THE EFFECTS OF BERYLLIUM METAL PARTICLES ON THE VIABILITY AND FUNCTION OF CULTURED RAT ALVEOLAR MACROPHAGES

Abstract — Rat pulmonary alveolar macrophages (PAM) were exposed in vitro to beryllium metal particles. The particles used were relatively large (Be-II) and small (Be-V) size fractions of beryllium metal obtained from an aerosol cyclone, and a beryllium metal aerosol generated by laser vaporization of beryllium metal in an argon atmosphere (Be-L). Glass beads (GB) were used as a negative control particle. The endpoints examined included cell killing (trypan blue dye exclusion) and phagocytic ability (sheep red blood cell uptake). Phagocytic ability was inhibited by beryllium particles at concentrations that did not cause appreciable cell killing. Results based on the mass concentration of particles in culture medium were transformed by the amount of specific surface area of the particles to permit expression of toxicity on the basis of amount of surface area of particles per unit volume of culture medium. On a mass concentration basis, the order of cytotoxicity was Be-L > Be-V ≈ Be-II > GB; for inhibition of phagocytosis, the cytotoxicity order was Be-L ≈ Be-V > Be-II > GB. On a surface area concentration basis, the order of toxicity for viability was altered to Be-II > Be-L ≈ Be-V (with GB indeterminant) and to Be-V > Be-II ≈ Be-L > GB for inhibition of phagocytosis. We conclude that there are factors in addition to specific surface area that influence the expression of toxic effects in cultured PAM.

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Although its toxicity has been recognized for many years, beryllium is being used in increasing quantities in various applications.[1] Depending on the amount and chemical form of beryllium compound inhaled and deposited in the respiratory tract, both acute and chronic toxic effects can be induced. Of particular concern is beryllium metal, which has been implicated in the induction of chronic berylliosis.[2]

Particles deposited in the lung are rapidly internalized by pulmonary alveolar macrophages (PAM). Because PAM can be readily obtained from the lung and maintained in culture, in vitro tests of PAM responses to toxic materials are valuable screening assays for evaluating potential inhalation hazards. In addition, such assays can provide information concerning the relative toxicities of various materials.[3]

This study was conducted to investigate the role of differing physical properties of beryllium metal particles on their cytotoxicity to PAM in culture. The experimental design was similar to another study that examined the effects of BeO and beryllium metal on cultured fibroblastic and epithelial cells (this report, pp. 305-310). We hypothesized that for beryllium metal, the most important factor influencing the expression of toxic effects to PAM is specific surface area. We used three physical forms of beryllium metal particles having differing sizes, morphologies, and specific surface areas. Endpoints examined included cell killing and inhibition of phagocytosis of test particles. As expected, phagocytosis (a measure of functional ability) was impaired at levels that did not cause appreciable cell killing. The order of toxicity could not be completely accounted for on the basis of specific surface area. Thus, we concluded that other factors were involved as well.

MATERIALS AND METHODS

Alveolar macrophages were obtained from whole lung lavage of equal numbers of male and female F344/N rats from the Institute's colony. Animals were 10 to 14 wk of age at the time of lavage; either 6 or 8 animals were used for each experiment. Animals were injected intraperitoneally with sodium pentobarbital, then sacrificed by cutting the hepatic artery. The heart/lung block was removed, and the lungs were lavaged 4 times (7 mL each for males, 5 mL each for females). Lavage fluids from the animals were pooled, centrifuged (1000 rpm for 10 min), and resuspended in RPMI-1640 culture medium twice. If necessary, red blood cells were lysed by resuspending cells in distilled water for 15 sec, then adding 2X concentrated saline to restore isotonicity.

To determine cell number and viability, an aliquot of the cell suspension was added to an equal volume of 0.4% trypan blue dye, then counted using a hemocytometer. An additional aliquot was centrifuged onto glass microscope slides and stained (Diff-Quik) to evaluate the distribution of cell types in the lavage fluid.

Cells were resuspended in culture medium consisting of RPMI-1640 supplemented with heat-inactivated 10% fetal bovine serum (Hyclone, Logan, UT) and 50 µg/mL gentamycin, to a final concentration of 0.5×10^6 viable cells per mL. Two mL of the cell suspension was added to each well of 6-well culture dishes, then cultures were incubated for 2 h in a 37°C, humidified, 5% CO_2/95% air atmosphere to permit PAM attachment. Following attachment, the medium containing nonadherent cells was withdrawn, fresh medium (with or without test compound) was added, and the cells were incubated for a 20 h exposure period.

The beryllium metal particles we examined included industrial beryllium metal powder (Brush-Wellman Corp., Elmore, OH) obtained from either stage II (Be-II) or stage V (Be-V) of an aerosol cyclone (operation described in 1986-87 Annual Report, LMF-120, pp. 45-49), and ultrafine chain-aggregate beryllium metal aerosols obtained from laser vaporization of beryllium metal in an argon atmosphere (Be-L; production described in 1985-86 Annual Report, LMF-115, pp. 39-42). Glass beads (Particle Information Services, Kingston, WA) were used in each experiment as negative control particles. Physical properties of the particles are described in Table 1. On each day

Table 1
Characteristics of Beryllium Metal Particles Used in
Cultured Rat Alveolar Macrophage Toxicity Studies

Particle Sample[a]	Size (µm)[b]	GSD[c]	Specific Surface Area ($m^2/g \pm SD$)[d]
Be Metal - II	2.4	1.9	7.6 ± 1.2
Be Metal - V	1.0	1.5	20.4 ± 4.0
Be Laser	0.05	1.2	62.1 ± 0.8
Glass Beads	1.67	1.26	2.6 ± 0.1

[a]For beryllium metal, Roman numeral refers to the stage number of the aerosol cyclone separator from which the sample was obtained.

[b]For glass beads, the count median diameter from electron micrographs is shown. For beryllium metal, the count median diameter (Feret's diameter) is given (1986-87 Annual Report, LMF-120, pp. 45-49). For laser-produced beryllium, the physical primary particle size is given (1985-86 Annual Report, LMF-115, pp. 39-42).

[c]Geometric standard deviation (GSD) in the size measurements.

[d]Measured using a nitrogen adsorption technique.[6]

that an experiment was performed, particles were suspended in fresh complete culture medium, then ultrasonically agitated for 30 min. Immediately before addition to PAM cultures, the suspension was stirred. Control cultures received fresh medium alone. Duplicate wells of each compound were tested for each concentration in each experiment. Experiments were repeated at least 3 times.

Following the 20-h exposure period, cells were removed from the culture dishes by scraping with a rubber scraper, placed into 15-mL centrifuge tubes, centrifuged, and resuspended in RPMI-1640. An aliquot was taken for dilution with trypan blue dye for viability determination, as described above. The ability of PAM to phagocytize sheep red blood cells (SRBC) was determined for selected cultures. A 1% suspension of antibody-sensitized SRBC in saline was prepared, then administered to PAM for 60 min in an incubator maintained at 37°C. Extracellular SRBC were lysed with distilled water, as described above, then an aliquot of the cell pellet was centrifuged onto glass slides, stained with Diff-Quik, and the number of SRBC internalized by each of 100 PAM was determined using oil-immersion light microscopy.

All viability and phagocytosis data were expressed as a percentage of control values for each experiment (percent viability and phagocytic index, respectively). Data were plotted against the logarithm of beryllium concentration, then a line was fitted to the portion of the curve where toxic effects were observed. The fitted line was used to determine the LC_{50} (lethal concentration for 50% of the cells) for viability, or EC_{50} (effective concentration that inhibits phagocytosis to 50% of control values) for phagocytosis. Error estimates were obtained from levels of uncertainty in the fitted parameters.

RESULTS

Table 2 summarizes total cell yields, viabilities, and cell types measured for each of seven experiments conducted to study the effects of beryllium particles on PAM. For all experiments, initial cell viability exceeded 93%, and PAM constituted greater than 91% of the cells obtained.

Table 2
Cell Yields, Viabilities, and Differential Cell Types from Lavaged Rat Lungs

Experiment Number	No. of Cells per Rat ($\times 10^6$)[a]	Percent Viability[b]	Differential Counts[c]			
			PAM	PMN	Lym	Epi
1	3.8	98.2%	94.3%	0.3%	2.3%	3.0%
2	6.9	98.6	96.2	0	3.5	0.3
3	6.8	98.1	96.2	0	2.8	1.0
4	4.3	96.6	98.0	0	1.0	1.0
5	6.3	95.9	91.6	0	4.2	4.2
6	4.8	94.0	97.2	0	1.0	1.8
7	6.3	93.0	95.2	0.2	2.4	2.2
Mean ± SEM	5.6 ± 0.5	96.3 ± 0.8	95.5 ± 0.8	0.1 ± 0.05	2.5 ± 0.5	1.9 ± 0.5

[a]Equal numbers of male and female rats per experiment.

[b]Viability determined by trypan blue dye exclusion.

[c]Cell types determined after staining in Diff-Quik; PAM = pulmonary alveolar macrophage, PMN = polymorphonuclear leukocytes, Lym = lymphocytes, and Epi = epithelial cells.

Because the number of SRBC phagocytized by each of 100 PAM cells per culture was counted, it was possible to describe the distribution of the phagocytosis data. Typical distributions for control and exposed cultures are shown in Figure 1. The number of SRBC per PAM appeared normally distributed for control cultures. However, for exposed cultures, the distribution was clearly not normal; large fractions of cells phagocytized $\leq$ 2 SRBC. We decided to use the median value rather than the mean to characterize the ability of cells to phagocytize SRBC in order to minimize the influence of the differences in distribution.

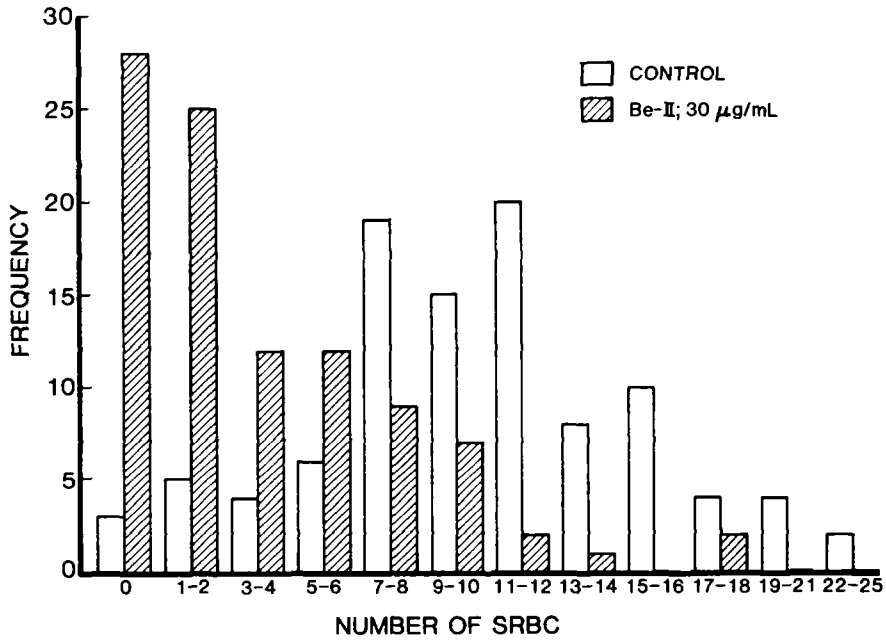

Figure 1. Frequency of PAM cells plotted against the number of sheep red blood cells phagocytized for 100 cells from either control or exposed (Be-II) cultures. The distribution of SRBC per PAM is approximately normal for control cultures and lognormal for exposed cultures.

Results describing the influence of beryllium particle concentration on viability are shown in Figure 2, and on phagocytosis in Figure 3. Also shown are lines fitted to the portion of the data where exposure-related effects were observed. Values for viability (LC_{50}) and phagocytosis (EC_{50}) determined from fitted parameters are shown in Table 3. In terms of the concentration of particles added, the order of toxicity (from greatest to least) for viability depression was Be-L > Be-II $\approx$ Be-V > GB. Minimal viability decrease was observed for GB even at the highest concentration tested (1 mg/mL). The order of toxicity for phagocytosis inhibition was Be-L $\approx$ Be-V > Be-II > GB. It is evident that EC_{50} values for phagocytosis inhibition are at least 10 times less than LC_{50} values for viability decrease.

When the results in Table 3 were normalized to the specific surface area concentrations of the particle samples, values of LC_{50} or EC_{50} in units of mm^2/mL of culture medium were obtained. The ranking of toxicity was changed; for viability, the order of toxicity became Be-II > Be-L $\approx$ Be-V, with GB indeterminant. For phagocytosis, the order was Be-V > Be-II $\approx$ Be-L > GB. The range of responses remained similar when expressed as mm^2/mL rather than μg/mL for viability, but decreased for phagocytosis.

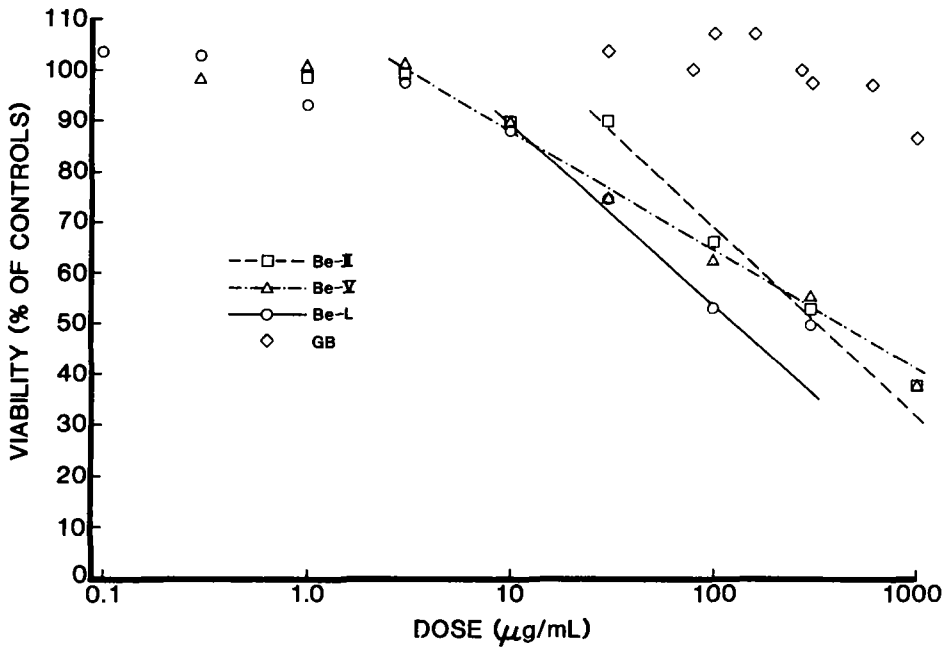

Figure 2. Viability of PAM cultures (expressed as a percentage of control cultures) as a function of particle concentration in the culture medium. Lines were fitted to the curves where cell killing occurred and were evaluated at the 50% point to give values of LC_{50}.

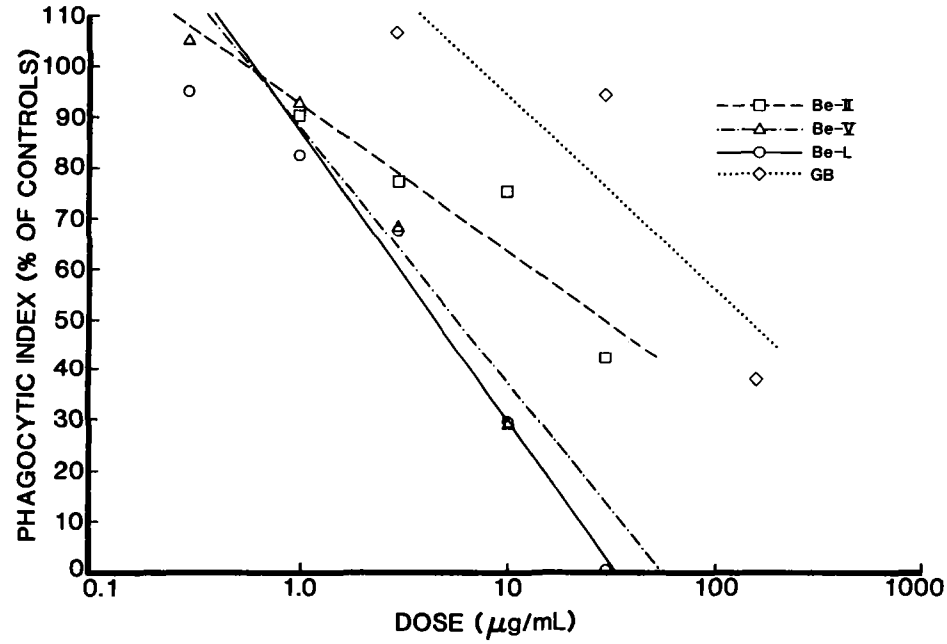

Figure 3. Phagocytic index of PAM that had internalized red blood cells (expressed as a percentage of control cultures) as a function of particle concentration in the culture medium. Lines were fitted to the curves where phagocytosis was inhibited and were evaluated at the 50% point to give values of EC_{50}.

Table 3

Effects of Beryllium Metal Particles on Viability and Phagocytic Ability of Cultured Rat Alveolar Macrophages as a Function of Mass and Surface Area Concentrations

Particle Sample[a]	Viability (LC_{50})[b]		Phagocytic Index (EC_{50})[c]	
	μg/mL	mm^2/mL	μg/mL	mm^2/mL
Be Metal - II	320 ± 60	2400 ± 400	28 ± 15	220 ± 110
Be Metal - V	420 ± 120	8500 ± 2400	5.7 ± 1.5	120 ± 30
Be Laser	130 ± 20	7900 ± 1500	4.4 ± 0.9	270 ± 60
Glass Beads	> 1000	> 2600	140 ± 40	370 ± 100

[a]For beryllium metal, Roman numeral refers to the stage of the aerosol cyclone separator from which the sample was obtained.

[b]Viability determined by trypan blue dye exclusion. Value shown is the LC_{50} determined from evaluation of the fitted line at the 50% point ± the standard error associated with the fitted parameters.

[c]Phagocytic index determined by phagocytosis of sheep red blood cells. Value shown is the EC_{50} determined from evaluation of the fitted line at the 50% point ± the standard error associated with the fitted parameters.

DISCUSSION

For all three beryllium metal particles tested, we observed that the value of LC_{50} for viability was greater than the EC_{50} for phagocytosis by one to two orders of magnitude. The observation that the functional capability of cultured PAM is decreased by toxicants at concentrations that do not cause appreciable cell killing has been described for other compounds.[3]

We used GB as a negative control particle. At the highest concentration tested, 1 mg/mL, cells were filled with beads, but viability was decreased only slightly compared with controls. Phagocytic ability was inhibited by 50% relative to control cultures at a concentration of 140 μg/mL; this was attributed to a particle loading effect.[3] At this concentration, cells contained numerous GB (data not presented), and presumably could not phagocytize as many SRBC because the cytoplasm was already packed, rather than because of any toxicity attributable to the beads.

The ranking of toxicity of viability depression (Be-L > Be-II ≈ Be-V > GB) and for inhibition of phagocytosis (Be-L ≈ Be-V > Be-II > GB) was approximately, although not exactly, as predicted. Transformation of the EC_{50} and LC_{50} values by specific surface areas of the particles also yielded toxicity rankings different than predicted based on results obtained using Chinese hamster ovary cells and rat lung epithelial cell cultures (this report, pp. 305-310). In addition, a retrospective analysis of values of LC_{50} for canine PAM exposed in vitro to BeO prepared at either 500° or 1000°C demonstrated that conversion from mass to surface area expression of dose decreased the difference between LC_{50} values for the two BeO particle types.[4]

We hypothesize that the expression of toxic effects involves phagocytosis followed by intracellular dissolution of beryllium within phagolysosomes. Because dissolution rate is a function of specific surface area (for relatively insoluble particles), it follows that once internalized, particles having equal amounts of surface should dissolve initially at similar rates. Once dissolved, beryllium ion would be available to complex with cellular constituents and thus induce toxic effects. One potential explanation for our results is that significant dissolution occurred within PAM phagolysosomes during the 20-h exposure. This is possible because of the acidic milieu of the phagolysosome, and dissolution of beryllium metal is enhanced in

acidic media.[5] Because dissolution of particles can result in alterations in their specific surface areas, it is conceivable that the specific surface areas of our test particles changed during the 20-h exposure period, resulting in a ranking of cytotoxicity different from that predicted on the basis of surface area alone.

Other potential factors are related to the particles themselves. The particles we tested, although all primarily beryllium metal, differed in oxygen content (1986-87 Annual Report, LMF-120, pp. 50-54) and in morphology. The potential influence of these differences on PAM are not known. Furthermore, because cells from different animals were pooled, heterogeneity of PAM could increase the uncertainty of results within experiments and cause variation between experiments.

In conclusion, we have demonstrated that for various beryllium metal particle samples, PAM phagocytosis is inhibited at concentrations that do not cause cell killing. The order of toxicity, in terms of either viability depression or phagocytosis inhibition, approximates but does not strictly correspond to the ranking by specific surface area of the particles. We conclude that although specific surface area has a major influence on the expression of cytotoxicity in this assay system, other factors appear to be important as well.

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