PRODUCTION AND TESTING OF ^{244}Cm-LABELED FLUORESCENT POLYSTYRENE LATEX MICROSPHERES

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Abstract — To provide a useful tool for studying the mechanisms of retention and translocation of respirable-sized, alpha-emitting particles, we have developed a method of incorporating ^{244}Cm into fluorescent polystyrene latex microspheres. The resultant particles contain alpha radioactivity comparable to μm-size (AMAD) $^{239}PuO_2$ particles, but are easily visible by fluorescent light microscopy. Preliminary testing of the particles with dog macrophages in vitro has shown that the initial uptake of these Cm-labeled particles is more rapid than uptake of unlabeled particles of similar size. We are continuing to develop procedures for achieving better particle yields, smaller dispersity of particle size distributions and improved retention of the Cm label by the particles.

Studies at this Institute and elsewhere have shown that insoluble alpha-emitting particles deposited in the alveolar region of the lung appear to be transported differently to lymph nodes and up the mucociliary escalator, at least at different rates, from presumably less toxic beta-emitting microparticles. The purpose of this study was to develop an alpha-emitting microsphere that could be observed directly using optical microscopy. Since $^{239}PuO_2$ particles in the respirable range are too small to be observed by light microscopy, we wished to produce an easily visible particle that contained alpha activity of 0.001 to 1 pCi/particle, which is similar to that contained by respirable $^{239}PuO_2$ particles. Our approach was to modify the technique of Henricks et al.[1] in which they prepared ^{51}Cr-labeled, monodisperse, polystyrene microspheres by incorporating high-specific-activity ^{244}Cm into the polystyrene, and, in addition, to incorporate a fluorescent label into the particle matrix to facilitate observation of the particles in tissue samples. This report summarizes the present state of development of this particle production technique and some preliminary in vitro data on the uptake of Cm-labeled and unlabeled particles by dog alveolar macrophages.

MATERIALS AND METHODS

The procedure of Henricks et al.[1] for labeling polystyrene latex microspheres (PLM) with ^{51}Cr involved the following general steps: (1) formation of Cr-diketonate complex; (2) extraction of the Cr complex in monomeric styrene; (3) addition of Cr-labeled styrene to a suspension of 1.0 to 2.0 μm PLM seed particles; and, (4) emulsion polymerization of Cr-styrene on the seed particles by successive steps involving high-dose, gamma-ray excitation and radical polymerization initiated with potassium persulfate.

Since selection of the appropriate Cm-diketonate complex was not obvious, three candidate ligands were selected, based on published results from other investigators. These were acetyl acetone[1] (AA), tetramethylheptane dione[2] (THD), and thenoyltrifluoroacetone[3] (TTFA). The TTFA was dissolved in distilled H_2O, while both the AA and THD were dissolved in a 67% ethanol-water mixture. Approximately 1 mCi $^{244}CmCl_3$ in 0.1 N HCl was combined with one of the three complexing

agents, shaken, and the pH raised to 7.5. The mixture was placed in a constant temperature water bath at 65°C for 3 h without shaking. After cooling, the respective complexes were extracted into benzene, and the extracted Cm complex dissolved in freshly distilled styrene monomer. Two mL of a suspension of 1.74 μm yellow-green fluorescent, polystyrene latex particles (Polysciences Inc. Fluoresbrite Microparticles; 8.7 x 10^9 particles/mL) were added to the Cm-styrene preparation and the mixture was agitated gently overnight (> 5 h). The samples were then irradiated with ^{60}Co gamma rays to a total dose of 13 Mrad at dose rates varying from 10-30 krad/min. The irradiations were performed at the Gamma Irradiation Facility, Sandia National Laboratory. Following irradiation, 10 mg of potassium persulfate radical initiator were added to each sample to further enhance binding of Cm and polymerization, and the samples were shaken for 5 h in a constant temperature water bath at 65-70°C. After polymerization was completed, the samples were purified by at least four sequential centrifugations. Finally, the particles were diluted to 0.25% in distilled water containing ~ 0.1% lauryl sulfate dispersant for storage.

The alpha radioactivity of the prepared particles was assayed by single-phase alpha liquid scintillation counting of dilute samples. Particles were sized by transmission electron microscopy. The leachability of the ^{244}Cm label was assayed *in vitro* using synthetic ultrafiltrate (SUF) as solvent.

Particle Uptake by Dog Alveolar Macrophages

Alveolar macrophages obtained by bronchoalveolar lavage of male Beagle dogs were washed once with saline, once with RPMI 1640, and resuspended in incubation medium consisting of RPMI 1640, 10% heat-inactivated fetal calf serum and 50 μg/mL gentamycin. Cells at a concentration of 10^6 cells/mL were plated on 22 x 22 mm glass cover slips in 35 mm culture dishes, and were incubated at 37°C for 2.5 h. After removal of nonattached cells, two mL of fresh incubation media containing ^{244}Cm-labeled or nonlabeled particles were added to the cultures. Glass coverslips with attached cells were removed at 10, 55, and 100 min and at 1 and 2 days. The cells were stained with Diff Quik, and the number of particles associated with the attached cells were enumerated using epifluorescent microscopy. In parallel with the above determinations, separate aliquots of the culture fluids were filtered through 0.20 μm Nuclepore filters and the filtrates assayed for alpha radioactivity by liquid scintillation counting. The amount of filterable ^{244}Cm, assumed to be dissolved Cm, was expressed as a fraction of the total ^{244}Cm activity in the culture fluid. To detect soluble, cell-bound Cm, the culture supernatants were replaced with 2 mL of 1% sodium dodecyl sulfate (SDS) in Dulbecco's phosphate buffered saline, the cultures were incubated overnight at 37°C, and 1 mL of the SDS digest was filtered through a 0.2 μm Nuclepore filter. Alpha radioactivity was again measured by alpha liquid scintillation counting.

RESULTS AND DISCUSSION

Cm Labeling of Polystyrene Latex Particles

Specific objectives of this method development were (1) to incorporate sufficient alpha activity into PLM to simulate that of respirable $^{239}PuO_2$ particles, (2) to obtain a fluorescent label of the particle that was robust enough to survive the production procedure and remain visible under fluorescence microscopy, (3) to yield a narrow particle size distribution similar to that of the original monodisperse particles used as seed microspheres, and (4) to obtain a high yield of particles that were uniformly and stably labeled. To date, the first two objectives have been successfully attained. Figure 1 shows an alpha autoradiograph of several particles that were labeled with ^{244}Cm and photographed under fluorescing light conditions. All three complexes of Cm were incorporated into PLM, but to varying degrees. The average activity per particle was 5.2 fCi for TTFA, 1.0 fCi for THD and 0.034 fCi for AA. The yield of particles was similar for all three

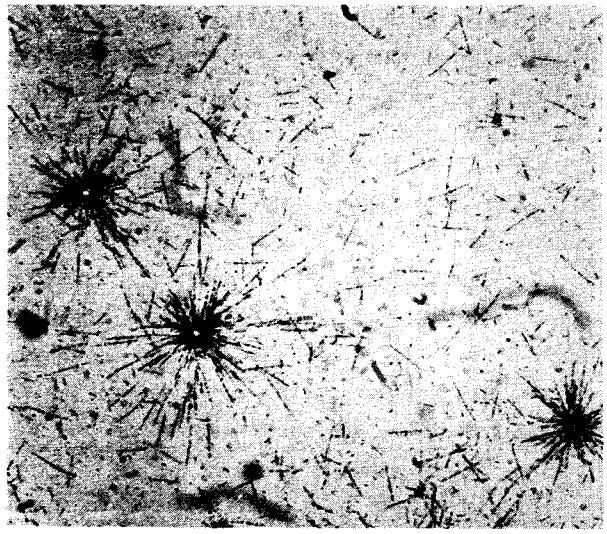

Figure 1. Alpha autoradiograph of ^{244}Cm-labeled fluorescent polystyrene latex particles (14-day exposure). Single tracks indicate either very small particles (< 0.1 μm) or solubilized ^{244}Cm.

complexes, 5.5% for TTFA, 8.4% for THD and 9.4% for AA. Most of the particle losses were due to coagulation. The in vitro solubility of Cm in synthetic ultrafiltrate (SUF) was higher than expected or desired. For both TTFA and THD groups, there was a 20% loss of Cm label in the first 2 h of dissolution; however, the leaching rate decreased rapidly thereafter, such that 75% of the original activity was still associated with the particles after four days of contact with solvent. For the AA group, 50% of the activity was leached from the particles in 2 h; only 32% of the Cm label was still associated with the particles after four days. Therefore, we have chosen TTFA as the preferred chemical form since this form produced the highest specific activity per particle with an adequate yield of particles, and did not require the use of ethanolic water solvent.

In terms of the fluorescence criterion, we have concentrated on using particles labeled with a yellow-green fluorescing dye. Pilot studies with an orange-red, rhodamine-based dye were unsuccessful, as the dye was apparently destroyed by the high-dose gamma irradiation. Although the yellow-green dye withstood the irradiation, there was significant leaching of the dye from the seed particles. This problem has been remedied by doping the styrene monomer with a dye, Coumarin 153, that fluoresces with wavelength characteristics similar to those used in the original seed particles. The resultant particles have adequate fluorescence to be easily seen with epifluorescent microscopy.

Several technical problems remain to be solved. At present, the yield of usable particles is quite low, ~ 10%. Attempts at raising the particle number concentration from 2.5% to 10% resulted in even lower recoveries, suggesting that more vigorous stirring, particularly during and after the gamma irradiations is needed. Secondly, the particle size distributions measured to date have been nonuniform, and multimodal. Better stirring may also ameliorate this problem.

The leachability of Cm from the particles also needs to be reduced. Previous levels of Cm leaching have not been acceptable, particularly since the leaching rates have been sustained over a several-day period. In the latest test run, however, the amount of Cm leached out of the particles was significantly less than before, being less than 0.3% per day after the first 24 h contact with the SUF solvent. However, 15-20% of the activity was leachable during the first 24 h.

Uptake of Cm-labeled PLM by Alveolar Macrophages

Two preparations of ^{244}Cm-labeled PLM were used in this study, one with an average activity per particle of 0.14 pCi, the other of 0.77 pCi. These two specific activities would correspond to $^{239}PuO_2$ particles with activity median aerodynamic diameters of 2.6 and 4.5 μm respectively. A

total of 0.15 μCi ^{244}Cm was added to each culture (0.08 μCi/10^6 added cells); with differing particle specific activities and fixed cell numbers per culture, the particle-to-cell ratios were 0.92 for the 0.14 pCi/particle group and 0.24 for the 0.77 pCi/particle group. We used two control groups with similarly sized, unlabeled fluorescent PLM at particle-to-cell ratios of 0.90 and 0.28, respectively. The uptake of particles by alveolar macrophages for the first 100 min incubation are shown in Figure 2, where the uptake is quantitated as the average number of particles per cell normalized by the particle-to-cell ratio for each specific experimental group. At 10 min, the particle uptake for the 0.77 pCi group was twice that of the other three groups, which were not different. By 55 min, the uptake for the 0.77 pCi group (0.29 particles/cell) was twice those of the control groups (0.15 particles/cell); that of the 0.14 pCi group was 60% higher than controls. Increased uptake by both groups treated with Cm-labeled PLM was also seen at 100 min, but the differences were somewhat decreased (~ 70% control values). At both one and two days, the uptakes for groups with labeled and unlabeled particles were no longer different. The increasing numbers of particles per dog macrophage with time of incubation was due to both increasing numbers of cells that ingested particles and to the increasing particle number concentrations within cells (data not shown).

Viabilities of cells on coverslips were between 89 and 94% after 100 min of incubation and > 90% at one and two days of incubation. There were no statistically significant differences in viability between cultures with radioactive vs. nonradioactive particles.

The "soluble" radioactivity in the culture supernatants was low at one and two days of incubation (Fig. 3) and showed no significant trend. Less than 5% of the initially added radioactivity passed a 0.2 μm filter at any time of measurement. The activity measured in the SDS-treated digests did not increase between 100 min and 2 days; their values were 20-30% of the activities measured in the filtered culture fluids containing particles without cells. At 100 min, about 30% of the particles in the cultures were inside cells; this value increased to about 60% by two days of incubation. Therefore, about 3-6% of the ^{244}Cm activity associated with the ingested particles could have been released by the macrophages. In general, it can be concluded that the Cm labels were reasonably stable inside cells for at least two days.

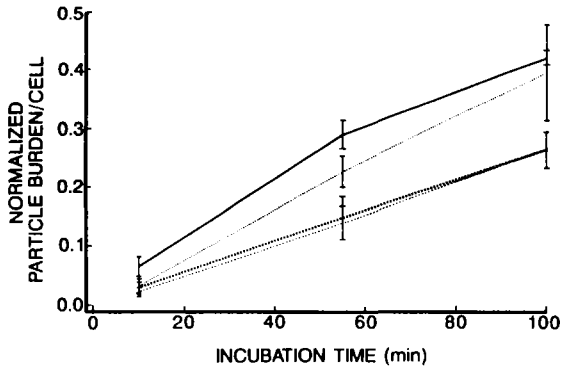

Figure 2. Average number of Cm-PLM per cell vs. incubation time normalized by the original particle-to-cell ratio for each study group. Curves are (———) 0.77 pCi/particles, (·····) 0.14 pCi/particle, (·····) 0.28 particles/cell, and (--------) 0.92 particles/cell. Error bars are standard deviations.

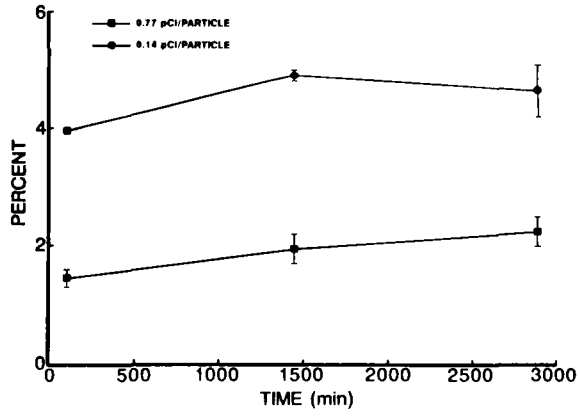

Figure 3. Fraction of ^{244}Cm added to cells that was filterable through 0.2 μm filters vs. time of incubation. Curves are (■) 0.77 pCi/particle and (●) 0.14 pCi/particle.

In summary, a technique has been developed to produce fluorescent polystyrene latex microspheres within the size range visible by ordinary light microscopy, and containing quantities of the alpha-emitting radionuclide ^{244}Cm that simulate the particle activities of inhalable $^{239}PuO_2$ particles. Further progress is needed to increase the yield of labeled particles, decrease the leachability of the incorporated Cm, and reduce the spread in particle size distribution. When Cm-labeled PLM are put into monolayer cultures of dog alveolar macrophages, they appear to be taken up initially more rapidly than are similar PLM that do not contain Cm. Although this observation may be an indication of the ability of random alpha particle irradiation of these cells to produce a nonspecific activation, its importance cannot be evaluated based on these limited data. From the point of view of phagocytosis, the initial rate of uptake may not be very important recognizing that by one day after exposure, > 95% of PuO_2 particles can be found in alveolar macrophages in vivo.

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