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SHORT-TERM IN VITRO SCREENING STUDIES RELATED TO THE INHALATION TOXICOLOGY OF POTENTIALLY TOXIC AEROSOLS

FINAL REPORT

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The toxicity of JCE001 particles was tested using the rabbit alveolor macrophage (RAM) test, hamster tracheal ~~ring organ~~ cultures ~~(in vitro assays)~~, and in vivo pulmonary bactericidal activity in mice.

In the RAM test, incubation of alveolar macrophages (AM) with the JCE001 particles decreased ~~($p < 0.05$)~~ AM viability, cellular adenosine 5'-triphosphate (ATP) and total protein levels. The lowest effective dose as determined by Williams' test was 50 ug/mL of JCE001 particles for all parameters examined. The presence of soluble cytotoxic components was established. The nature and rate of release of soluble toxic components from the JCE001 particles were important contributing factors in the mechanism by which these particles produced their toxic effects.

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A single 1-hr in vitro exposure of hamster tracheal ring organ cultures to the JCE001 particles was conducted to determine the dose response on ciliated respiratory epithelium. JCE001 particles caused a significant decrease in cilia beating frequency and an increase in cytopathological alterations when tested at 250 µg/mL and above. Morphological alterations in the tracheal epithelium were observed with light microscopy and scanning electron microscopy. An inhibitory effect of JCE001 particles on RNA synthesis, protein synthesis, and succinic dehydrogenase activity was not demonstrated. Explants treated with 1000 µg/mL of JCE001 particles showed an inhibition of DNA synthesis.

The effect of the JCE001 particles on the ability of AM to kill inhaled [^{35}S]K. pneumoniae was also measured. No statistical main effect of treatment was observed although the bactericidal activity of all groups treated with the JCE001 particles was markedly reduced compared to that of controls.

In addition, the effects of the Asbury #650 and Dixon KS-2 graphite particles on the pulmonary bactericidal activity of mice to inhaled [^{35}S]K. pneumoniae were measured. Significantly decreased bactericidal activities were observed in the 250 µg-treatment groups compared to both naive and vehicle controls. Intratracheal instillation of Asbury #650 and Dixon KS-2 particles did not affect the pulmonary bactericidal activity.

FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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INTRODUCTION

JCE001, Asbury #650 and Dixon KS-2 particles were tested to determine their toxicity. In vitro tests, including rabbit alveolar macrophage viability, adenosine 5'-triphosphate (ATP) content, total protein, and particle solubility, etc., were performed on rabbit and alveolar macrophages to determine the specific toxicity of the JCE001 particles. Ciliary-beating frequency and histopathology of tracheal organ cultures were also investigated in hamsters with the JCE001 particles. Further testing was done on pulmonary bactericidal activity in mice for all particles.

PART 1. THE IN VITRO EFFECTS OF JCE001 PARTICLES IN THE RABBIT ALVEOLAR MACROPHAGE TEST

OBJECTIVE

The rabbit alveolar macrophage (RAM) test was used to determine the effects of JCE001 particles on alveolar macrophage (AM) viability, levels of ATP, and total protein.

MATERIALS AND METHODS

Test Particles

Atlantic brass, Richgold 1800, designated as JCE001 particles, was provided by the U.S. Army Medical Research and Development Command and was stored at ambient temperature. According to the data sheet that accompanied the test particles, the particles had a mass median aerodynamic diameter (MMAD) of 2.3 µm, with a geometric standard deviation of 1.61. The elemental analysis showed 65 to 74% copper, 26 to 35% zinc, and traces of lead, arsenic, aluminum, and various fatty acids.

The RAM Test

Male 2- to 3-kg New Zealand albino rabbits (Hazelton Dutchland Laboratories, Denver, PA) were killed by injection of sodium pentobarbital (75 mg/kg) in the marginal ear vein. AM obtained by tracheobronchial lavage were centrifuged and washed in Hanks' Balanced Salt Solution (HBSS). Cell viability and total and differential cell counts were determined.[1] An AM suspension and a particle suspension were prepared in Medium 199/HBSS supplemented with serum and antibiotics at twice the projected exposure concentrations, and equal volumes of the two suspensions were mixed. A constant concentration of 10^6 AM/mL was maintained in the test suspension; the concentration of the particles was increased serially from 10 to 1000 µg/mL. The test suspensions were incubated in wells of disposable plastic cluster dishes placed on a rocker platform for 20 hr at 37°C in a humidified 5% carbon dioxide (CO_2) atmosphere.

The test parameters included the percentage of AM viability, micrograms of protein per milliliter and femtograms of ATP x 10^6 per microgram of total protein. Immediately after incubation, the percentage of AM viability was determined by Trypan blue exclusion technique. The test suspensions were subsequently washed, centrifuged, and resuspended in HBSS before monitoring total protein and ATP levels. An aliquot of the cell suspension was treated with sodium deoxycholate, the resulting cell lysate centrifuged at 10,000 x g, and the supernatant used for the Lowry protein assay.[2] After extraction from the cells with dimethyl sulfoxide (DMSO) taken from another aliquot, ATP was determined using the luciferin-luciferase reaction in a DuPont 760 Luminescence Biometer.[3]

Soluble Cytotoxic Components

To determine the extent to which leachable components contribute to the total cytotoxic effect, samples of the test particles were incubated in the culture medium at 1000 µg/mL as described above, omitting AM and serum. After incubation, the particles were removed from the media by ultracentrifugation at 10,000 rpm x 15 min, filtered using 0.22 µm filters (Falcon Co.), and resuspended in fresh medium. Then the filtered supernatant, the resuspended preincubated particles, and the original unseparated particle suspension after preincubation were tested separately for cytotoxicity using only AM viability.

Statistical Analysis

Total protein values are given as micrograms per 1.0 mL of the initial incubation sample. ATP levels are expressed in femtograms of ATP per microgram of total protein (experimentally determined), or per milliliter of the initial incubation sample. Changes in total protein and ATP levels are expressed as a percentage of the controls. Because viability of the control AM is not affected by the incubation period, viability values are reported directly as a percentage of the total AM in the experimental sample. One-way analysis of variance (ANOVA) was used to evaluate the effects of the particles on the various parameters tested. Williams' test [4, 5] was used to determine the lowest effective dose.

Experimental Design

Because of difficulties in suspending the test particles in the aqueous incubation medium used for the assay, a concentrated (10,000 µg/mL) stock suspension was prepared in aqueous medium containing 1% isopropyl alcohol (IPA), and the test concentrations were obtained by diluting this stock. Based on the results of a range-finding assay, the particles were tested at 10, 50, 250, 500, and 1000 µg/mL. Three to four culture wells with 10^6 AM/mL were used at each concentration with AM pooled from two to four rabbits as needed to obtain an adequate number of cells for the assays. Two replicate experiments were conducted. The particles were screened for soluble cytotoxic components prior to conducting the main RAM test.

RESULTS

Test Particles

The JCE001 particles are very hydrophobic and floated on the aqueous Medium 199, coating the container walls without entering suspension. An appropriate wetting agent had to be used to disperse the particles in the culture medium. Ethyl alcohol, acetone, IPA, glycerine, and DMSO were tested. IPA proved to be the best; a good suspension was obtained, and relatively few of the test particles coated the container walls.

Soluble Cytotoxic Components

Before assessing the in vitro effects of the JCE001 particles suspended with AM in the liquid maintenance medium, it was necessary to determine whether any toxicity could have been contributed by material solubilized from the particles under the exposure conditions. Results were obtained in examining the test sample for potentially toxic soluble components. The data show that the filtered supernatant produced a marked decrease (38%) in AM viability relative to the unexposed controls (93.9%). This clearly demonstrates that soluble cytotoxic components were released from the JCE001 particles during incubation with the cells. The resuspended preincubated particles remained cytotoxic (19.2%), as well as the original unseparated particle suspension after preincubation (29%). Thus, the nature and rate of release of soluble toxic components from the JCE001 particles were important contributing factors in the mechanism by which these particles produced their toxic effects.

Cytotoxicity of Particles

The results for the two replicate experiments are summarized in Table 1. The viability values were recorded as the percentage of total AM in each experimental sample, whereas the data for total protein and ATP were expressed as the percentage of the corresponding control responses.

Table 2 shows the combined means and standard errors calculated from the two experiments. Inspection of the data reveals significant effects on all parameters within the concentration range of 50 to 1000 µg/mL. The effects on ATP and total protein levels were more pronounced than the effect on AM viability. Dose-response decreases in the response parameters with increased particle exposure were seen only in the 10- to 500-µg/mL concentration range. Exposure concentrations of 500 and 1000 µg/mL appear to be equally toxic.

Statistical analysis of the data by one-way ANOVA indicated a main effect ($p \leq 0.05$) of exposure to the JCE001 particles for all end points. Subsequently, Williams' test, which requires a monotonic relationship between exposure concentration and the measured response, was used to determine the dose at which an end point became significantly different ($p \leq 0.05$) from control. The results shown in Table 2 demonstrate that the lowest effective dose for all parameters tested was 50 µg/mL of JCE001 particles.

SUMMARY

The RAM test was used to determine the effects of the JCE001 particles on the percentage of AM viability, levels of ATP, and total protein. The presence of soluble cytotoxic components was also examined. Incubation with the particles produced significant effects ($p \leq 0.05$) in AM viability, cellular ATP, and total protein levels. The lowest effective dose among all doses tested was 50 µg/mL. The presence of soluble cytotoxic components was established. The nature and rate of release of soluble toxic components from JCE001 particles were important contributing factors in the mechanism by which these particles produced their toxic effects.

PART 2. THE EFFECTS OF JCE001 PARTICLES ON PULMONARY BACTERICIDAL ACTIVITY

OBJECTIVE

The purpose of the study was to determine the effects of the JCE001 test particles on the ability of AM to kill inhaled [^{35}S]Klebsiella pneumoniae.

MATERIALS AND METHODS

Test Particles

Atlantic brass, Richgold 1800, designated as JCE001 particles, was provided by the U.S. Army Medical Research and Development Command and was stored at ambient temperature. According to the data sheet that accompanied the test particles, the particles had a MMAD of 2.3 µm, with a geometric standard deviation of 1.61. The elemental analysis showed 65 to 74% copper, 26 to 35% zinc, and traces of lead, arsenic, aluminum, and various fatty acids.

Because of difficulties encountered in suspending the JCE001 particles for intratracheal instillation, the particle suspensions were made by diluting a 10,000 µg/mL stock suspension. The particles were first mixed and wetted completely with IPA, then diluted in Medium 199 so that the IPA was diluted to 1% of the total volume. The suspension was then sonicated/homogenized for 3 min using a PT 10 sawtooth probe generator with a PT10-35 Brinkmann homogenizer at power setting six to break up the large agglomerates and produce a uniform suspension. Suspensions (10, 50, 100, and 200 µg) were made in 1% IPA in HBSS, which was also the diluent intratracheally instilled in the control mice.

TABLE 1. EFFECTS OF 20-HR IN VITRO EXPOSURE AT 37°C TO JCE001 PARTICLES IN RAM TEST[a]

Exposure Concentration (μg/10⁸ AM/mL)	Experiment	Viability (%)	Total Protein		ATP			
			μg/mL[b]	% of Control	fg×10⁸/mL[b]	% of Control	fg×10⁶/μg Protein	% of Control
0	1	96.2 (1.2)	169.7 (25.5)	–	8.00 (1.37)	–	4.68 (0.13)	–
	2	93.9 (0.8)	254.1 (4.7)	–	14.36 (1.30)	–	5.65 (0.47)	–
10	1	81.9 (1.1)	165.9 (6.6)	97.6 (3.9)	7.88 (1.13)	98.5 (14.1)	4.72 (0.55)	112.0 (5.0)
	2	89.3 (0.3)	255.2 (3.9)	100.5 (1.5)	16.63 (1.61)	104.7 (4.1)	6.51 (0.60)	104.9 (2.8)
50[c]	1	57.3 (1.0)	68.7 (9.7)	40.5 (5.7)	1.53 (0.34)	19.2 (4.3)	2.18 (0.53)	46.6 (5.7)
	2	72.8 (0.8)	126.0 (5.4)	49.6 (2.1)	3.45 (0.68)	24.1 (4.8)	2.69 (0.41)	47.7 (7.2)
250	1	23.6 (1.6)	23.1 (2.9)	13.6 (1.7)	0.03 (0.00)	0.4 (0.0)	0.13 (0.03)	2.8 (0.7)
	2	33.1 (2.6)	42.6 (0.7)	16.3 (0.3)	0.04 (0.01)	0.3 (0.1)	0.09 (0.02)	1.6 (0.4)
500	1	15.2 (1.4)	23.0 (2.4)	13.5 (1.4)	0.11 (0.01)	1.4 (0.1)	0.51 (0.05)	10.8 (1.1)
	2	21.0 (1.1)	41.7 (4.4)	16.4 (1.7)	0.18 (0.02)	1.3 (0.1)	0.44 (0.05)	7.9 (0.9)
1000	1	15.8 (2.3)	37.5 (0.7)	22.1 (0.4)	0.29 (0.01)	3.7 (0.1)	0.78 (0.02)	16.7 (0.3)
	2	22.0 (1.6)	50.5 (4.2)	19.9 (1.7)	0.19 (0.10)	2.4 (0.5)	0.25 (0.18)	5.6 (2.6)

a Mean (±SE) are calculated from quadruplicate incubation samples at each exposure concentration for each parameter for Experiments 1 and 2, respectively. For each replicate experiment, the cells from a separate rabbit pool were used.

b. Of incubation sample.

c. Lowest effective dose ($p \leq 0.05$) for all parameters measured was determined by Williams' test.

TABLE 2. COMBINED EFFECTS OF 20-HR IN VITRO EXPOSURE AT 37°C TO JCE001 PARTICLES IN THE RAM TEST[a]

Exposure Concentration (μg/10^6AM/mL)	Viability (%)	Total Protein (μg/mL)	ATP	
			fg×10^8/mL	fg×10^6/μg Protein
0	95.0 (1.6)	211.9 (59.7)	11.18 (4.49)	5.16 (0.69)
10	85.6 (5.3)	210.4 (63.3)	12.25 (6.18)	5.61 (1.27)
50	65.1 (10.9)*	97.3 (40.6)*	2.49 (1.36)*	2.44 (0.36)*
250	28.4 (6.7)*	32.9 (13.8)*	0.03 (0.01)*	0.11 (0.03)*
500	18.1 (4.1)*	32.3 (13.2)*	0.15 (0.05)*	0.48 (0.04)*
1000	18.9 (4.4)*	44.0 (9.2)*	0.24 (0.08)*	0.57 (0.30)*

a. Mean (±SE) are calculated from Experiments 1 and 2 in Table 1.
* Significantly different from control ($p \leq 0.05$) determined by two-tailed Williams' test.

Animals

Thirty-day-old female CD-1 mice weighing 19-21 g (Charles River Breeding Laboratories, Inc., Kingston, NY) were quarantined for two weeks and were randomized into treatment groups at the initiation of the study.

Intratracheal Instillation

Intratracheal instillation was performed as described by Aranyi et al.[6] Briefly, mice anesthetized with Metofane gas received 10-μL intratracheal injections of Medium 199/1% IPA with or without the test compound. Suspensions of the particles were prepared by diluting each dose level. Aseptic procedures were used, and the suspensions were checked for sterility using standard microbiological procedures. The suspensions were used for intratracheal instillation within one week of preparation to avoid agglomeration of the particles before injection.

Pulmonary Bactericidal Activity

Aerosols of [^{35}S]K. pneumoniae disseminated with a Retec X-70 disposable nebulizer were used for the bactericidal activity assay. Radiolabeled K. pneumoniae were grown in a medium in which the sulfate requirement of the bacteria was provided by [^{35}S]sodium sulfate. Pulmonary bactericidal activity was determined in the lungs of the individual animals from both exposed and control groups that simultaneously inhaled aerosols of the viable radiolabeled bacteria. The ratio of the viable bacterial counts to the radioactive counts in each animal's lungs provided the rate at which bacteria were destroyed 3 hr after infection. Thus,

$$\% \, Bactericidal \, Activity = \left(1 - \frac{R_3}{K_o}\right) 100$$

where R_3 is the ratio of bacterial-to-radioactive counts in the lungs of individual mice at 3 hr, and K_o is an average determined from the same ratios in the lungs of four or five mice per group killed immediately after inhaling the bacteria.

To achieve balanced exposure times to the particles from each dose level (10, 50, and 100 μg/mouse), a rotation schedule was used whereby one mouse from each dose group was injected in sequence. Eighteen mice per dosage level received the particle suspension by intratracheal

instillation. Twenty to 24 hr later, all mice were given simultaneously an inhalation challenge with an aerosol of [^{35}S]K. pneumoniae. Four randomly selected mice per group were killed immediately after the challenge to determine the labeling ratio of the bacteria. The remaining 14 mice per group were killed 3 hr later and used to determine the pulmonary bactericidal activity.

Statistical Analysis

A three-factor mixed-model ANOVA was performed to examine the differential fixed effects of test particles and dosage on bactericidal activity controlling for the random effect of replication. Bactericidal activity originally expressed as a percentage was natural log transformed to better approximate the assumed normality inherent in the use of this statistical model.[8]

RESULTS

Twenty to 24 hr after intratracheal instillation of the JCE001 particles, the mice were challenged with an aerosol of [^{35}S]K. pneumoniae for the determination of bactericidal activity. The results are shown in Table 3. Although the bactericidal activity for all groups that received the JCE001 particles was reduced compared to the controls, there was no main effect of treatment ($p = 0.27$). From these data, it appears that an increased sample size would be required to bring the observed reversed dose-response to statistical significance.

TABLE 3. PULMONARY BACTERICIDAL ACTIVITY OF CD-1 MICE TO INHALED [^{35}S]K. PNEUMONIAE 20 HR AFTER INTRATRACHEAL INSTILLATION OF JCE001 PARTICLES

JCE001 µg/Mouse	% Inhaled [^{35}S]K. pneumoniae Killed in 3 hr
0[a]	76.6 ± 2.9 (12)[b]
10	55.6 ± 8.4 (14)
50	67.0 ± 5.3 (13)
100	71.3 ± 4.6 (12)

a. The controls received 1% IPA in Medium 199.
b. Mean ± SE (number of mice).

SUMMARY

The effects of the JCE001 particles on the ability of AM to kill inhaled [^{35}S]K. pneumoniae were measured. JCE001 particles (0, 10, 50, or 100 µg/mouse) were suspended in 1% IPA in Medium 199 and intratracheally instilled in female CD-1 mice 20 to 24 hr before aerosol challenge with the [^{35}S]K. pneumoniae. No statistical main effect of treatment was observed although the bactericidal activity of all groups treated with the JCE001 particles was reduced compared to that of controls.

PART 3. THE EFFECTS OF JCE001 PARTICLES IN HAMSTER TRACHEAL RING ORGAN CULTURE ASSAYS

OBJECTIVE

The objective of this study was to determine the toxic effects of JCE001 particles on tracheobronchial epithelium, as measured by cilia-beating frequency, morphological alterations, and biochemical changes.

MATERIALS AND METHODS

The toxic effects of JCE001 particles on tracheal epithelium were assessed in hamster tracheal ring organ cultures.[9] Tracheal ring explants were allowed to stabilize for 24 hr prior to use. JCE001-containing medium was prepared at concentrations of 10, 50, 250, and 1000 µg/mL. Because of the hydrophobic nature of the test material, the powder did not go into suspension. DMSO was then used to prepare the suspensions. At 0 and 1 hr of exposure to JCE001-containing medium, the tracheal specimens were examined for ciliary activity, cytotoxicity, and biochemical determinations. Specimens from each group were fixed and prepared for light microscopy and scanning electron microscopy.[10]

RESULTS

Ciliary Activity and Cytopathology

The cilia-beating frequency of hamster tracheal rings exposed in vitro for 1 hr to various concentrations of JCE001 and observed immediately after is shown in Table 4. In initial experiments, ring explants were exposed to JCE001 particles in L-15 culture medium. Exposure to a concentration of JCE001 particles greater than 250 µg/mL resulted in a significant decrease in cilia-beating frequency. When explants were exposed to test material suspended in DMSO-medium, the average cilia-beating frequency for each concentration was lower than particles in medium alone. A significant reduction in ciliary activity was seen in explants exposed to a JCE001 concentration greater than 50 µg/mL.

TABLE 4. MEAN CILIA-BEATING FREQUENCY IN HAMSTER TRACHEAL RING ORGAN CULTURES EXPOSED 1 HR TO JCE001 PARTICLES

Concentration of Test Particle (µg/mL)	Cilia Beating Frequency (Beats/Min)			
	L-15 Medium		L-15 and DMSO Medium	
	Mean[a]	SE	Mean[a]	SE
Baseline	1253[b]	4	1239[b]	5
Untreated control	1213[b]	5	1216[b]	6
10	1195[b,c]	5	1189[b,c]	4
50	1166[b,c]	5	1156[c,d]	5
250	1144[d]	6	1125[c,d]	6
1000	1132[d]	12	1101[d]	23

a. Mean data from 16 separate determinations.

b,c,d. Values with the same superscript letter(s) within each column are not significantly different ($p \leq 0.05$; Dunnett's test).

Tracheal epithelium of explants exposed to medium or DMSO-medium showed similar cytological alterations (Table 5). Significant alterations in the epithelium were seen with 250 and 1000 µg/mL of JCE001. In areas of the epithelium not affected, beating cilia were observed. Focal areas of ciliostasis were seen in epithelium of explants exposed to a JCE001 concentration of 1000 µg/mL.

TABLE 5. PERCENTAGE OF NORMAL EPITHELIUM IN HAMSTER TRACHEAL RING ORGAN CULTURES AFTER A 1-HR IN VITRO EXPOSURE TO JCE001 PARTICLES

Concentration of Test Particle (µg/mL)	Percentage of Normal Epithelium[a]			
	L-15 Medium		L-15 and DMSO Medium	
	Mean[b]	SE	Mean[b]	SE
Baseline	96[c]	1	95[c]	2
Untreated control	93[c,d]	1	90[c,d]	2
10	89[c,d]	1	89[c,d]	1
50	85[c,d]	2	65[c,d]	6
250	65[e]	6	65[e]	6
1000	59[e]	6	63[e]	6

a. Normal epithelium is defined as a smooth luminal surface with beating cilia.
b. Mean data from eight separate determinations.
c,d,e. Values with the same superscript letter(s) within each column are not significantly different ($p \leq 0.05$; χ^2 distribution test).

Histopathology (Light Microscopy)

Specimens exposed to the test material in culture medium did not get the full impact of the particles because they were hydrophobic and floated on the medium surface. Tracheal explants exposed to all concentrations exhibited no morphological changes as observed by light microscopy. The epithelium resembled that of control explants maintained in culture medium without test particles.

The JCE001 particles were then prepared in spectrophotometric-grade DMSO and added to culture medium. Histopathological examinations of untreated (DMSO-medium) control tracheas showed ciliated, columnar epithelium, similar to that of normal hamster trachea immediately after removal from the animal. The epithelium of tracheal explants exposed to 10 and 50 µg/mL exhibited occasional vacuolization. Exposure to greater than 50 µg/mL resulted in moderate pathological alterations. The epithelium showed some loss of ciliated cells, intracellular gaps, and vacuolization. Approximately 50% of the epithelium was not affected.

Scanning Electron Microscopy

Untreated control trachea explants showed an epithelial surface consisting of ciliated and microvillous cells (Figures 1A and 1B). In JCE001-treated specimens, particles were observed resting on the tips of the cilia (Figures 2A and 2B). A second reaction was also observed involving the test particles and the microvillous cells. Particles were occasionally observed attached to the microvillous surface (Figure 3A). In other instances, particles detached from the microvillous surface leaving crater-like structures (Figure 3B).

Figure 1A. Ciliated and microvillus cells of epithelium of control trachea explants, 1100X.

Figure 1B. Ciliated and microvillus cells of epithelium of control trachea explants, 2200X.

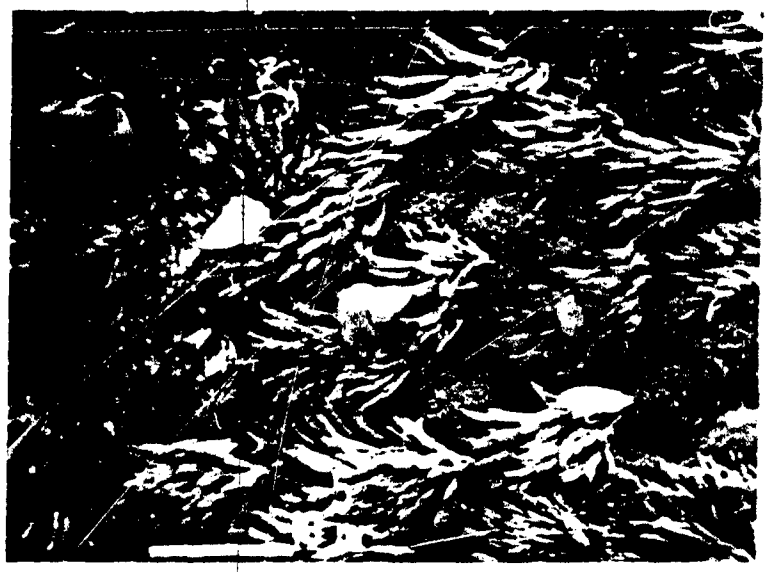

Figure 2A. Particles resting on tips of cilia in JCE001-treated tracheal explants, 2200X.

Figure 2B. Particles resting on tips of cilia in JCE001-treated tracheal explants, 1100X.

Figure 3A. Particles attached to microvillus surface in JCE001-treated tracheal explants, 2200X.

Figure 3B. Crater-like structure in microvillous surface of JCE001-treated tracheal explants, 5500X.

Autoradiography

Tritiated Thymidine Incorporation

Silver grains relating to incorporated tritiated thymidine were seen over the nuclei of epithelial cells in control (untreated) ring explants and in the epithelium of tracheal rings treated with 10, 50, and 250 µg/mL of JCE001 particles. This reaction indicates no inhibition of DNA synthesis. Inhibition of DNA synthesis was observed in explants treated with 1000 µg/mL of JCE001 particles.

Tritiated Uridine Incorporation

Silver grains over the nuclei and to a lesser extent over the cytoplasms were seen in the control explants. Incorporation of tritiated uridine appeared in both ciliated cells and mucous cells, with heavier labeling indicating more RNA in the ciliated cells. Epithelia of tracheal rings treated with the JCE001 particles were also labeled similar to those in controls.

Tritiated Leucine Incorporation

Silver grains corresponding to incorporated tritiated leucine were seen over the cytoplasm in both the untreated control ring cultures and rings treated with all four concentrations of JCE001 particles, indicating no inhibition of protein synthesis.

Succinic Dehydrogenase Activity (Histochemistry)

Untreated controls and JCE001-treated tracheas incubated in medium with the tetrazolium salt, succinate, edetic acid, and buffer showed succinic dehydrogenase activity as evidenced by a blue formazan stippling in the cytoplasm of epithelial cells.

SUMMARY

A single 1-hr in vitro exposure of hamster tracheal ring organ cultures to JCE001 particles was conducted to determine the doss-response on ciliated respiratory epithelium. JCE001 particles caused a significant decrease in cilia-beating frequency and an increase in cytopathological alterations when tested at 250 and 1000 µg/mL. Morphological alterations in the tracheal epithelium were observed with light microscopy and scanning electron microscopy. An inhibitory effect of JCE001 particles on RNA synthesis, protein synthesis, and succinic dehydrogenase activity was not demonstrated. Only explants treated with 1000 µg/mL of JCE001 particles showed an inhibition of DNA synthesis.

PART 4: THE IN VIVO EFFECTS OF GRAPHITE PARTICLES ASBURY #650 AND DIXON KS-2 ON PULMONARY BACTERICIDAL ACTIVITY

OBJECTIVE

The study was conducted to determine the effect of suspensions of two natural graphite samples administered by intratracheal instillation on the pulmonary bactericidal activity of mice to inhaled [^{35}S]K. pneumoniae.

MATERIALS AND METHODS

Test Particles

Two natural graphite samples, designated Asbury #650 and Dixon KS-2, were provided by the U.S. Army Medical Research and Development Command and stored at ambient temperature. Each particulate sample first was mixed and wetted completely with IPA, then diluted in sterile saline so that the final IPA concentration was 5% of the total volume. The suspensions were then dispersed for 25 sec using a Model W185 sonicator (Sonifier Cell Disrupter Heat Systems - Ultrasonics, Inc.,

Plainview, NY) with a microtip at power setting six to break up the large agglomerates. The 5% IPA in sterile saline was also used as a vehicle control for intratracheal instillation in control mice.

Animals

Thirty-day-old female CD-1 mice weighing 16-18 g (Charles River Breeding Laboratories, Inc., Portage, MI) were quarantined for two weeks and randomized into treatment groups at the initiation of the study.

Intratracheal Instillation

Intratracheal instillation was performed as described by Aranyi et al.[6] Briefly, mice anesthetized with Metofane gas received intratracheal injections of 10 µL of 5% IPA/sterile saline with or without the graphite particles. Suspensions of the particles were prepared by dilution for each dose level. Aseptic procedures were used, and the suspensions were checked for sterility using standard microbiological procedures. The suspensions were used for intratracheal instillation within one week of preparation to avoid agglomeration of the particles before injection.

Pulmonary Bactericidal Activity

Aerosols of [^{35}S]K. pneumoniae disseminated with a Retec X-70 disposable nebulizer were used for the bactericidal activity assay.[7] Radiolabeled K. pneumoniae were grown in a medium in which the sulfate requirement of the bacteria was provided by [^{35}S]sodium sulfate. Pulmonary bactericidal activity was determined in the lungs of the individual animals from both exposed and control groups that simultaneously inhaled aerosols of the viable radiolabeled bacteria. The ratio of the viable bacterial counts to the radioactive counts in each animal's lungs provided the rate at which bacteria were destroyed 3 hr after infection. Thus,

$$\% \, Bactericidal \, Activity = \left(1 - \frac{R_3}{K_o}\right) 100$$

where R_3 is the ratio of bacterial-to-radioactive counts in the lungs of individual mice at 3 hr, and K_o is an average determined from the same ratios in the lungs of mice killed immediately after inhaling the bacteria.

Statistical Analysis

A three-factor mixed-model ANOVA was performed to examine the differential fixed effects of graphite articles and dosage on bactericidal activity controlling for the random effect of replication. Bactericidal activity originally expressed as a percentage was natural log transformed to better approximate the assumed normality inherent in the use of this statistical model.[8]

Experimental Design

The concentrations of the intratracheally injected test suspensions were selected based on the results of preliminary LD_{50} studies. Each graphite sample was tested at 25 and 250 µg/animal. The two samples were tested simultaneously. Twelve mice per group were used with a total of six treatment groups in each replicate experiment: two for each of the two graphite samples, one IPA/saline vehicle-control group, and one naive-control group that received no intratracheal instillation. Within 24 hr after intratracheal instillation of the graphite particles, mice from all treatment groups were given a simultaneous inhalation challenge with an aerosol of [^{35}S]K. pneumoniae. Two randomly selected mice per group were killed immediately after the challenge to determine the K_o labeling ratio of the bacteria. The remaining 10 mice per group were killed 3 hr later and used to determine the pulmonary bactericidal activity. This experimental design was repeated two more times to increase the sample size for statistical analysis.

RESULTS

A preliminary intratracheal instillation LD_{50} study was conducted using the Asbury #650 and Dixon KS-2 particles, each at 25, 100, 250, and 500 µg/mouse, along with the respective 2.5, 5, and

10% IPA/saline vehicle controls. None of the mice died within 48 hr of the instillation. Mice that received 500 µg of either particle appeared somewhat ruffled after 24 hr. The corresponding 10% IPA/saline controls appeared normal. The dosage levels of 25 and 250 µg/mouse administered in 5% IPA/saline were selected for both graphite samples in the final experimental design for the tests for bactericidal activity.

The results of the bactericidal activity assays are summarized in Table 6. Statistical analysis for comparing the two graphite samples was not significant (i.e., there was no main effect of test article or test article by dosage interaction). Analysis of the data for the Asbury #650 particles showed a main effect ($p \leq 0.01$) of treatment. Individual post hoc comparisons indicated the main effect was caused by decreased bactericidal activity in the 250-µg treatment group relative to both control groups. The main effect of treatment for the groups that received the Dixon KS-2 particles approached significance ($p \leq 0.07$). Once again, it was the 250-µg group whose bactericidal activity was significantly decreased relative to the naive controls ($p \leq 0.02$) and the vehicle controls ($p \leq 0.03$). Interpretation of the marginal main effect of treatment for the Dixon KS-2 groups is reasonable given the absence of a significant test particle by dosage interaction. Overall, the main effect of dosage in the total sample (i.e., both test particles combined) was significant ($p \leq 0.025$), and the two treatment groups of 25 and 250 µg also were significantly different ($p \leq 0.025$).

TABLE 6. PULMONARY BACTERICIDAL ACTIVITY IN CD-1 MICE TO INHALED [^{35}S]K. PNEUMONIAE 24 HR AFTER INTRATRACHEAL INSTILLATION OF ASBURY #650 AND DIXON KS-2 PARTICLES

Test Particle	µg/Mouse	% Inhaled K. pneumoniae Killed in 3 hr
Naive controls[a]	0	85.5 ± 1.3 (29)[b]
Vehicle controls[c]	0	85.4 ± 1.5 (28)
Asbury #650	25	84.3 ± 2.0 (30)
Asbury #650	250	79.1 ± 2.6 (30)**
Dixon KS-2	25	84.2 ± 1.8 (29)
Dixon KS-2	250	81.0 ± 2.4 (30)*

a. The naive controls received no intratracheal instillation.
b. Mean ± SE (number of mice).
c. The vehicle controls received 5% IPA in sterile saline.
* $p \leq 0.05$.
** $p \leq 0.01$.

SUMMARY

The effects of the Asbury #650 and Dixon KS-2 graphite particles on the pulmonary bactericidal activity of mice to inhaled [^{35}S]K. pneumoniae were measured. The particles (0, 25, or 250 µg/mouse) were suspended in 5% IPA/sterile saline and intratracheally instilled in female CD-1 mice 24 hr before aerosol challenge with the [^{35}S]K. pneumoniae. Both samples produced significantly decreased bactericidal activities in the 250-µg treatment groups compared to both naive and vehicle controls. There was no significant difference between the effects of the intratracheally instilled Asbury #650 and Dixon KS-2 particles on the pulmonary bactericidal activity of mice.

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