The Effect of Repeated Exposure to Particulate Air Pollution (PM_{10}) on the Bone Marrow

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Studies have shown that exposure to ambient particulate matter is related to an increased cardiopulmonary morbidity and mortality. The present study was designed to measure the effect of repeated exposure to urban air particles (PM_{10}) on the rate of production and release of polymorphonuclear leukocytes (PMN) from the bone marrow into the peripheral blood. Rabbits exposed to PM_{10} (5 mg) twice a week for 3 wk, were given a bolus of 5′-bromo-2′-deoxyuridine (BrdU) to label dividing cells in the marrow that allows us to calculate the transit time of PMN in the bone marrow mitotic and postmitotic pools. The PM_{10} exposure ($n = 8$) causes a persistent increase in circulating band cells ($p < 0.05$) and a shortening of the transit time of PMN through the postmitotic pool in the marrow (64.4 ± 2.2 h to 56.3 ± 2.2 h, $p < 0.05$) if compared with vehicle-exposed control subjects ($n = 6$). PM_{10} exposure increases the bone marrow pool of PMN particularly the mitotic pool of PMN ($p < 0.05$). The PM_{10} were distributed diffusely in the lung and caused a mild mononuclear inflammation. The percentage of alveolar macrophages containing PM_{10} correlated significantly with the bone marrow PMN pool size (total pool $r^2 = 0.56$, $p < 0.012$, mitotic pool $r^2 = 0.61$, $p < 0.007$) and the transit time of PMN through the postmitotic pool ($r^2 = -0.42$, $p < 0.043$). We conclude that repeated exposure to PM_{10} stimulates the bone marrow to increase the production of PMN in the marrow and accelerate the release of more immature PMN into the circulation. The magnitude of these changes was related to the amount of particles phagocytosed by alveolar macrophages.

Epidemiological studies have shown that particulate air pollution (particles smaller than 10 μm, PM_{10}) at relative low concentrations produces adverse health effects (1–5). Time series analysis of PM_{10} pollution and mortality suggests that the effect of increasing PM_{10} by 10 $\mu g/m^3$ above the minimally accepted value increases total daily mortality by 1.0%, respiratory daily mortality by 3.4%, and cardiovascular daily mortality by 1.8% (3). Furthermore, elevated PM_{10} levels correlate with a decline in several indicators of pulmonary function in a more consistent fashion than gaseous pollutants such as ozone and sulfates (3, 6). Residents of communities exposed to high levels of PM_{10} showed faster rates of lung function decline, chronic respiratory disease (6), and hospital admissions for pneumonia and chronic obstructive pulmonary disease (COPD) (6–9) after adjusting for several individual risk factors including smoking (6). Despite consistent evidence of adverse respiratory and cardiovascular health effects related to PM_{10} air pollution, the biological mechanisms by which ambient PM_{10} pollution exerts these adverse health effects are not clear.

Weiss and colleagues have shown that an increase in leukocyte count is a predictor of total mortality, independent of smoking in large population-based studies (10). This is supported by other independent longitudinal studies linking elevations of the peripheral blood leukocyte count to increased mortality (11–13). Recent work from our laboratory has shown that the deposition of inert fine carbon particles in the lung results in a leukocytosis that is associated with bone marrow stimulation in animals (14). During an acute episode of air pollution in South Asia in 1997, Tan and colleagues have shown that military recruits who performed combat training outdoors have a leukocytosis that returned to normal after the pollution cleared (15). These findings suggest that acute exposure to particulate matter air pollution induces a systemic inflammatory response that includes bone marrow stimulation.

This study was designed to measure the bone marrow response to repeated PM_{10} exposure and test the hypothesis that an important part of the systemic response to chronic PM_{10} exposure is stimulation of the bone marrow with the release of immature polymorphonuclear leukocytes (PMN) into the circulation. Rabbits were exposed to PM_{10} collected over a major Canadian city for 3 wk and the bone marrow response was measured using the thymidine analogue 5′-bromo-2′-deoxyuridine (BrdU) to label the dividing myeloid cells in the bone marrow (15–17). This technique allows us to identify new cells released from the bone marrow and calculate the transit time of myeloid cells through the bone marrow and measure the size of the different pools of granulocytes in the marrow.

METHODS

Experimental Groups

Female New Zealand White rabbits ($n = 17$; weight, 2.2 to 3.0 kg) were used in this study. All studies were approved by the Animal Experimentation Committee of the University of British Columbia.

Experimental Protocol

Animals were exposed to well-characterized PM_{10} (EHC-93, 18, 19). Briefly, these urban particulate matter (EHC-93) was recovered from bag-house filters of the single-pass air filtration system of the Environmental Health Centre in Ottawa (100% outdoor air) and elemental and organic contents have been reported before (18). The dispersed particles have a mass median diameter of 4–5 μm and 20% of the mass is associated with the $PM_{2.5}$ fraction based on chemical profile and size distribution (19). The particles have a low direct cytotoxicity to lung macrophages (20) and contain endotoxin levels well below those detected in similar preparations (21).

The animals were exposed by intrapharyngeal instillation ($n = 8$) and compared with control (saline)-exposed animals ($n = 6$). Briefly, rabbits were anesthetized with 5% halothane and 1 ml of normal saline or PM_{10} (5 mg EHC-93 mixed with 1 ml of saline) was instilled twice

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a week for 3 wk. This was done by placing a pediatric nasogastric tube (15 G) into the nasopharynx above the vocal cord and injecting the 1 ml of saline or 1 ml of saline mixed with PM_{10}. Rabbits were rotated from side to side to promote diffuse distribution of the aspirated solution. In preliminary experiments, this procedure was compared with nasal instillation (n = 3) for efficiency of PM_{10} deposition in the lung.

Blood samples. Blood samples were obtained from the central ear artery just before each instillation, in order to measure the total circulating white blood cell (WBC), PMN, and band cell counts.

To label dividing cells in the bone marrow, 100 mg/kg of BrdU (Sigma Chemical, St. Louis, MO) was administered 24 h before the fifth instillation by infusion through the marginal ear vein at a concentration of 15 mg/ml in normal sterile saline over a period of 15 min (17). To measure the transit time of PMN through the bone marrow, blood samples were obtained just before the fifth instillation and at 3, 6, 9, 12, 24, 36, 48, 72, 96, 120, 144, and 168 h after the fifth instillation. Blood (1 ml) was collected in standard vacutainer tubes containing ethylenediaminetetraacetic acid (Becton Dickinson, Rutherford, NJ) for leukocyte counts, 1 ml was collected in tubes containing acid-citrate dextrose (ACD) for the detection of BrdU-labeled PMN (PMN^{BrdU}). Fentanyl (0.02 mg/kg) and droperidol (1.0 mg/kg) were given subcutaneously as sedation to assist blood collection.

WBC counts were determined on a model SS80 Coulter Counter (Coulter Electronics, Hileah, FL). Differential counts were obtained by counting 100 leukocytes in randomly selected fields of view on Wright's stained blood smears and 100 PMN were evaluated in randomly selected fields of view to determine the changes in the number of band cells. Blood collected in ACD was used to obtain leukocyte-rich plasma (LRP). Erythrocytes in the ACD blood sample were allowed to sediment for 25–30 min after the addition of an equal volume of 4% dextran (average molecular weight, 162,000) (Sigma) in PMN buffer. The resulting LRP was cytospun onto 3-aminopropryl-triethoxysilane-coated slides by cytocentrifugation at 180 × *g* with a Cytospin 2 (Shandon Lab Products, Chestire, UK) for 5 min. The cytospin specimens were air dried and stained using the alkaline phosphatase and anti-alkaline phosphatase (APAAP) method (22) to determine the fraction of PMN^{BrdU} in each specimen.

Immunohistochemical Detection of PMN^{BrdU} cells

Cytospin specimens were stained for the presence of nuclear BrdU by using the APAAP technique (23). Briefly, the slides were fixed in methanol for 10 min and then digested at 37° C for 15 min in a 0.004% pepsin solution acidified to pH 2.5. DNA in the samples was denatured in 2 N HCl at 37° C for 60 min. The 2 N HCl was neutralized by washing the slides three times with 0.1 M borate buffer, pH 8.5, each for 10 min. After nonspecific binding sites were blocked by incubation with 5% normal rabbit serum for 15 min, the specimens were incubated with 2 μg/ml mouse anti-BrdU antibody (DAKO Laboratories, Copenhagen, Denmark) for 60 min in a humidity chamber at room temperature. Nonspecific mouse immunoglobulin G (IgG) (5 μg/ml) was used as a negative control. Incubation in a 1:20 dilution of rabbit anti-mouse IgG (DAKO) for 30 min was followed by 30 min in a 1:50 dilution of a mouse monoclonal APAAP complex (DAKO). All antibodies were prepared in 50 mM Tris hydrochloride and 150 mM NaCl, pH 7.6 (TBS) with 1% bovine serum albumin, and slides were washed in 0.1% Tween 20 (Fisher Scientific, Fair Lawn, NJ) in TBS twice for 5 min between each antibody application. The alkaline phosphatase was developed for 20 min in 50 ml TBS at pH 8.7 after addition of a mixture of 0.25 ml of 4% sodium nitrate, 0.1 ml of 5% fuchsin (Merck, Rahway, NJ) in 2 M HCl, and 25 mg naphthol-AS-B1 phosphate (Sigma) dissolved in 0.3 ml *N*,*N*-dimethylformamide. Endogenous alkaline phosphatase was blocked by addition of 50 μl levamisole (Sigma) to color reaction. The preparations were counterstained with Mayer's hematoxylin for 2 s.

Evaluation of PMN^{BrdU}

PMN^{BrdU} were evaluated as previously described in detail (17). Briefly, PMN with any nuclear stain were counted as BrdU-labeled. PMN^{BrdU} were divided into three groups according to the intensity of nuclear staining, using an arbitrarily designated grading system; weakly positive (staining of less than 5% of the nucleus: G1), moderate positive (staining of 5 to 80% of the nucleus: G2), and highly positive (staining of more than 80% of the nucleus: G3). This grading system was designed to evaluate the transit time of the myeloid cells that were in their last division in the mitotic pool when exposed to BrdU (G3), those that were in the middle (G2), and those that were in their first division (G1). These slides were coded and examined without knowledge of the group or sampling time. Fields were selected in a systematic randomized fashion, and 200 cells were evaluated per specimen. All PMN of interest in a selected field were evaluated, except if the cell was broken or overlapping with other cells.

PMN Transit Time from Bone Marrow to Circulation

Transit time of PMN^{BrdU} through the bone marrow was calculated as previously described (17). Briefly, the number of PMN^{BrdU} was corrected for the disappearance ($t_{1/2}$) of cells in the circulation. In previous studies, we have reported that the half-life ($t_{1/2}$) of PMN^{BrdU} in rabbits is 270 min or 4.5 h, using a whole blood transfusion method (23). We have applied this rate of exponential loss of PMN^{BrdU} from the circulation to calculate the number of PMN^{BrdU} released from the bone marrow and the transit time through the different pools in the bone marrow in the following manner:

$$\Delta N(\Delta t) = Nt_j - Nt_i \exp^{-(k_{\Delta t})}$$

where N is the relative number of labeled cells, t; t_i and t_j are the initial and successive times, $\Delta t = t_j - t_i$, and $k = In2/t_{1/2}$.

These calculations were made for each 6-h interval, and a histogram was drawn showing the distribution of the PMN^{BrdU} released from the bone marrow during each 6-h interval. The mean transit time for all the PMN^{BrdU} and the different populations of PMN^{BrdU} (G1, G2, and G3 cells) were calculated individually in each rabbit.

Distribution of PM_{10} in the Lung

Animals were sacrificed 2 d following the last (sixth) instillation with an overdose of sodium pentobarbitone. The thorax was opened rapidly, the base of the heart ligated, and the lungs removed and inflated at 25 cm H_2O by intratracheal instillation of 10% phosphate-buffered formalin for histological evaluation.

After fixation, the lungs were cut into five slices perpendicular to the gravitational field, and random samples of tissue were obtained from each slice and embedded in paraffin. Tissue blocks were sectioned (3 μm thick) and stained with hematoxylin and eosin. Random fields of view of the tissue sections chosen by computer-generated coordinates of a mechanical stage were examined ×400 magnification using a light microscope (Nikon, Tokyo, Japan). A total of 400 alveolar macrophages (AM) were evaluated and assigned to categories where macrophages contained no particles in their cytoplasm (negative), < 5% of the AM cytoplasm surface area contained PM_{10}, or > 5% of the AM cytoplasm surface area contained PM_{10}.

To determine the relevance of the particle exposure of the rabbits to human exposure, we determine the particle load in alveolar macrophages of human lung tissue using the same method as described above. Lung sections from lungs resected for small peripheral tumors (n = 10) were evaluated. The sections were obtained from a noninvolved segment or lobe of the resected lung. A group of smokers and lifelong nonsmokers were evaluated.

Statistical Analysis

All values are expressed as mean ± SEM. Data were analyzed using a two-way analysis of variance (ANOVA) for repeated measures and Bonferroni's corrections were done for multiple comparisons. Transit times of PMN^{BrdU} were compared between groups using unpaired Student's *t* test. The correlation between parameters was examined by Spearman's rank correlation test. A value of $p < 0.05$ was accepted as significant.

RESULTS

Distribution of PM_{10} in the Lung

The PM_{10} exposure caused a diffuse mild inflammatory response both in the small airways (Figure 1F) and the alveoli (Figure

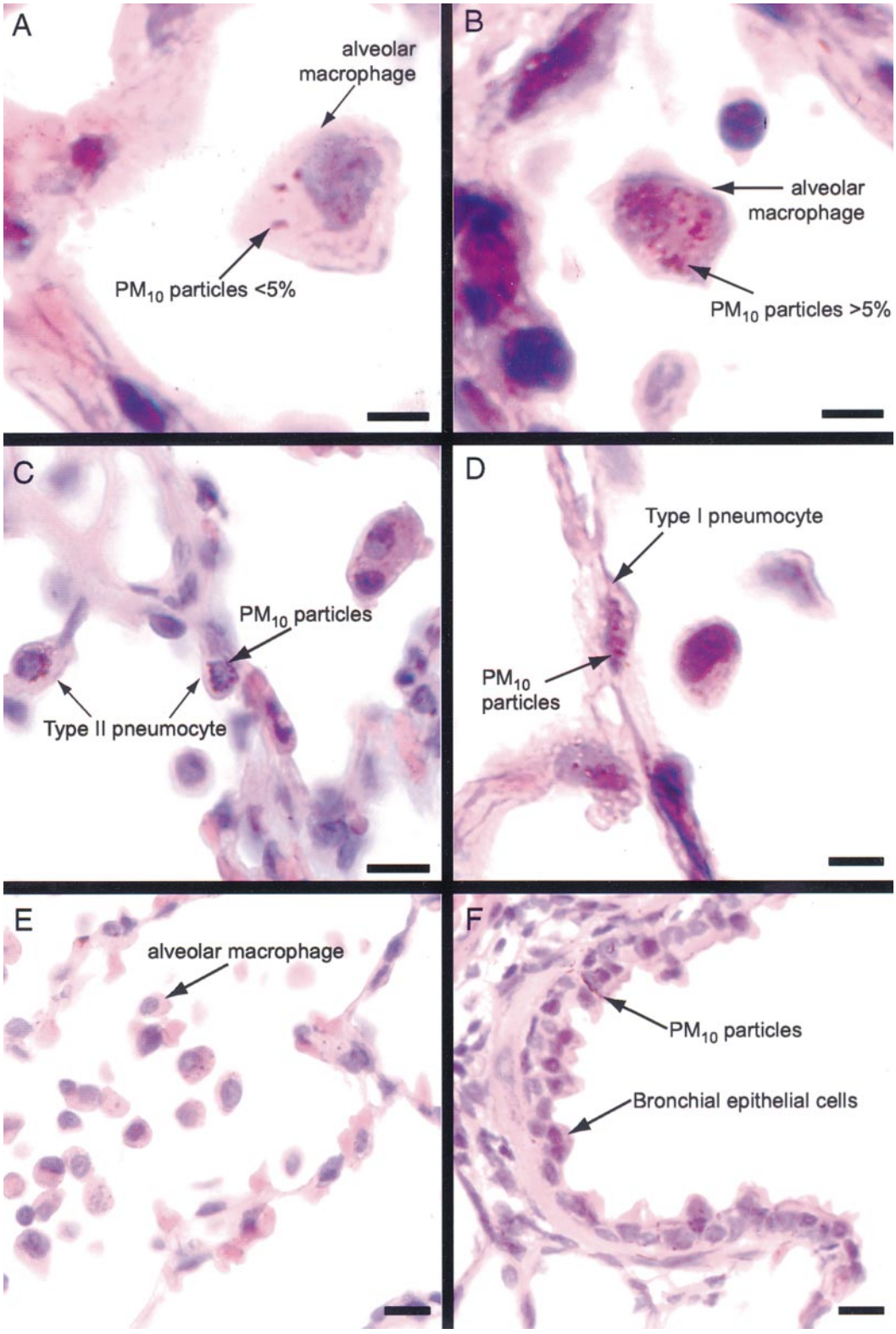

Figure 1. Photomicrographs of formalin-fixed, paraffin-embedded rabbit lung tissue stained with hematoxylin and eosin. (*A*) An alveolar macrophage with a few PM_{10} particles in the cytoplasm (< 5% of the cytoplasm surface area) and (*B*) an AM with many particles in the cytoplasm (> 5% of the cytoplasm surface area). Particles were also present in pneumocytes (*C* and *D*) and bronchial epithelial cells (*F*). The PM_{10} exposure caused a mild mononuclear alveolitis (*E*). The bar represents 10 μm.

1E). Inflammatory cells present in the alveoli were predominantly mononuclear (Figure 1E). PM_{10} particles were diffusely distributed throughout the lung and were present in AM (Figures 1A and 1B), and occasionally observed in type II pneumocytes (Figures 1C and 1D) and in the airway walls (Figure 1F). Alveolar macrophages containing PM_{10} were distributed diffusely in all lung regions. Most positive AM contained just a few particles (< 5% of their cytoplasm containing PM_{10}). The intrapharyngeal instillation caused a higher percentage of positive alveolar macrophages compared with the intranasal instillation ($p < 0.02$) (Figure 2). There were no significant differences between the smokers and nonsmokers in their age (52 ± 5.2 versus 60 ± 2.9 yr), sex, and lung-diffusing capacity (88 ± 5.1 versus 84 ± 7.6, % predicted) but a lower FEV_1 (95 ± 3.1 ver-

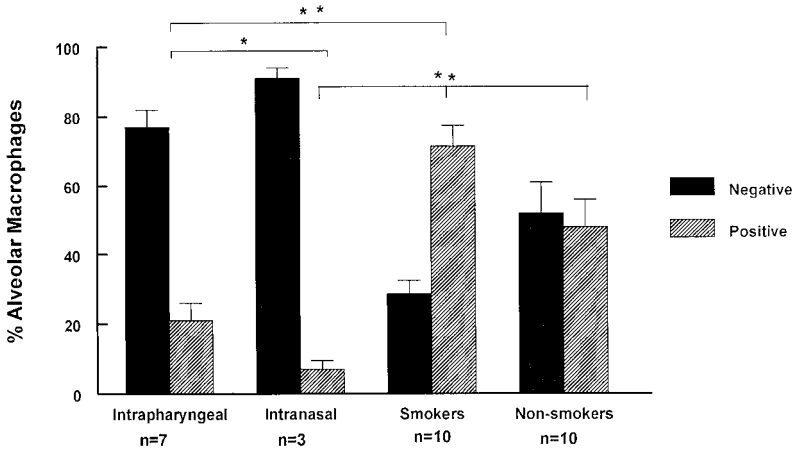

Figure 2. Percentage of alveolar macrophages that have phagocytosed PM_{10} in rabbit lungs after intrapharyngeal and intranasal instillation of PM_{10} twice a week for 3 wk and in human smokers and nonsmokers. The intrapharyngeal instillation caused a higher percentage of positive cells compared with the intranasal instillation and human alveolar macrophages have more particles that rabbits. *$p < 0.02$ (comparing intrapharyngeal and intranasal instillation). **$p < 0.05$ (comparing human and rabbit AM).

sus 76 ± 6.5, % predicted) and VC (96.8 ± 3.1 versus 85 ± 4.4, %predicted) in smokers compared with nonsmokers. The percentage of positive alveolar macrophages in human lung tissue was higher in smokers than nonsmokers but both groups were higher than the intrapharyngeal instillation group (Figure 2).

Effect of PM_{10} on the Release of PMN from the Bone Marrow

Leukocyte in the circulation. Neither intranasal (data not shown) nor intrapharyngeal instillation of PM_{10} changed the circulating leukocyte counts compared with saline controls (Fig-

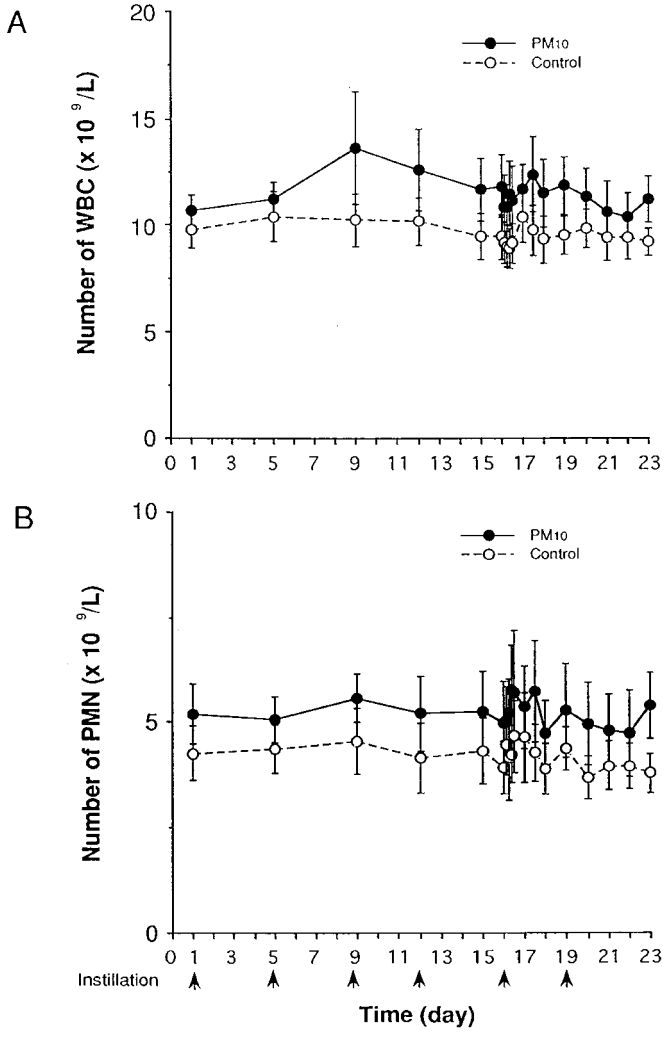

Figure 3. The circulating white blood cell (WBC) counts (*A*) and polymorphonuclear leukocyte (PMN) counts (*B*) of rabbits exposed to PM_{10} for 3 wk (n = 8) or saline (control) (n = 6). Values are means ± SEM.

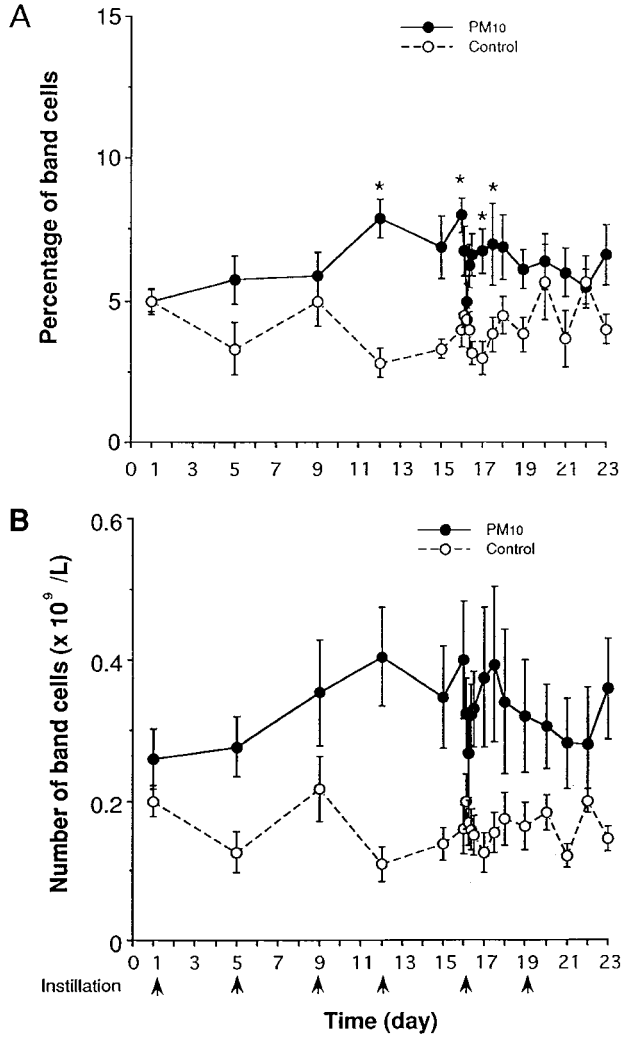

Figure 4. The percentage (*A*) and number (*B*) of band cells in the circulation of rabbits exposed to PM_{10} for 3 wk (n = 8) or saline (control) (n = 6). Values are means ± SEM. *$p < 0.05$ (compared with control group).

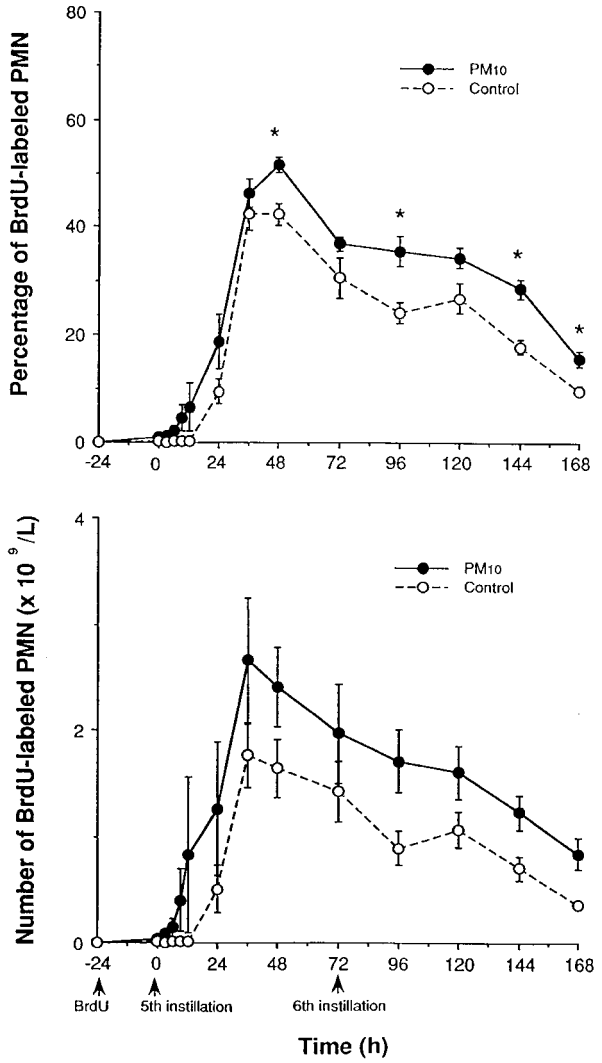

Figure 5. The percentage (*top panel*) and number (*bottom panel*) of all BrdU-labeled PMN in the circulation of rabbits exposed to PM_{10} for 3 wk (n = 8) or saline (control) (n = 6). Values are means ± SEM. *$p < 0.05$ (compared with control group).

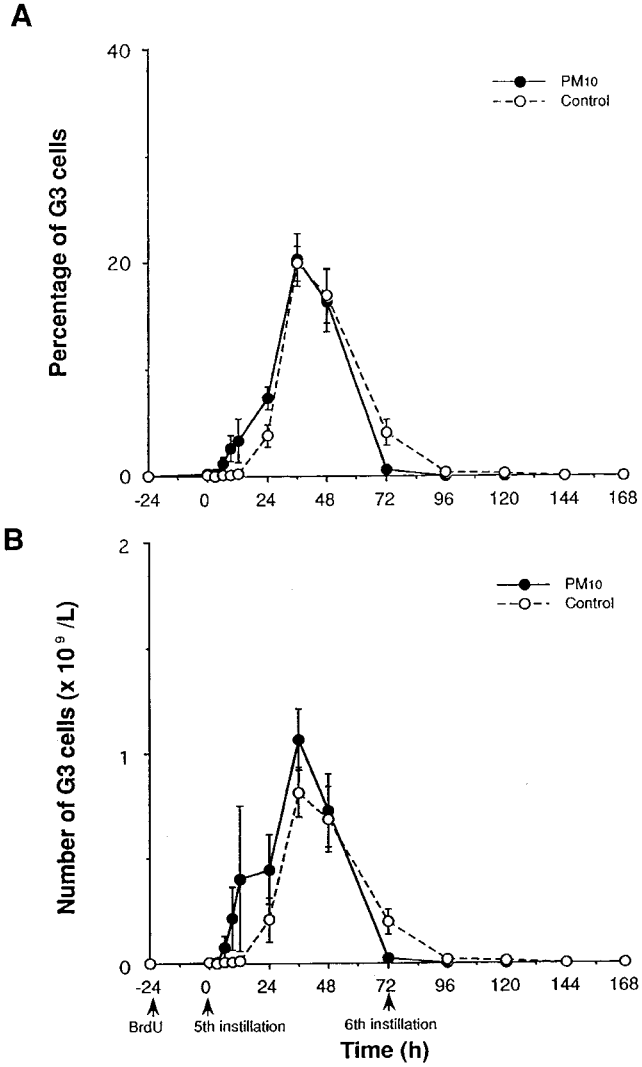

Figure 6. The percentage (*A*) and number (*B*) of G3 cells (*see text*) in the circulation of rabbits exposed to PM_{10} for 3 wk (n = 8) or saline (control) (n = 6). Values are means ± SEM.

ures 3A and 3B). However, the percentage circulating band cells (expressed as a percentage of PMN) (Figure 4A) or the absolute number of circulating band cells (Figure 4B) increased within the second week of exposure. Band cell counts did not change with intranasal instillation of PM_{10} and there was no significant difference in the percentage or number of mononuclear cells among groups (data not shown).

BrdU-labeled PMN in the circulation. Twenty-four hours after the BrdU labeling the percentage and total number of BrdU-labeled cells increased rapidly in the circulation and peaked between 36 and 48 h in both the PM_{10}-exposed (intrapharyngeal) and control groups (Figure 5). The percentage of PMN^{BrdU} in the peripheral blood of the PM_{10}-exposed group was higher at 48, 96, 144, and 168 h than that in the control group ($p < 0.05$) (Figure 5, *top panel*). Highly stained PMN^{BrdU} (G3 cells) were released into the circulation more rapidly in the PM_{10}-exposed compared with the saline control group (Figures 6A and 6B). The percentage of weakly stained PMN^{BrdU} (G1 cells) (Figures 7A and 7B) was higher in the PM_{10}-exposed group at 120, 144, and 168 h compared with the control group. There was no significant difference in the percentage and the number of PMN^{BrdU} in the circulation between intranasal instillation of the PM_{10} group and control group (data not shown).

To evaluate the size of the different bone marrow pools, the cumulative number of BrdU-labeled PMN in the circulation was calculated as previously described (23). Figure 8 shows the cumulative frequency distribution of all BrdU-labeled PMN, G3 cells, and G1 cells. PM_{10} exposure causes a significant increase in the size of the bone marrow pool of myeloid cells (all BrdU-labeled PMN, $p < 0.05$) and the mitotic pool (G1 cells, $p < 0.01$). A significant correlation was observed between the percentage of alveolar macrophages positive for PM_{10} and the size of bone marrow pools of PMN (all BrdU-labeled PMN, $r = 0.56$, $p < 0.012$ [Figure 9a] or G1 cells, $r = 0.61$, $p < 0.007$ [Figure 9b]).

Transit time of PMN^{BrdU} through the bone marrow. Table 1 shows the calculated transit time of all the PMN^{BrdU} and the different subpopulations of PMN^{BrdU} (G3, G2, and G1 cells). PM_{10} exposure shortened the transit time of PMN in the postmitotic pool (G3 cells, $p < 0.05$), but not in the mitotic pool. Intranasal instillation of PM_{10} produced transit times of PMN

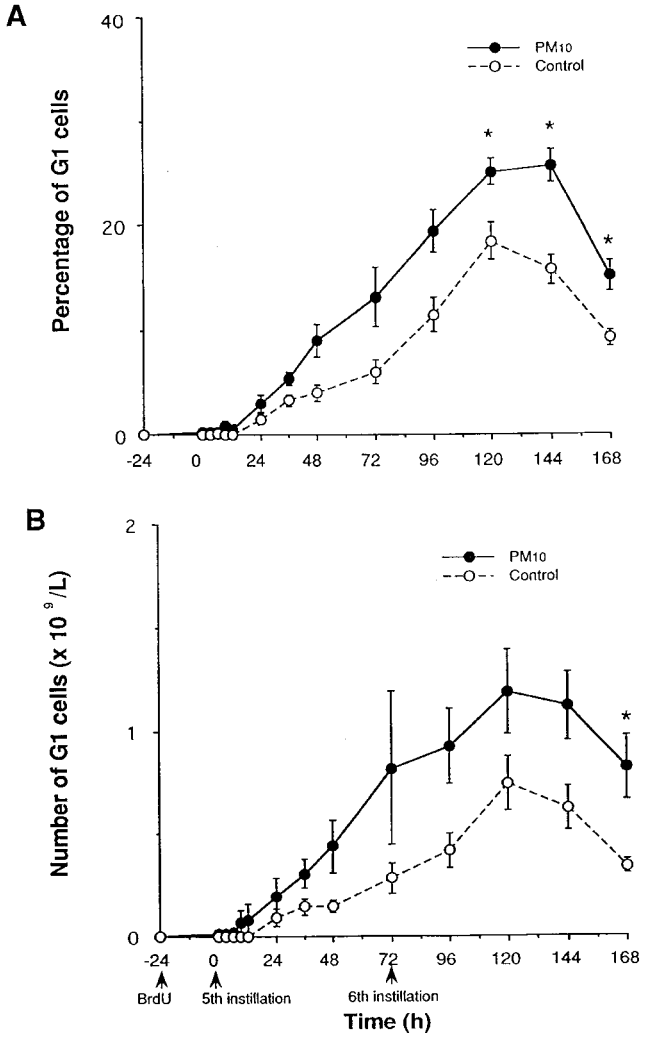

Figure 7. The percentage (*A*) and number (*B*) of G1 cells (*see text*) in the circulation of rabbits exposed to PM_{10} for 3 wk (n = 8) or saline (control) (n = 6). Values are means ± SEM. *p < 0.05 (compared with control group).

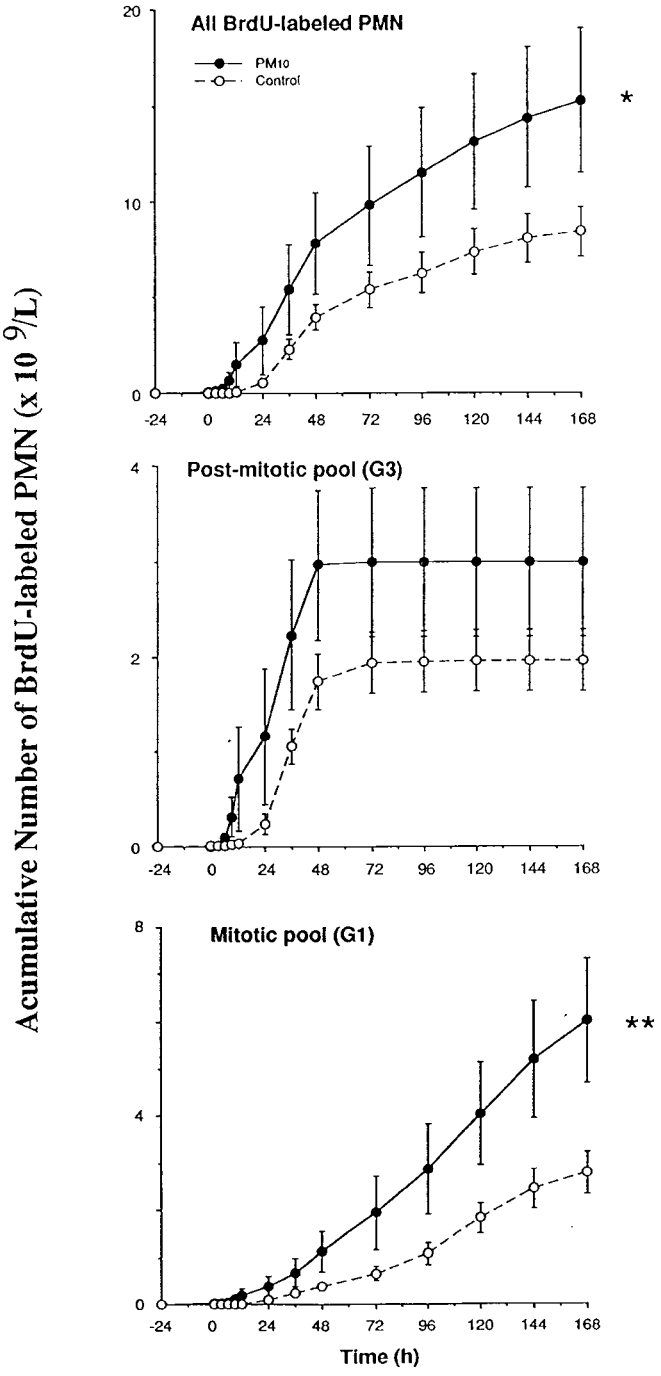

Figure 8. Cumulative number of BrdU-labeled PMN in the circulation of rabbits exposed to PM_{10} for 3 wk (n = 8) or saline (control) (n = 6). The total bone marrow pool size (all BrdU-lableled PMN) and the mitotic pool size (G1 cells) were significantly larger in the PM_{10}-exposed (n = 8) rabbits if compared with saline-exposed (n = 6) animals. Values are means ± SEM. *p < 0.05, **p < 0.01 (compared with control group).

through the bone marrow that were similar to control values (all PMN 97.0 ± 4.9 versus 101.6 ± 1.2 h; G3 cells 58.8 ± 4.6 versus 64.4 ± 2.2 h; G2 cells 81.5 ± 11.8 versus 88.6 ± 2.5 h; G1 cells 126.3 ± 7.3 versus 131.7 ± 2.5 h [intranasal instillation of PM_{10} versus controls]). A significant correlation was observed between the percentage of alveolar macrophages containing PM_{10} and the transit time of G3 cells (Figure 9c, $r = -0.42$, $p < 0.043$).

DISCUSSION

This study shows that repeated exposure to ambient PM_{10} causes an accelerated release of immature PMN from the bone marrow. This was associated with an increase in the bone marrow turnover of PMN with a shortening of their transit time through the postmitotic pool of the marrow. PM_{10} exposure also increased the bone marrow pool of myeloid cells, particularly the mitotic pool size. The magnitude of stimulation of the bone marrow by PM_{10} exposure was related to quantity of particles phagocytosed by alveolar macrophages. These results show that chronic PM_{10} exposure induces a systemic inflammatory response that includes stimulation of the bone marrow and we postulate that this marrow stimulation is initiated by mediators released from the lung.

Exposure to particulate air pollution has adverse health effects due to an increase in the morbidity and mortality of respiratory and cardiovascular diseases (1–6) but the biological

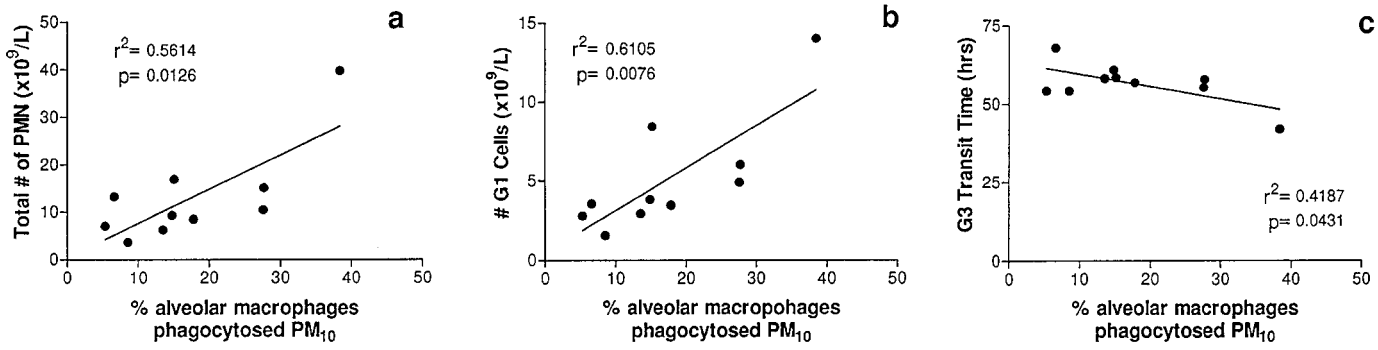

Figure 9. Correlation between the percentage of alveolar macrophages that have phagocytosed PM_{10} and the total bone marrow pool size (*a*, $r^2 = 0.56$, $p = 0.012$) and mitotic pool size (G1 cells) (*b*, $r^2 = 0.61$, $p < 0.007$) and transit time of PMN through the postmitotic pool in the marrow (*c*, $r^2 = 41$, $p = 0.043$).

mechanisms responsible for this association are not clear. Seaton and colleagues proposed the hypothesis that the inhalation of fine particles provokes a low grade inflammatory response in the lung that causes an exacerbation of lung disease such as asthma and COPD and change blood coagulability that results in increased pulmonary and cardiovascular deaths (24). A previous study from our laboratory (14) showed that a single instillation of small inert carbon particles directly into the lungs of rabbits stimulates the bone marrow and shortens the transit time of PMN through bone marrow. Furthermore, Tan and colleagues have demonstrated a leukocytosis and an increase in circulating band cells in young military recruits exposed to an acute episode of air pollution during forest fires of Southeast Asia in the summer of 1997 (15), suggesting that an episode of acute exposure to PM_{10} causes bone marrow stimulation in humans. The present study extends these findings by showing that the repeated deposition of low levels of particulate matter in the lung causes a systemic response that includes stimulation of the bone marrow.

The pattern of bone marrow stimulation with the repeated PM_{10} exposure reported here is distinctly different from that described with acute exposure to particles. Acute exposure caused an acute leukocytosis with a rapid release of PMN from the marrow and an accelerated transit time through all the bone marrow pools (14). In contrast, repeated PM_{10} exposure does not result in a leukocytosis and has a smaller effect on the bone marrow transit times (Table 1). However, it increased the size of the bone marrow pools and released more immature PMN (band cells) from the bone marrow. This distinct bone marrow response following repeated particle exposure could be due to a difference in the inflammatory mediators released from the lung following repeated exposure as compared with acute exposure. Interestingly, rabbits exposed to cigarette smoke on a daily basis over a 2-wk period of time developed a similar (albeit larger) bone marrow response than the PM_{10} exposure reported here (16). This suggests that chronic low grade exposure to PM_{10} stimulates the bone marrow through mechanism(s) that are similar to chronic cigarette smoking and that particle exposure is important in this response.

TABLE 1

TRANSIT TIMES OF POLYMORPHONUCLEAR LEUKOCYTES (PMN) THROUGH THE BONE MARROW

Group	n	All PMN (*h*)	G3 (*h*)	G2 (*h*)	G1 (*h*)
PM_{10}	8	103.5 ± 2.4*	56.3 ± 2.2†	87.4 ± 3.1	130.0 ± 2.8
Control	6	101.6 ± 1.2	64.4 ± 2.2	88.6 ± 2.5	131.7 ± 2.5

Definition of abbreviations: All PMN = the total transit time of 5′-bromo-2′-deoxyuridine (BrdU)-labeled PMN; G1 to G3 = the transit times of different subpopulation of BrdU-labeled PMN; G3 = transit time of PMN through postmitotic pool; G1 = transit time of PMN through the mitotic and postmitotic pool; PM_{10} group = intrapharyngeal instillation of UPM_{10} group.

* Values are mean ± SEM.

† $p < 0.05$ versus control group.

Animals were exposed to PM_{10} by either intranasal or intrapharyngeal instillation of particles. The percentage of alveolar macrophages that phagocytosed PM_{10} in the intrapharyngeal instillation group was about three times higher than that in the intranasal instillation group (Figure 2), suggesting that intrapharyngeal instillation of PM_{10} is a more efficient way of delivering particles to alveolar macrophages compared with intranasal instillation. Interestingly, the percentage of alveolar macrophages that was positive for PM_{10} particles ($\sim$ 20%) with the repeated intrapharyngeal instillation over 3 wk is similar to a single exposure of 1 mg (carbon particles) instilled directly into the lung using fluoroscopy (14). This suggests significant clearance of particles from the lung in this model of repeated exposure. Intrapharyngeal instillation also induced stronger bone marrow stimulation. The correlation between the percentage of alveolar macrophages that has phagocytosed PM_{10} and the bone marrow pool size as well as the transit time of G3 cells suggests that the deposition of PM_{10} in the lung stimulates the bone marrow in a dose-dependent manner. This is consistent with previous findings from our laboratory showing that human alveolar macrophages exposed to PM_{10} *in vitro* produce tumor necrosis factor-α (TNF-α) in a dose-dependent manner (25).

The relevance of the dose of exposure in our study to ambient human exposure is clearly important. With the intrapharyngeal instillation of EHC-93, we have estimated that 20% of the delivered dose is aspirated into the lung of which $\sim$ 4% reached the alveolar surface. With an estimated 5.9 m^2 alveolar surface for a 2.5-kg rabbit the calculated alveolar exposure was 4.3 ng/cm^2 for each dose or 25.8 ng/cm^2 over the experimental period. This exposure compares well with previous experiments using rats in exposure chambers (20) and is similar to a human exposed to 150 μg/m^3 for 20 d. This magnitude exposure is similar to exposure of humans during the Southeast Asia forest fires of 1997 (15). Furthermore, the particle load of alveolar macrophages in human smokers and nonsmokers was significantly higher than animals in this experiment (Figure 2), suggesting that the dose of particles we have used in this experiment is comparable with other animal experiments and relevant to human exposure.

The mechanisms that control the release of PMN from the bone marrow and proliferation of PMN in the bone marrow after deposition of PM_{10} in the lung are incompletely understood. Previous studies using a similar rabbit model showed that small inert carbon particles instilled into the lung are phagocytosed by alveolar macrophages, and that mediators produced during this phagocytotic process resulted in bone marrow stimulation (14). Furthermore, human alveolar macrophages incubated with PM_{10} (EHC-93) produced mediators that have the ability to stimulate the bone marrow and accelerate the transit time of PMN through the bone marrow (25). Because alveolar macrophages are important in processing airborne particles, we suspect that they are an important source of inflammatory mediators responsible for the bone marrow stimulation following PM_{10} exposure. Human airway epithelial cells also produce inflammatory mediators when exposed to PM_{10} (26), and we have observed PM_{10} particles in large and small airway epithelial cells and alveolar epithelial cells (Figure 1). Although particles in epithelial cells were rarely seen, we cannot exclude the possibility that inflammatory mediators released from epithelial cells contribute to the bone marrow stimulation either independently or following stimulation by mediators released from alveolar macrophages.

Maturation of PMN in the postmitotic pool of the bone marrow is associated with an increase in the mobility, deformability, and chemotactic responsiveness of these cells (27, 28). The shortening of the transit time of PMN through the postmitotic pool and increased number of band cells in the circulation induced by repeated PM_{10} exposure indicate that this exposure causes an increase in the number of immature cells present in the circulation. This increase in circulating immature PMN could be important in the pathogenesis of the heart and lung diseases associated with PM_{10} exposure. Our laboratory has shown that immature PMN released from the bone marrow by acute pneumococcal pneumonia (29, 30), endotoxemia (31), and cigarette smoke exposure (32) preferentially sequester in the pulmonary microvessels and are slow to migrate out of the capillaries into an inflammatory site (29, 30, 33). We postulate that activation of these immature PMN trapped in the lung microvessels by circulating or local inflammatory mediators results in the release of hydrolytic enzymes and the production of oxygen radicals and damage to the alveolar walls from within the capillaries. We speculate that the increased burden of immature PMN in the circulation could contribute to the observed decrease in lung function associated with chronic exposure to particulate air pollutants (34–36).

In summary, our results show that repeated PM_{10} deposition in the lung parenchyma stimulates the bone marrow to expand its pools, accelerates their transit through the postmitotic pool, and increases the release of immature PMN into the circulation. We also show that this bone marrow stimulation is related to the amount of particles phagocytosed by alveolar macrophages in the lung. We suspect that this systemic inflammatory response to PM_{10} plays an important role in the pathogenesis of the cardiopulmonary diseases associated with particulate air pollution.

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