larger in our previous study (30). In contrast, the same exposure was associated with an accelerated monocyte transit time through the marrow and an increase in their release into the circulation with no change in marrow pool size (Figs. 3 and 4A and Table 1). Together, these results confirm our previous reports on the behavior of PMN after particulate exposure (18, 30) and extend these observations by showing a different pattern of monocyte release following the same stimulation. Moreover, our results show a correlation between the percentage of AM that phagocytosed PM10 and the bone marrow transit time of monocytes (Fig. 5A), suggesting that the deposition of PM10 in the lung accelerates the release of monocytes from the marrow. The present data also show that the monocyte bone marrow pool size is independent of PM10 exposure (Fig. 4A), which supports the concept that newly formed monocytes enter the circulation immediately without undergoing a maturation process in the marrow (12, 15, 36).

Work from several laboratories, including our own (9, 16, 35), has shown that AM and lung epithelial cells both secrete cytokines when exposed to PM10 and that this production is enhanced by interaction between these cell types (9). We have also shown that several of these mediators are capable of stimulating the bone marrow as part of a systemic inflammatory response (19, 29, 32). The hematopoietic growth factors, granulocyte/macrophage (GM)-CSF and monocyte colony-stimulating factor, IL-6, and the β-chemokines, all mediators produced by AM and lung epithelial cells, are thought to be important mediators for the production and mobilization of monocytes from the bone marrow (20). For example, the importance of IL-6 in monocytopoiesis was demonstrated in vitro in serum-deprived bone marrow cultures where the addition of exogenous IL-6 to cultures stimulated with GM-CSF resulted in increased numbers of monocytic colonies (14). We previously reported that human AM exposed to PM10 in vitro produce tumor necrosis factor (TNF-α) in a dose-dependent manner (17, 35). An early event in the pathogenesis of atherosclerosis is the adherence of monocytes to the arterial endothelium followed by migration into the lesion where they become lipid-taken foam cells (10, 24). TNF-α is known to upregulate the secretion of MCP-1 by endothelial cells (23) to promote the migration of monocytes into atherosclerotic lesions and activate arterial endothelium to increase L-selectin-dependent monocyte adhesion (11).

Monocyte accumulation in the inflammatory sites is induced by the local production of chemotactic factors (8). Schratzberger and colleagues (25) showed that interaction between PMN and the endothelium causes the release of active MCP-1, which assists in the recruitment of monocytes into atherosclerotic lesions. Interestingly, our data also show that the monocyte bone marrow transit times of the control WHHL rabbits with atherosclerosis are shorter than those of NZW rabbits without atherosclerosis (30.4 ± 1.9 h vs. 35.2 ± 0.9 h, P = 0.041, Table 1). This supports the concept that atherosclerosis is an inflammatory process that includes a systemic inflammatory response. Our data suggest that as part of this systemic inflammatory response, the bone marrow monocyte production is increased and that this production is further accelerated by the deposition of atmosphere particulates in the lung. These results support the hypothesis that PM10 exposure induces the release of monocytes into the circulation, and we speculate that they may contribute to the accelerated atherogenesis associated with exposure to particulate matter air pollution.

In summary, our results show that the monocyte transit times are shorter in WHHL rabbits with atherosclerosis, and chronic PM10 deposition in the lung further accelerates the release of monocytes from the bone marrow. These monocytes are released into the circulation earlier than PMN in response to PM10, and the number released is related to the amount of particles phagocytosed by AM in the lung. We postulate that these newly released monocytes may play a critical role in the accelerated atherogenesis associated with particulate air pollution.

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