Alveolar Macrophage Kinetics after Inhalation of ^{239}PuO$_2$ by CBA/Ca Mice: Changes in Synthesis of DNA

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For workers in the nuclear industry, the primary route for the entry of radioactive materials into the body is by inhalation, and the rate of clearance of particles from the pulmonary region of the lung is an important factor in determining radiation dose. It is the function of alveolar macrophages (AM) to maintain the sterility of the lung and to remove insoluble particles from the respiratory surfaces and airways. The AM population is not static, and under normal conditions the loss of macrophages from the alveoli via the conducting airways is balanced by renewal. Studies of the effects of external irradiation on the kinetics of AM are numerous, but to date little is known about the effects of inhaled radioactive particles. In this investigation the effects of inhaled ^{239}PuO$_2$ (plutonium dioxide) particles on the synthesis of DNA by AM were studied at times up to 77 days after exposure. We also measured the number of cells recovered by bronchoalveolar lavage and the incidence of AM with nuclear aberrations. The latter provides a sensitive indicator of the effects of radiation.

One of the earliest effects observed after exposure to ^{239}PuO$_2$ is a reduction in the number of AM recovered by lavage. This reduction is associated with a 3-fold reduction in the proportion of AM undergoing DNA synthesis at early times after exposure. The overall mean pulse labeling index of AM recovered from sham-exposed mice is 1.68%, and no trend is observed with time. At later times after exposure there is a concurrent increase both in the number of AM recovered by lavage and the proportion of AM in the S-phase of the cell cycle. This repopulation of the AM pool is associated with an increase in the incidence of AM with nuclear aberrations. The results of this study are consistent with the theory of an intrapulmonary pool of proliferating macrophages. The depletion of the AM pool and the latency in the induction of nuclear aberrations after exposure to ^{239}PuO$_2$ can be attributed to a radiation-induced inhibition of cell division in addition to interphase death of AM.

Introduction

For workers in the nuclear industry, the primary route for the entry of radioactive materials into the body is by the inhalation of airborne particles. When deposited on the respiratory surfaces and airways, such particles are readily phagocytized by alveolar macrophages (AM). Once inside macrophages, even relatively insoluble particles can be dissolved due to the low pH of the phagolysosomes (1). This dissolution provides a mechanism not only for the clearance of insoluble particles from the lung, but also for the translocation of radioactive materials to other organs. In addition, the movement of AM within the lungs causes particles to become redistributed. For AM containing radioactive particles, such movement alters the temporal pattern of radiation dose, which has important implications for the dosimetry of α-emitters in the lung. The rate of macrophage-mediated clearance of radioactive particles from the pulmonary region is therefore an important factor in determining not only the total dose but also the temporal pattern of radiation dose to the lung.

The AM population is not static, and under normal conditions loss of macrophages from the alveoli by death and clearance via the conducting airways is balanced by renewal. This system is resilient, and when challenged by an increased particle load the lung responds with an influx of new AM. As yet the mechanism by which the lung maintains the size of the AM pool is not understood. There is some evidence that renewal of the AM population is achieved by the recruitment of monocytes from the bloodstream (2-5), but others have implicated either the proliferation of AM precursors within the interstitial tissues of the lung (6) or division of AM in situ (7-14). Impairment of the normal mechanism for the renewal of AM leads to a depletion of macrophages in the alveoli, which might alter the rate of macrophage-mediated clearance of particles deposited in the lung.

Studies of the effects of external irradiation on the kinetics of AM are numerous (15-20), but to date little is known of the effects of inhaled radioactive particles. Previous studies have demonstrated a dose-dependent decrease in the size of the AM pool after exposure to ^{239}PuO$_2$ (plutonium dioxide) particles (21-24); however, it is not certain whether this decrease was due

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to interphase death (i.e., an increased output of AM from the lungs) or a radiation-induced inhibition of AM renewal (i.e., a decreased input of new AM). The accumulation of radioactive particles in AM after exposure to aerosols of radionuclides has precluded the use of [^{3}H]thymidine and autoradiography (25) to identify dividing AM. Consequently, few studies of the effects of inhaled actinides on AM kinetics have been reported. Therefore, an alternative technique was used in this study that involves the incorporation of bromodeoxyuridine (BrdU), an analogue of thymidine, into the DNA of dividing AM. An immunocytochemical technique was then used to identify BrdU-substituted DNA as a probe for S-phase cells (26–28).

The purpose of the present study was to determine the effects of inhaled $^{239}PuO_2$ particles on the kinetics of AM and to relate any changes observed to the size of the AM pool. In addition, the relationship between AM kinetics and the induction of nuclear aberrations in AM after the administration of $^{239}PuO_2$ to mice was studied. Evidence for the absence of a diurnal variation in the mitotic activity of AM from CBA/Ca mice is also presented.

Materials and Methods

Radioactive Materials

$^{239}PuO_2$ was prepared by calcination of the oxalate at 550°C for 3 hr in air and stored dry. A sized respirable fraction of $^{239}PuO_2$ was prepared by water sedimentation (29) and suspended in dilute ammonia solution (pH 10.4). An aerosol was generated from the suspension using an Acorn nebulizer (Medic-aid Ltd., Pagham, Sussex, UK) to give airborne particles with an activity median aerodynamic diameter (AMAD) of approximately 1.5 μm and geometric standard deviation of 1.2–1.3.

Animals and Exposures

Barrier-bred female CBA/Ca mice (Harlan Olac Ltd., Bicester, Oxon, UK) were used and allowed pelleted food (Diet 41B, Pilsbury Ltd., Birmingham, UK) and chlorinated water *ad libitum*. The mice were maintained at 20°C, ± 1°C and a relative humidity of 55% ± 5%. A 12-hr light/dark cycle was in operation. On arrival at the laboratory the mice were ear tagged and allocated randomly to one of four groups.

Two groups of 28 mice were exposed (30) at 12 weeks of age to an aerosol of $^{239}PuO_2$ to give planned mean initial alveolar deposits (IAD) at 24 hr after inhalation of 60 Bq and 400 Bq. At 24 hr after the start of the exposure, four mice from each group were killed and the ^{239}Pu content of their lungs determined radiochemically by liquid scintillation counting (31). An additional group of 24 mice was sham exposed to the carrier solution only in the same exposure system.

Recovery of Free Cells by Bronchoalveolar Lavage

At 7, 14, 21, 28, 35, and 77 days after exposure, four mice were taken at random from each group and injected (IP) 1 hr before sacrifice with 50 mg/kg body weight of 5-bromo-2'-deoxyuridine. One hour later the mice were killed by an injection (IP) of Sagatal (RMB Animal Health Ltd., Dagenham, Essex, UK) and exsanguination via the brachial artery. Free cells were recovered from the lungs by bronchoalveolar lavage (BAL) *in situ*

as described by Moores et al. (21), which involves 10 sequential washes with 0.5 mL sterile physiological saline (Polyfusor; Boots, Nottingham, UK) at room temperature. The combined washes were collected in preweighed plastic universal containers (Sterilin, Middlesex, UK), kept on ice, and the volume of fluid recovered determined by weight. The concentration of AM in the lavage fluid was measured with a Fuchs-Rosenthal hemacytometer.

Preparation of Cytospins and Determination of DNA Synthesis

Six cytospins were prepared from each sample of lavage fluid with a cytocentrifuge (Cytospin II, Shandon Southern, Runncorn, Cheshire, UK) operated at 1000 rpm for 5 min. The volume of lavage fluid used to prepare each cytospin was adjusted so that the same density of cells was obtained on all slides. Two slides for each animal were fixed in absolute methanol for 10 min and the remainder was fixed in 70% ethanol for 30 min.

The ethanol-fixed slides were used to determine the proportion of AM in DNA synthesis. To obtain maximum binding of the antibody to BrdU, the DNA was partially denatured to the single stranded form by immersing the slides in a fresh solution of absolute ethanol and 0.1 M sodium hydroxide (2:5 v/v) for 4 min at room temperature (26). After denaturation the slides were washed in two changes of phosphate-buffered saline, rinsed in distilled water, and air dried. BrdU incorporated into DNA was then visualized by incubating the deposit of cells on the cytospins with 20 μL of undiluted fluorescein-conjugated anti-BrdU monoclonal antibody (clone B44, mouse IgG, Becton Dickinson, Oxon, UK) for 1 hr at room temperature in a dark, humidified box (28). After incubation the slides were rinsed three times in water and air dried.

Before examination, the slides were counterstained by immersion in a solution containing 0.1 mg/L of propidium iodide (Sigma, Poole, Dorset, UK) for 30 sec, followed by a brief rinse in distilled water. The slides were cover slipped with one drop of fluorescent mounting medium (Sigma) and at least 2000 cells on each slide were examined using a UV microscope (Laborlux-K, Leitz, Luton, Bedfordshire, UK). Each field (400 × magnification) was examined using epifluorescent illumination and both the total number of cells and the number of cells stained with the anti-BrdU monoclonal antibody were counted. The pulse labeling index (I_s), the percentage of labeled cells after the injection of BrdU, was calculated for each animal.

Differential Cell Counts

Slides fixed in methanol were stained with May-Grünwald/Giemsa, and differential leukocyte counts were made on more than 1500 cells. In addition the frequency of nuclear aberrations in the AM population was also assessed. Macrophages were classified as either normal, micronucleate (MiAM), binucleate (BiAM), with both types of aberration (BiMiAM), or in metaphase. Micronuclei were identified according to the criteria of Countryman and Heddle (32).

Diurnal Variation in DNA Synthesis

In an additional experiment, we used a group of 72 female CBA/Ca mice to investigate the possibility of diurnal fluctuations

in the number of AM in DNA synthesis. The I_s of AM recovered by BAL from CBA/Ca mice was measured in groups of six mice selected randomly every 2 hr during a period of 24 hr. The procedures used to recover the free cell population and to determine the proportion of AM in S-phase were the same as those described earlier.

The Student's t-test was used to compare the means of actinide-exposed and sham-exposed groups for all parameters measured. The null hypothesis was rejected at a significance level of 5% or greater. The single-factor ANOVA test was used to compare the variation in labeling index. Values of F greater than the 5% value of known probability indicate a significant difference between population means.

Results

Initial Alveolar Deposits of ^{239}Pu

The mean IADs based on the radiochemical analysis of lungs from the two groups of actinide-exposed mice were 67 Bq $\pm$ 6 Bq (mean $\pm$ SEM) and 424 Bq $\pm$ 16 Bq. Based on previous experiments (22,33), these IADs correspond to cumulative absorbed doses, averaged over the whole lung, of approximately 1.8 Gy and 11.7 Gy after 77 days.

Recovery of Free Cells and Differential Counts

The mean recoveries of AM from mice up to 77 days after exposure are given in Figure 1. For sham-exposed mice, between 24.1 × 10^4 and 46.9 × 10^4 AM were recovered by BAL, and these values agree with those of previous studies (21,22,33). After exposure to 424 Bq of ^{239}PuO$_2$ there was a reduction in the number of AM recovered by BAL, reaching a nadir between 20 and 30 days after exposure; at this time the mean recovery of AM was approximately 2.5 times lower than for sham-exposed mice. No significant reduction was observed in the recovery of AM from mice exposed to 67 Bq of ^{239}PuO$_2$, but the mean recoveries for this group were generally lower compared to sham-exposed

mice. Although not significant statistically, some variation was observed in the recovery of AM from mice within the same group. Similar variations have been reported for the recovery of radioactive particles (22,34) and have been attributed to differences in the efficiency of lavage between different operators. In most mice, AM accounted for more than 98% of the cells recovered by lavage. Lymphocytes and neutrophils accounted for approximately 0.17% and 0.33% of the cells, respectively; however, occasionally a modest increase was observed in the percentage of these cells. Monocytes accounted for less than 0.3% of all cells and eosinophils were extremely rare.

Changes in DNA Synthesis

The mean pulse labeling index (I_s) of AM recovered at times up to 77 days after exposure is shown in Figure 2. No significant change in I_s was observed in AM recovered from sham-exposed mice, and the overall mean I_s for the group was 1.68%. A 3-fold reduction in the percentage of AM in DNA synthesis was observed at 7 and 14 days after exposure to 424 Bq of ^{239}Pu. After 14 days there was a rapid increase in the I_s of AM toward normal, and by 21 days the I_s approached the lower values measured in sham-exposed mice. At 28 days after exposure, the mean I_s for the 424 Bq group was approximately 30% higher than that of corresponding sham-exposed mice, and although not significant statistically, this might represent a slight overshoot in AM proliferation before returning to normal. No significant trend in the I_s of AM recovered from mice exposed to 67 Bq of ^{239}Pu was observed, and at no time after exposure was the mean I_s of this group significantly different from that of corresponding sham-exposed mice.

The number of mitotic figures in the AM population was also measured and invariably accounted for less than 1 in 1000 AM. No significant changes in the percentage of AM in mitosis wee seen after exposure to ^{239}PuO$_2$.

Figure 3 shows the proportion of AM in DNA synthesis during a period of 24 hr. These data illustrate the considerable in-
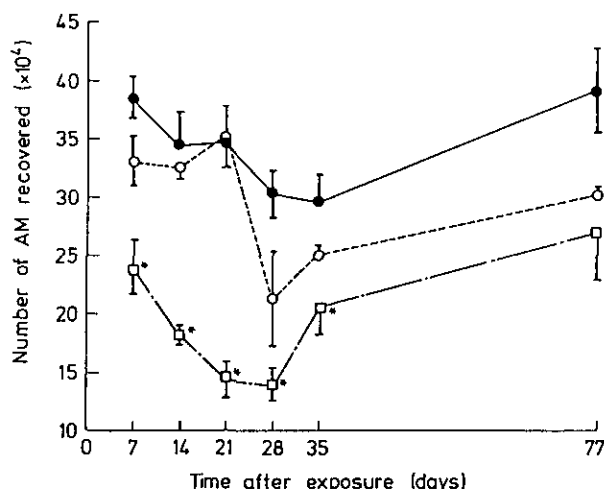

FIGURE 1. The recovery of alveolar macrophages from sham-exposed mice (●) and mice exposed to 67 Bq (○) and 424 Bq (□) of ^{239}Pu at times up to 77 days after exposure, means ± SEM. (*) Significant at 5% probability level or greater.
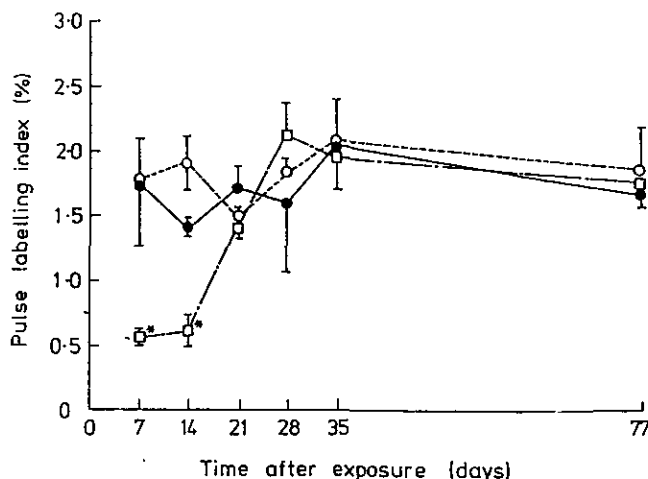

FIGURE 2. The pulse labeling index of alveolar macrophages recovered by lavage from sham-exposed mice (●) and mice exposed to 67 Bq (○) and 424 Bq (□) of ^{239}Pu at times up to 77 days after exposure, means ± SEM. (*) Significant at 5% probability level or greater.
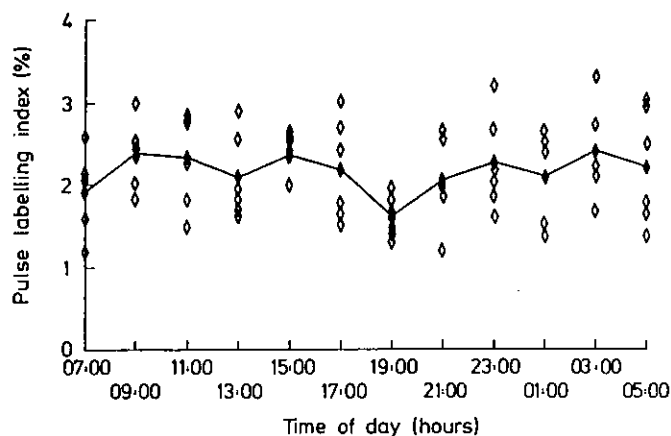
FIGURE 3. The pulse labeling index of alveolar macrophages recovered from the mouse lung by lavage during a period of 24 hr. Each point (◊) is the pulse labeling index of an individual animal. Curve plotted through the mean value (♦) at each time.

teranimal variation in the I_s of AM recovered from the lungs of CBA/Ca mice, which has also been observed in previous studies (35). Analysis of variance reveals that the scatter in the I_s for individual mice within the same group is significant at a probability level of 5% ($F = 3.69$) and is due to a real interanimal variation rather than imprecision in the technique used. Generally, the difference between replicate measurements of the same sample was small ($F = 0.74$) and is not significant by the analysis of variance. The overall mean I_s of AM from mice in this study was 2.15% ± 0.07% (mean ± SEM), and the mean I_s for most groups was within two standard deviations of this value. At 1900 hr the mean I_s was 1.61%; however, this is not significantly different from other times by the analysis of variance. No significant trend in the I_s of AM recovered from CBA/Ca mice was therefore observed with time.

Incidence of Aberrant Alveolar Macrophages

The incidence of nuclear aberrations in the AM population was assessed by examing stained cytospins, and the results of these measurements are presented in Figure 4. The spontaneous incidence of MiAM in sham-exposed mice was low (less than 0.2%), and no significant change was observed with time. In mice exposed to $^{239}PuO_2$, the incidence of MiAM increased rapidly after 14 days to reach peak values of 3.0 and 1.7% at 35 and 28 days for the high and low IAD groups, respectively. After 11 weeks, the incidence of MiAM decreased to between 1 and 2% of all AM for both the high and low actinide-exposed groups but remained significantly higher than in corresponding sham-exposed mice.

The spontaneous incidence of BiAM in sham-exposed mice was generally twice that measured for MiAM, and a slight, but nonsignificant, increase in incidence was observed with time. In contrast to the induction of the micronuclei, increase in incidence of BiAM did not display a well-defined peak, and the percentage of BiAM after exposure to $^{239}PuO_2$ increased steadily over the course of the experiment.

Macrophages with both types of aberration (BiMiAM) were rare and accounted for less than 0.02% of all AM in sham-
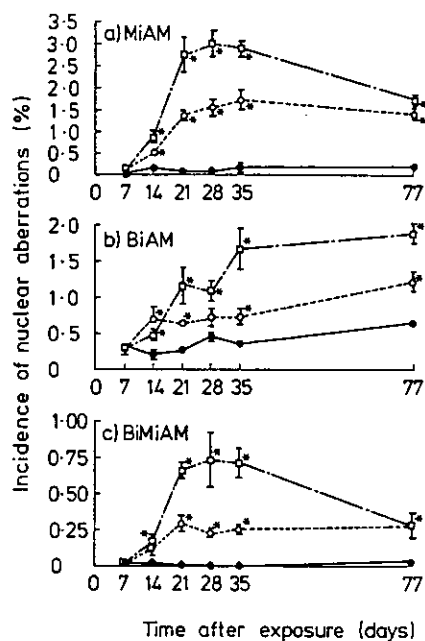

FIGURE 4. Incidence of alveolar macrophages with (a) micronuclei (MiAM); (b) two nuclei (BiAM); (c) both types of aberration (BiMiAM) recovered by lavage from sham-exposed mice (●) and mice exposed to 67 Bq (○) and 424 Bq (□) of ^{239}Pu at times up to 77 days after exposure, means ± SEM. (*) Significant at 5% probability level or greater.

exposed mice. After exposure to ^{239}Pu the incidence of BiMiAM increased and the pattern of induction was similar to that seen for MiAM. On average, the incidence of BiMiAM was five times lower than the incidence of MiAM at corresponding times.

Discussion

The origin of AM is still under debate; there is some evidence that AM are derived from precursors in the bone marrow (36–38), but more recent studies indicate that the AM population is largely self-sustained by proliferation in situ (7–14). Studies of the effects of external irradiation on the lungs are numerous and have demonstrated a decrease both in the size of the AM pool and in the proportion of AM undergoing DNA synthesis (15–19). The accumulation of radioactive particles by AM has precluded the use of [^3H]thymidine and autoradiography to identify AM in DNA synthesis after exposure to airborne actinides. Consequently, few systematic studies of the effects of inhaled actinides on AM kinetics have been reported, and our knowledge of how the lung responds when challenged by radioactive particles is limited. The introduction of new immunocytochemical techniques to identify S-phase cells will overcome the problems encountered heretofore and make such studies more practicable.

One of the earliest effects observed in the present study was a reduction in the number of AM recovered by BAL after exposure to ^{239}Pu. Such observations have been reported previously in mice (21), rats (23), and baboons (24) and also after exposure to other actinides (22,33). In this study the number of AM recovered by BAL from the high IAD group decreased by almost 69% in the first 3 weeks after exposure. It seems unlikely that this

decrease can be attributed to the effects of radiation on progenitor cells in the bone marrow because such cells are distal to the site of radiation, and Morgan et al. (39) showed that very little plutonium is translocated to the bone at early times after exposure to $^{239}PuO_2$. The loss of AM from the mouse lung by mucociliary clearance occurs at the rate of approximately 1% per day (2), and although this may increase after exposure to $^{239}PuO_2$ particles, it cannot account for the decrease in the recovery of AM observed in the present study. Clearly, an alternative mechanism must exist for the depletion of AM after the administration of $^{239}PuO_2$, and such an observation is consistent with a pool of radiosensitive proliferating macrophages or macrophage precursors in the lung.

The results of this study confirm earlier observations (21,28) that, under normal conditions, between 1 and 2% of AM recovered by lavage are in the S-phase of the cell cycle. The significance of such cells is uncertain; some authors have suggested that they are "transitional" cells derived from a pool of proliferating, interstitial macrophages that undergo a final division in the alveoli before maturing into functional AM (40,41). Others believe that they are a true subpopulation of free macrophages that divide in the alveoli to maintain the size of the AM pool (42). Both of these mechanisms involve an intrapulmonary pool of dividing cells that would be sensitive to the effects of radiation. Diurnal fluctuations in the synthesis of DNA have been demonstrated in various tissues of the mouse and other species (43,44). Lambert et al. (45) demonstrated a peak and nadir in the I_s of AM from BALB/c mice occurring around midnight and 1000 hr, respectively, and attributed these fluctuations to diurnal variations in the activity of mice. Such variation would be an important consideration for workers aiming to model the kinetics of the AM population. The data presented here show that, although there is a significant variation in I_s between individual animals, no diurnal fluctuations in DNA synthesis by AM were measured. In conclusion, it seems that in CBA/Ca mice the rate of renewal of AM is constant during the day and night.

One criticism of the present study is that although AM have been demonstrated to undergo DNA synthesis, an observation that has been confirmed by flow cytometry (8), this does not provide direct evidence of AM division. Adamson et al. (6) suggested that there was no evidence of an increase in DNA synthesis by AM following phagocytosis; moreover, the presence of mitotic figures in the AM population implies that AM are capable of progression through the cell cycle. Mitotic AM are rare and in the present investigation accounted for less than 0.1% of all AM recovered by lavage. Others have suggested a similarly low basal rate of mitosis, which has been shown to increase after exposure to NO_2 (46) and carbon (6), further evidence for the ability of AM to divide. No significant change in the mitotic activity of AM was observed after exposure to $^{239}PuO_2$.

A 3-fold decrease in the percentage of AM in DNA synthesis was observed up to 14 days after exposure to 424 Bq of ^{239}Pu. This reduction was followed by a rapid decrease in the number of AM recovered by lavage, and it is likely that these observations can be attributed to an inhibition of AM division or mitotic death of AM after irradiation. Further credence is given to this argument by an increase in the number of AM after 28 days, which was preceded by an increase in the I_s of AM. The results of the present study would be weakened if it was shown either that

lavage was selective with respect to the recovery of S-phase AM or that the efficiency of lavage changes after exposure to $^{239}PuO_2$. Talbot et al. (34) showed that there was no change in the recovery of ^{169}Yb particles after the inhalation of actinides, and it therefore seems unlikely that the reduction in the number of AM after exposure to $^{239}PuO_2$ is due to changes in the efficiency of lavage. An implicit assumption in most studies using lavage is that the AM recovered are representative of the whole pulmonary AM population. The ability to model the kinetics of AM would be weakened if it was shown that lavage was selective with respect to AM kinetics. Studies at this laboratory (R. J. Talbot, personal communication) have shown that even when the lungs of mice are lavaged exhaustively with up to 50 washes, the labeling index of AM was unchanged, which confirms that lavage is a suitable method of obtaining cells for studies of AM kinetics.

The induction of nuclear aberrations in the AM population of the rodent lung after exposure to airborne actinides is well documented (22,35,47). These studies have demonstrated a dose-dependent increase in both the incidence and total number of aberrant AM after exposure to $^{239}PuO_2$. Micronuclei are thought to arise from acentric chromosome fragments that are not incorporated into the daughter nuclei during cell division (8,49) or whole chromosomes that lag behind during mitosis due to a failure in the spindle apparatus (50). The frequency of this type of aberration depends on both the rate of chromosome damage and the rate of cell division and is a sensitive indicator of damage to DNA. Talbot et al. (22) suggested that the formation of micronuclei in AM was proof of the ability of AM, or their precursors, to divide in the pulmonary region of the lung.

The pattern for the induction of MiAM is characteristic of exposure to actinides. Typically there is a latent period of approximately 2 weeks after which the incidence of MiAM increases rapidly to reach peak values between 30 and 40 days after exposure. It is likely that the latency in the induction of MiAM is due to a reduction in the number of dividing AM at early times after the inhalation of $^{239}PuO_2$. At times after 14 days the percentage of AM in DNA synthesis returned to normal and the frequency of micronuclei in the macrophage population increased. Not surprisingly, other studies have demonstrated a decrease in the yield of aberrant AM per unit dose with increasing IAD of $^{239}PuO_2$ (51). At least one cell division is required before micronuclei are expressed, and it is likely that after exposure to high levels of ^{239}Pu the yield of MiAM will be reduced not only due to interphase death but also because of an inhibition of mitosis in the AM population.

Summary

The use of BrdU provides a rapid and convenient alternative to autoradiography to determine the effects of inhaled radioactive materials on the cell kinetics of AM. Using this technique it has been established that the AM is not a terminally differentiated cell incapable of further division and that in normal mice approximately 2% of the AM population recovered by lavage are undergoing DNA synthesis. Furthermore, the recovery of AM in mitosis and the induction of micronuclei in AM after exposure to $^{239}PuO_2$ is additional evidence for the ability of AM to divide. In contrast to other studies (43–45), the data presented here provide no evidence to suggest a significant diurnal variation in the

synthesis of DNA by AM, and it appears that the rate of renewal of AM is constant throughout the day and night.

A decrease in the I_s of AM was seen at early times after exposure to 424 Bq of ^{239}Pu. It is likely that this decrease is due to an inhibition of proliferation in a pool of radiosensitive, intrapulmonary precursors of AM and may in part explain the decrease in the size of the AM pool after exposure to ^{239}PuO$_2$. Finally, the repletion of the macrophage pool was preceded by a recovery in the number of AM in DNA synthesis; at this time there was a rapid increase in the incidence of AM with nuclear aberrations, particularly micronuclei, and it appears that the pattern for the induction of nuclear aberrations can in part be attributed to changes in the kinetics of AM after exposure to ^{239}PuO$_2$.

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