Pu contamination/Lung distribution/Alveolar macrophages/Inflammatory mediators.

The physico-chemical form in which plutonium enters the body influences the lung distribution and the transfer rate from lungs to blood. In the present study, we evaluated the early lung damage and macrophage activation after pulmonary contamination of plutonium of various preparation modes which produce different solubility and distribution patterns. Whatever the solubility properties of the contaminant, macrophages represent a major retention compartment in lungs, with 42 to 67% of the activity from broncho-alveolar lavages being associated with macrophages 14 days post-contamination. Lung changes were observed 2 and 6 weeks post-contamination, showing inflammatory lesions and accumulation of activated macrophages (CD68 positive) in plutonium-contaminated rats, although no increased proliferation of pneumocytes II (TTF-1 positive cells) was found. In addition, acid phosphatase activity in macrophages from contaminated rats was enhanced 2 weeks post-contamination as compared to sham groups, as well as inflammatory mediator levels (TNF-\(\alpha\), MCP-1, MIP-2 and CINC-1) in macrophage culture supernatants. Correlating with the decrease in activity remaining in macrophages after plutonium contamination, inflammatory mediator production returned to basal levels 6 weeks post-exposure. The production of chemokines by macrophages was evaluated after contamination with Pu of increasing solubility. No correlation was found between the solubility properties of Pu and the activation level of macrophages. In summary, our data indicate that, despite the higher solubility of plutonium citrate or nitrate as compared to preformed colloids or oxides, macrophages remain the main lung target after plutonium contamination and may participate in the early pulmonary damage.

INTRODUCTION

Plutonium (Pu) is potentially one of the most hazardous radionuclides associated with the nuclear energy industry and pulmonary contamination may occur at the workplace. In addition to insoluble oxides, Pu nitrate (Pu(NO_3)_4) is widely encountered in nuclear fuel fabrication and reprocessing. Due to the short range of \(\alpha\) rays in tissue, actinide contamination leads to heterogeneous and chronic irradiation, as a consequence of the long term retention in tissues. Nitrate is considered as a moderately soluble form of Pu, which tends to be more diffusely distributed in the lungs than the insoluble oxide form, and thus results in the irradiation of a higher number of target cells. Long term pulmonary damage has been demonstrated after inhalation of \(^{239}\)Pu(NO_3)_4 in cynomolgus monkeys and dogs where a higher lung cancer risk for the nitrate form of Pu was found as compared to oxides.\(^1\)\(^-\)\(^3\) These results are in accordance with the hypothesis that one of the parameters influencing the risk of lung tumor development is the distribution of activity within the lungs, the more homogeneous the \(\alpha\) dose delivery, the higher the rate of tumor incidence.\(^2\)\(^-\)\(^5\) Another consequence of the higher solubility is the significant quantities of Pu translocated to the bone and liver where tumors can also occur.\(^6\)\(^-\)\(^8\)

We have recently shown in the rat that inhalation of PuO_2 induces early inflammatory responses in the lungs and that alveolar macrophages are involved in this process.\(^9\) Following inhalation of insoluble matter, lung macrophages play a crucial role in the distribution and in the clearance of particles. However, the involvement of macrophages to the response of more soluble compounds needs to be addressed. Therefore, in the present study our goals are two-fold 1-
to study the early biokinetic behavior of Pu as well as the role of macrophages in the retention of Pu according to solubility properties of the contaminant and 2- to evaluate lung damage and alveolar macrophage activation following Pu contamination administered as compounds with various solubility properties, from the most soluble (citrate) to the insoluble (oxide).

**MATERIALS AND METHODS**

**Animals and pulmonary contaminations**

Male Sprague Dawley rats, mean body weight of 250 g at the time of exposure, were obtained from Charles River Laboratories (France), quarantined at least 6 days prior exposure, and housed 5 or 6 to a cage. They were maintained at constant temperature (22 ± 1°C) on a 12:12 h light-dark cycle. They received commercial rodent chow and water *ad libitum*. Housing and experiments were carried out in compliance with the French regulation for animal experimentation (European act 2001-486, June 6, 2001).

Plutonium was administered by intratracheal administration in rats after light gaseous anesthesia (isoflurane, Baxter, France). Sham animals were manipulated as rats contaminated with Pu, but were not exposed to the radionuclide.

**Plutonium compounds**

For the experimentations, a range of working Pu solutions were used, differing both in isotopic composition and physico-chemical properties as listed in Table 1. These Pu solutions will be designated later in the paper as “Citrate”, “Nitrate 238”, “Nitrate 238,239” and “Colloid”. They were prepared from two stock solutions of Pu(NO₃)₄ in concentrated HNO₃: one composed of 99% ²³⁹Pu in α activity, the other composed of 12.5% ²³⁹Pu and 86% ²³⁸Pu. The differences in specific activity of the two compounds gave about a 10 fold difference in mass for similar activity. Following evaporation of stock solutions, the “Citrate”, “Nitrate 238” and “Nitrate 238,239” compounds, were made so final concentrations were 20 mM citrate, 25 mM nitrate and 25 mM nitrate respectively. For the “Colloid” compound, colloids were formed *in vitro* as follows. After evaporation of the stock solution of 2.5% ²³⁹Pu and 86% ²³⁸Pu, Pu was diluted so Pu concentration was 25 μM and nitrate concentration was 20.8 mM then the pH was elevated at 11 with NaOH. After incubation overnight, the final pH (8.5), nitrate (9.7 mM) and Pu concentrations were adjusted.

For all working solutions, the final pH ranged from 4-8.5, which is compatible with the physiology of the lungs, and the concentration allowed an administered activity of either 10 or 25 kBq in a final volume of 200 μl. The physical state of these solutions was determined by their filterability through a 10 nm filter.

Some of the results were compared to those obtained after inhalation of PuO₂ as described in a previous paper.²¹ Two types of PuO₂ were used, named “PuO₂₉⁶” and “PuO₂₉⁷”, referring to their percentage in americium which were respectively approximately 46% and 4.5% in activity. For these experiments, the initial lung deposits ranged from 14 to 30 kBq.

**Animal follow up and euthanasia**

The initial Pu deposit after intratracheal administration was defined as the total α activity of the solution administered, determined by scintillation counting of an aliquot of the solution.

Animals were maintained in metabolism cages for the first day following contamination and urines were collected. Rats were euthanized under deep anaesthesia (pentobarbital, 40 mg.kg⁻¹, Sanofi-Santé, France) 4 hs, 3, 7, 10 or 14 days post-contamination for Pu behavior studies and lung distribution of activity, or 2 and 6 weeks after contamination for evaluation of lung damage. The lungs, both femurs and the liver were removed. The transfer of Pu from lungs to systemic compartments was estimated through activity measurement in urine and main extra-pulmonary organs of retention, i.e. skeleton and liver.

Biological samples were successively dry-ashed and wetashed with nitric acid and H₂O₂ until clear solutions were obtained. Total α activity was measured by liquid scintillation counting (Packard counter) in Instagel (Packard). The skeletal retention of activity was estimated assuming that the two femurs represent 10% of the whole skeleton.²⁰

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### Table 1. Properties of the Pu compounds

<table>
<thead>
<tr>
<th>Isotopes</th>
<th>pH</th>
<th>Masses administered</th>
<th>10 nm filterability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate</td>
<td>99%²³⁹Pu, 1%²³⁸Pu</td>
<td>7</td>
<td>59 ng (10 kBq)</td>
</tr>
<tr>
<td>Nitrate 238</td>
<td>99%²³⁹Pu, 1%²³⁸Pu</td>
<td>4–4.5</td>
<td>59 ng (10 kBq)</td>
</tr>
<tr>
<td>Nitrate 238,239</td>
<td>86%²³⁹Pu, 12.5%²³⁸Pu, 1.5%²⁴¹Am</td>
<td>4–4.5</td>
<td>558 ng (10 kBq)</td>
</tr>
<tr>
<td>Colloid</td>
<td>86%²³⁹Pu, 12.5%²³⁸Pu, 1.5%²⁴¹Am</td>
<td>8.5</td>
<td>558 ng (10 kBq)</td>
</tr>
</tbody>
</table>
**Distribution of activity within lungs**

The main bronchus of the left lung was clamped with forceps, and Broncho Alveolar Lavages (BAL) were carried out on the right lung with 4 sequential washes to a total of 12 ml of warmed sterile PBS (pH 7.4). The total number of nucleated cells in BAL was determined. Macrophages represented more than 95% of the total cells. Aliquots of BAL and isolated macrophages were kept for liquid scintillation counting of the total α activity. The left lung was isolated from each exsanguinated rat and expanded to inspiratory volume by intratracheal instillation of 10% neutral buffered formalin (Carlo Erba, France). After 48 to 72 hs fixation in formalin, lungs were dehydrated and embedded in paraffin for histology and in situ autoradiographic distribution studies. For this purpose, lung sections were covered with photographic emulsion (NTB 2K-5, Kodak, France) and exposed for 7 days. Slides were then counterstained with hemalun or Hematoxylin-eosin (H&E).

**Histology and immunohistochemistry**

Lung sections were deparaffinized and dehydrated in graded alcohols solutions. For routine pathology, sections were hydrated and stained with H&E. For immunolabeling studies, slides were incubated in citrate buffer 0.01 M, pH 6 for 15 min at 98°C and kept for 1 h at room temperature in the presence of mouse anti-α CD68 (clone ED1, from Serotec, France) used at 25 μg/ml or overnight at 4°C in the presence of anti-α TTF-1 (Thyroid-Transcription Factor-1, from Santa Cruz biotechnology) used at 2 μg/ml. For the detection of proliferative cells, the monoclonal mouse anti-rat Ki-67 antigen, (Clone Mi3-5 from Dako) was used at 10 μg/ml. After incubation in triton x100 0.1% for 10 min, followed by 20 min at 98°C in citrate buffer, lung sections were kept overnight at 4°C in the presence of anti-Ki67 antibody. For immunodetection, a commercially available ABC-technique (Vectastain, France) was used according to the manufacturer’s instructions. Visualization of peroxidase localization was performed using 3,3’diaminobenzidine as a substrate. The immunostained sections were counterstained with methyl green, or covered with photographic emulsion for autoradiographs, as described above. Specificity of immunohistochemical staining was demonstrated by the absence of staining products using an irrelevant isotype-matched mouse immunoglobulin (mouse IgG1 MOPC 21, Sigma-Aldrich, France).

Quantitative assessment of immunoreactivity was done on computerized image analysis using Histolab software (Microvision, France). Cells positive for TTF-1 were scored under 40X magnification on 50 randomly chosen fields per section. The scoring was realized from 2 to 3 rats per group.

**Cytokine and chemokine measurements**

After centrifugation of BAL, cells were resuspended in HAM’S F12 medium containing 15% FCS, 200 mM L-Glutamine, 1M HEPES, 10^4 IU/ml penicillin and 10 mg/ml streptomycin, all obtained from Sigma Aldrich (France). Cells were plated into 24 well dishes at a concentration of 10^5 cells/well and kept at 37°C in 5% CO₂ incubator. In order to evaluate macrophage activation, supernatants from 24 h-cell cultures were assessed for cytokine and chemokine content by specific ELISA as recommended by the manufacturer (R&D systems, France for TNF-α and CINC-1, Biosource, France for MIP-2 and Amersham, France for MCP-1). Detection limits were 12.5 pg/ml for TNF-α, 7.8 pg/ml for CINC-1, 10 pg/ml for MIP-2, and 38 pg/ml for MCP-1. The choice of this pro-inflammatory cytokine and these chemokines was based on their role in inflammatory processes, and particularly in pulmonary damage following PuO₂ inhalation.⁹

**Acid phosphatase activity measurement**

Cell lysates were obtained from 10⁶ macrophages after successive cycles of freezing in liquid nitrogen and thawing, followed by homogenization with ultrathurax® grinding. The lysosomal enzyme acid phosphatase is classically used as a marker of lung damage, with the measurement of enzyme activity from activated lyzed macrophages. The spectrometric determination of acid phosphatase activity is based on the ability to hydrolyse 4-nitrophenyl phosphate, using an assay kit from Sigma-Aldrich (France).

**Statistical analysis**

Data are presented as means from different animals ± sd. Normality of distribution was tested on each set of data. Means were compared using the Student t test or non-parametric Mann-Whitney test when comparing two groups or with one way ANOVA when comparing multiple groups. The significance level was defined as p < 0.05.

**RESULTS**

**Plutonium behavior after pulmonary contamination**

Variation in isotopic composition of Pu under the same chemical form (nitrate) was used to obtain differences in mass between the two compounds. Due to the high specific activity of ^238Pu, a 10 times difference in mass between Nitrate 238 and Nitrate 238,239 was obtained for a similar α activity, owing to a lower specific activity of ^239Pu and thus to the higher mass of Pu needed to attain same activities. Thus, due to the increased probability to form colloids with the higher mass, higher lung retention was expected. Although not significant after 4 hs due to the dispersion of results, 3 days post-contamination the bone deposit was almost 2 fold lower in the animals receiving Nitrate 238,239 as compared to Nitrate 238 Pu (p < 0.01) which can be related to the ability to form colloids for Nitrate 238,239 Pu more readily than Nitrate 238 (Table 2). No statistically significant difference was observed in the urinary excretion.

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Another set of experiments was carried out to evaluate the mass effect on transfer from lungs to the systemic compartment, using Nitrate 238,239 administered at two different activities: 10 and 25 kBq per rat. The significantly higher urinary excretion (2.3 fold) and bone deposit (1.5 fold) suggest a higher transfer obtained for the lower activity, thus the lower mass, of Pu (Table 3).

Different compounds of Pu, both in terms of isotopic composition and chemical form were used in a same experiment. The in vitro behavior of Pu after pulmonary administration was evaluated through the Day 14 retention in bone, liver and lungs, as well as by urinary excretion at Day 1 (Fig. 1). The in vivo behavior does not fully reflect the in vitro solubility properties. In vitro, both Citrate and Nitrate 238 show a high filterability (close to 80%, Table 1), whereas 94% of Colloid are retained on the 10 nm pore filters. However, a higher proportion of Pu citrate is transferred from lungs to blood as compared to nitrate and colloids, illustrated by the higher retention in bone and liver (Figs. 1A and B), the higher urinary excretion (Fig. 1D) as well as the lower activity in lungs (Fig. 1C), with only 16.1% ± 3.1 of the administered activity retained in lungs after 14 days as compared to 40% after colloid contamination. These results confirm that, as opposed to Citrate, nitrate compounds are able to form colloids at physiological pH.

Table 2. Percentage of activity in urine one day post-contamination or in bone from 4 hs to 14 days post-contamination with Pu nitrate/activity administered (10 kBq). Data are mean ± sd.

<table>
<thead>
<tr>
<th>Activity administered</th>
<th>% activity/administered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Urine</td>
</tr>
<tr>
<td>One day</td>
<td></td>
</tr>
<tr>
<td>4 hours</td>
<td>1.0 ± 0.7</td>
</tr>
<tr>
<td>(n = 11)</td>
<td></td>
</tr>
<tr>
<td>3 days</td>
<td>45.8 ± 2.4</td>
</tr>
<tr>
<td>(n = 5)</td>
<td></td>
</tr>
<tr>
<td>14 days</td>
<td>29.7 ± 2.4</td>
</tr>
<tr>
<td>(n = 8)</td>
<td></td>
</tr>
<tr>
<td>Nitrate 238</td>
<td>0.7 ± 0.4</td>
</tr>
<tr>
<td>(n = 8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>26.8 ± 1.6</td>
</tr>
<tr>
<td>(n = 4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>26.8 ± 1.6</td>
</tr>
<tr>
<td>(n = 5)</td>
<td></td>
</tr>
<tr>
<td>Nitrate 238,239</td>
<td>0.7 ± 0.4</td>
</tr>
<tr>
<td>(n = 11)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>26.8 ± 1.6</td>
</tr>
<tr>
<td>(n = 5)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Percentage of activity in urine from Day 1(D1) or in bone and liver at Day 14 (D14) /activity administered. Initial deposit was 10 or 25 kBq of Nitrate 238,239. Data are mean ± sd. P value determined with Student’s t test, NS: not significant.

<table>
<thead>
<tr>
<th>Activity administered</th>
<th>10 kBq (n = 8)</th>
<th>25 kBq (n = 15)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine D1 (% administered)</td>
<td>0.7 ± 0.4</td>
<td>0.3 ± 0.1</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Bone D14 (% administered)</td>
<td>22.4 ± 6.7</td>
<td>14.9 ± 6.5</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>Liver D14 (% administered)</td>
<td>1.7 ± 0.3</td>
<td>2.0 ± 0.8</td>
<td>NS</td>
</tr>
</tbody>
</table>

Fig. 1. Urinary excretion and retention of Pu with various solubility properties. Fourteen days post-administration of 10 kBq Pu under various forms (Citrate, nitrate or colloid), bone (A), liver (B) and lungs (C) were harvested and activity measured for Pu retention assessment. Urine excretion was determined 1 day post-exposure (D). Results are expressed as percentage of activity administered determined as mean ± sd from 4 rats. *: p < 0.05 between groups as indicated (ANOVA).
Distribution of activity within lungs

The distribution of $\alpha$ activity in whole lungs was evaluated qualitatively by autoradiography studies, and quantitatively by liquid scintillation counting in different lung compartments, i.e. in BAL, containing both macrophages and the epithelial lining fluid and isolated alveolar macrophages.

At the organ level, no aggregates of cells containing Pu were observed on autoradiography studies, resulting in a rather uniform distribution of activity. As shown on Figs. 2, 4 hs post-administration of Nitrate 238,239, many single $\alpha$ tracks were seen in lung interstitium, even though accumulation of tracks associated with macrophages were also observed (Fig. 2A). Accumulation of activity within macrophages became more obvious with time (Figs. 2B and C). Fourteen days post-Pu nitrate administration, some isolated tracks remained visible in tissue, but activity was concentrated in macrophages, as agglomeration of $\alpha$ tracks, indicating that the Pu colloids formed $in$ $vivo$ at physiological pH, can be quickly taken up by macrophages. At this same time point, differences are observed between the various forms administered. Even with the most soluble form of Pu, i.e. the citrate form, (Fig. 2D) some activity seems to be associated with macrophages, although only few tracks per macrophages are seen. The qualitative observations made both from lung slides and isolated macrophages indicate that the number of tracks per macrophage increase with the insolubility

![Fig. 2](image-url)
of Pu, leading to smaller number of macrophages associated with Pu for the less soluble compounds, but with a higher level of activity per macrophage (Figs. 2 D–I).

Autoradiographs of lung sections after immunolabeling with the marker of macrophage activation, CD 68 (Fig. 3A) confirm that macrophages represent the main cellular retention compartments in the lungs. In addition, autoradiographs after TTF-1 immunolabeling indicated that although some activity can be found associated to pneumocytes II, a co-localization of Pu and type II pneumocytes did not occur frequently (Fig. 3B).

Quantitative study of retention of activity in alveolar macrophages was carried out by liquid scintillation counting. The proportion of activity associated with alveolar macrophages recovered in BAL showed only moderate differences (less than 2 fold) considering the differences on their solubility properties (Table 4). However, the compounds showing the higher solubility (Citrate and Nitrate 238) also have the lower percentages of macrophage-associated activity, whereas similar macrophage retention was obtained after Colloid or Nitrate 238,239 contaminations. Similarly, rats receiving 10 or 25 kBq of Nitrate 238,239 do not differ in the percentage of activity associated with macrophages compared to total activity in BAL (respectively 76.4% ± 17.2 and 57.8% ± 7.4).

**Inflammatory changes and macrophage activation**

BAL cell and lung histological analyses provided little evidence of an acute inflammatory response. In all BAL samples, the recovered cells were >95% macrophages. However, a slight increase in the number of neutrophils was seen 14 days after contamination with Nitrate 238,239, which became significant 6 weeks post-contamination, with 0.07 ± 0.16% neutrophils of the total BAL cell population in sham-contaminated rats vs 1.1±1.1% in Pu contaminated rats (p < 0.05, Mann Whitney). A significant increase in the number of multi-nucleated macrophages was also seen in the Pu contaminated rats 6 weeks post-exposure (1±1% in the sham group vs 2.9 ± 2.8% in the Pu contaminated one, p < 0.05, Mann-Whitney).

Histological analyses were carried out on lungs from contaminated rats. As early as 2 weeks post-contamination, foci of inflammatory lesions were observed in Pu nitrate contaminated rats, such as congestion and occlusion of alveoli with exudates, as well as macrophagic alveolitis (Fig. 4B). Such lesions were more commonly seen 6 weeks post-exposure with the presence of higher number of foci and larger areas of damaged tissues (Fig. 4C).

We have previously shown that PuO₂ induced early activation of macrophages. To determine if similar reaction was observed after Pu nitrate contamination, macrophage activation was evaluated by CD68 expression and measurement of cytokine and chemokine production. Only few CD68 positive cells are observed in sham contaminated animals (Fig. 4D). Pulmonary contamination with Nitrate 238,239 led to an increase in the number of CD68 positive cells as well as the intensity of labeling. This feature was observed 14 days post-contamination (Fig. 4E) but was more obvious 6 weeks post-exposure, where a high number of activated macrophages was observed throughout the lungs.

### Table 4. Percentage of activity associated with macrophages/activity recovered in BAL, from 4 hours to 14 days post-intratracheal contamination with the different Pu compounds (10 kBq). ND: not done. Data are mean ± sd.

<table>
<thead>
<tr>
<th>Activity in macrophages/activity in BAL (%)</th>
<th>4 hours</th>
<th>3 days</th>
<th>7 days</th>
<th>10 days</th>
<th>14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate</td>
<td>ND</td>
<td>57.6 ± 4.5 (n = 3)</td>
<td>43.9 ± 13.4 (n = 3)</td>
<td>ND</td>
<td>42.0 ± 9.8 (n = 4)</td>
</tr>
<tr>
<td>Nitrate 238</td>
<td>13.4 ± 4.1 (n = 8)</td>
<td>38.4 ± 17.4 (n = 5)</td>
<td>ND</td>
<td>41.2 ± 4.4 (n = 8)</td>
<td></td>
</tr>
<tr>
<td>Nitrate 238,239</td>
<td>7.9 ± 1.9 (n = 5)</td>
<td>42.9 ± 8.7 (n = 3)</td>
<td>60.0 ± 9.6 (n = 11)</td>
<td>ND</td>
<td>76.4 ± 17.2 (n = 17)</td>
</tr>
<tr>
<td>Colloid</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>67.4 ± 8.2 (n = 6)</td>
<td>63.0 ± 6.2 (n = 4)</td>
</tr>
</tbody>
</table>
as well as the presence of foci of dense labelling (Fig. 4F).

Cultured macrophages from Nitrate 238,239 contaminated rats produced higher inflammatory mediator levels (TNF-α, MCP-1, MIP-2 and CINC-1) than those from sham-contaminated rats. Two weeks post-contamination, inflammatory mediator production was significantly enhanced for rats receiving Pu, either 10 or 25 kBq. A more pronounced effect was observed for the higher activity administered (25 kBq), with approximately a 2 fold increase as compared to the 10 kBq contaminated rats (Fig. 5). At 6 weeks post-contamination, only MCP-1 levels remained higher than controls.

The results obtained 14 days after contamination with Pu nitrate were compared to previous data obtained after similar levels of contamination with Pu oxide (Table 5). Both Pu nitrate forms caused an increase in alveolar macrophage cytokine and chemokine production, and with comparable levels of increase for the two forms. However, the level of enhancement remained lower than those of oxides. Both types of PuO2 induced an elevated inflammatory mediator production, with a more pronounced effect for PuO2R for similar initial lung deposit, confirming previously published data.9) Due to the difference of solubility between the moderately soluble forms (nitrate) and the insoluble oxides, the activity associated with macrophages varies considerably between oxide and nitrate forms for similar initial lung deposit. At this particular time point, the amount of activity associated with macrophages did not differ between the 2 oxides (273 Bq/10⁶ cells for PuO2R and 237 Bq/10⁶ cells for PuO2P). However, it is approximately 5 times lower for Nitrate 238,239 as compared to oxides, and 13 times for Nitrate 238.

As a marker of activation, a lysosomal enzyme activity (acid phosphatase) was measured in the lysate of macrophages from sham- or Pu-contaminated rats, both after nitrate and oxide contamination. Results indicate that all forms of Pu studied led to an increase in acid phosphatase activity as compared to sham groups, with a higher tendency for oxide (6 fold) than for nitrate (2.5 fold, Table 5).

To evaluate the consequences of solubility properties of Pu on macrophage activation, chemokine production was evaluated on macrophages issued from the experiment conducted with Pu compounds of various solubility properties.

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**Fig. 4.** H&E staining (A–C) and immunostaining with anti-CD68 antibody (D–F) of rat lung sections. Lungs were obtained from rats 14 days (A, B, D, E), or 42 days (C, F), after sham-contamination (A) or Nitrate 238,239 contamination (B–F). Slides were processed for H&E staining or incubated with anti-CD68 antibody. Panel D shows the immunolabeling with control IgG. Original magnification: × 200. Bars represent 50 μm.
As shown on Table 6, although the level of activity associated with macrophages is over 3 times higher in 238 Nitrate and Colloid groups, the concentration of cytokine is only slightly different. However, it has to be noted that the activity associated with macrophages represents an average activity per 10⁶ cells and does not indicate neither the percentage of macrophages containing activity nor the level of activity per macrophage, which both might differ between groups.

It has been proposed that plutonium-induced epithelial lesions and neoplasms in the rats might originate from alveolar type II pneumocytes, therefore, we evaluated the number of TTF-1 positive cells in lung sections from contaminated rats as compared to sham groups, 2 and 6 weeks post-contamination. No difference was observed between...
Table 6. Chemokine production by alveolar macrophages in culture and α activity associated with macrophages 14 days post-contamination with 10 kBq of various forms of Pu. Data represent mean ± sd from 4 rats.

<table>
<thead>
<tr>
<th></th>
<th>MCP-1 (pg/ml)</th>
<th>MIP-2 (pg/ml)</th>
<th>Bq/10⁶ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate</td>
<td>638.5 ± 367.3</td>
<td>530.9 ± 186.1</td>
<td>78.0 ± 4.1</td>
</tr>
<tr>
<td>Nitrate 238</td>
<td>630.1 ± 193.2</td>
<td>671.9 ± 161.6</td>
<td>75.8 ± 43.9</td>
</tr>
<tr>
<td>Colloid</td>
<td>532.3 ± 182.3</td>
<td>623.4 ± 131.2</td>
<td>71.6 ± 29.0</td>
</tr>
</tbody>
</table>

Table 7. Number of TTF-1 positive cells in a field (0.1 mm²) as determined using image analysis after immunostaining of rat lung sections with anti-TTF-1 antibody. Lungs were harvested for paraffin inclusion 14 days or 6 weeks after PuO₂ inhalation, Nitrate 238,239 contamination or sham contamination. Data represent mean ± sd of 50 fields per lung section obtained from 2-3 rats per group.

<table>
<thead>
<tr>
<th></th>
<th>Number of TTF-1 positive cells/field</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
</tr>
<tr>
<td>14 days</td>
<td>14.2 ± 2.5</td>
</tr>
<tr>
<td>6 weeks</td>
<td>11.4 ± 0.6</td>
</tr>
</tbody>
</table>

sham treated animals and Pu contaminated rats 2 weeks post-contamination. A slight increase in the number of type II pneumocytes was found 6 weeks after PuO₂ inhalation (Table 7).

In addition, we used the proliferation marker Ki67 to assess proliferative status of lung cells after Pu contamination. Up to 6 weeks post-contamination, no foci of proliferative cells were seen (data not shown).

**DISCUSSION**

Importance of actinide lung distribution with regard to the risk of pathology and tumor development has been the subject of discussion for many years.²,¹³ However, a compromise seems to emerge with the more homogeneous distribution resulting in higher risk.³ Early after inhalation, and before fibrotic lesions occur, insoluble materials distribute uniformly in the lungs at the organ scale but not at the cellular level. Thus, due to the short range of α rays in tissue, irradiated cells consist of cells in close contact to the deposition of activity following lung contamination with insoluble actinides. After PuO₂ inhalation, macrophages represent the main target in terms of irradiation, due to phagocytic properties. More soluble materials such as nitrate are believed to lead to a more homogeneous distribution within lungs and thus, α irradiation, and consequently to increase probability of target cells (epithelial cells) to be hit and functionally affected. The mechanisms underlying radiation tumorigenesis are not yet elucidated and interactions between the wide variety of cells and molecules present in the lungs might be involved as it is the case in cancers from other origins.

In addition, although the early consequences of insoluble actinides in lungs have been described,⁴⁻⁶ no available data concern early damage following pulmonary contamination with more soluble compounds. Since similar to inhalation of PuO₂, pulmonary contamination with ²³⁸,²³⁹Pu(NO₃)₄ induces radiation pneumonitis and fibrosis,⁷ the aim of the present work was to study the early inflammatory response following pulmonary contamination with Pu compounds of various solubility, in relation to lung distribution and pulmonary clearance.

In the present study, the *in vivo* behavior of plutonium was studied with Pu compounds of various solubility properties following 3 approaches. First, Pu was administered as nitrate compounds with different isotopic composition (Nitrate 238 and Nitrate 238,239), leading to a difference of about 10 fold in mass for a similar activity. Second, Nitrate 238,239 was administered at two different activities to obtain a mass difference for similar isotopic composition. And third, different preparation modes were performed to obtain various *in vitro* solubility properties.

The percentage of activity transferred from lungs to blood is measured by urinary excretion and bone and liver retention measurements. Differences were observed between groups, indicating that, as demonstrated in earlier studies (ICRP 1994), the soluble compounds have a higher transfer rate than less soluble compounds, and thus lower lung retention. This was shown following our three approaches. It is likely that nitrate has formed colloids at the physiological pH of the lungs, since a lower transfer to blood is observed as compared to citrate, and lung retention at day 14 is not statistically different from the one of the colloid form. However, the differences in Nitrate 238,239 and Nitrate 238 were seen only at early time point. This suggests that despite the ability for Nitrate 238,239 to form colloids more readily than Nitrate 238 for similar activity levels, Nitrate 238 rapidly becomes as poorly soluble as Nitrate 238,239. The colloids formed at physiological pH are rapidly taken up by alveolar macrophages and, two weeks post-exposure, plutonium was distributed in the same lung compartment, i.e. mainly in macrophages, whatever the chemical form used for contamination. However, Nitrate 238,239 and Colloid, where colloids are present prior contamination, show a higher retention in macrophages than Nitrate 238 and Citrate. The low solubility of Nitrate 238 observed, and demonstrated by the high pulmonary retention at Day 14 (similar to Colloid) might suggest that other sites of retention within lungs are involved, such as interstitial macrophages or some components of the epithelial lining fluid. Overall, despite the higher solubility of Pu administered as nitrate or colloids as compared to the oxide form, an important fraction of the initially deposited activity remained in the lungs over months, confirming previous studies²⁰,²¹ and phagocytic cells repre-
sent the main target for irradiation over time.

Histological analyses and BAL contents indicated a mild pulmonary inflammatory response. Inflammatory lesions appeared to be similar to those reported in the literature for external irradiation and to what is described after Pu contamination.\(^9,22\) In addition, the number of CD68 positive cells increased in Pu nitrate contaminated rats 2 weeks after contamination, and large foci of immunolabelled cells were seen 6 weeks post-contamination indicative of macrophage alveolitis.

In addition to the increased expression of CD68, activation of macrophages has been also shown with the increased cytokine and chemokine production in macrophage cultures from rats contaminated with plutonium. TNF-\(\alpha\) production in the microenvironment of target cells for tumorigenesis, such as pneumocytes II may offer conditions that increase susceptibility to cancer.\(^23\) Furthermore, chemokines, in addition to their role as inflammatory cell recruiters in the damaged tissue, contribute to cancer progression through their anti-apoptotic and angiogenic properties.\(^24,25\) Two weeks after contamination with Pu nitrate, TNF-\(\alpha\) and chemokines were produced in higher quantities by macrophages than after sham-contamination. However, this effect was transient since only MCP-1 remained elevated 6 weeks post-exposure. Following Pu nitrate contamination the activity associated with alveolar macrophages drops off approximately 6 times between week 2 and week 6 post-contamination. MCP-1 sustained production correlates with the higher number of activated macrophages in the lungs, which could have been recruited through the production of MCP-1.\(^26\)

When comparing inflammatory mediator production by macrophages from rats contaminated with oxides or nitrates for similar initial lung deposits, the fold increases as compared to sham animals, are PuO\(_2\)\(^8\) > PuO\(_2\)\(^R\) \(\geq\) Nitrato 238,239 = Nitrate 238. It has to be noted that PuO\(_2\)\(^R\) is the compound with the lower lung clearance\(^27\) and the nitrate compounds have the highest of the four compounds tested here. Thus, the inflammatory mediator production is consistent with the dose-response relationship we previously described after PuO\(_2\)\(^R\) inhalation.\(^9\) Although significant, the differences between oxide and nitrate groups remain modest relative to their difference in activity levels (12 fold difference between Nitrate 238 and oxides, and 5 fold between Nitrate 238,239 and oxides). The final absorbed dose received by macrophages is difficult to assess due to the continual redistribution of activity within lungs. However, it is likely that macrophages received lower doses after contamination with nitrate than oxide, as a consequence of the more rapid initial clearance when plutonium is administered as nitrate, and thus to the lower retention of activity in the lungs. In the experiment conducted with Pu of different solubility, no differences were observed in inflammatory mediator production by macrophages, even though the levels of activity in these cells differ between groups. However, it is not known if the difference in the distribution of activity in macrophages might explain the absence of difference in cytokine and chemokine production. In a study conducted by Talbot et al.,\(^28\) macrophage damage, in terms of cell numbers, nuclear abnormalities and \(\beta\)-glucuronidase activity correlated with the distribution of \(\alpha\) activity, the more uniform distribution, the greater the effect on macrophages. However, this apparent discrepancy could result from two differences in the protocols used: 1- In their study, Talbot et al. contaminated the animals with very different levels of activity to reach “similar 100-day cumulative doses” in the different groups of mice. In the present study, rats were contaminated with similar levels of activity, which lead to different doses at a given time point, due to the differences in the solubility in the Pu compounds 2- the parameters measured by Talbot et al. (cell numbers, nuclear abnormalities and \(\beta\) glucuronidase activity) are different from those evaluated in the present study (CD68 expression and cytokine/chemokine production).

Although most indicators of macrophage activation returned to basal levels 6 weeks post-contamination with Pu nitrate, histological evidence of inflammatory lesions remained visible. Epithelial cells represent a source of inflammatory mediator production after irradiation.\(^29\) Since after exposure to Pu nitrate a higher number of epithelial cells are likely to be hit as compared to oxide inhalation, a higher production of cytokine by irradiated cells may occur. Such a response may participate to overall inflammatory damage expression and to macrophage activation, due to a crosstalk between macrophages and epithelial cells.\(^30,31\) We do not have any clear explanation regarding the sustained presence of macrophages overexpressing CD68 in lungs whereas cytokine and chemokine production from macrophages in culture returned to basal levels. CD68 expression relates to the involvement of macrophages in oxidative burst and inflammatory mediator production might involve different activation pathways.

Type II pneumocytes may represent one of the cell type from which tumors originate.\(^11,32\) Therefore, we investigated whether plutonium contamination led to an early increase in the proliferation of pneumocytes II though the immunolabeling of TTF. However, up to 6 weeks post-contamination, no increase in the number of type II cells was observed. These results are in accordance with previous studies, where authors showed a Pu induced proliferation of pneumocytes II in mouse but not in rats.\(^33\)

In conclusion, our findings show that whatever the solubility properties of Pu compounds, macrophages remain the main target cell and participate in the early pulmonary inflammatory response.

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