ENVIRONMENTAL RESEARCH 40, 313–320 (1986)

Lifetime Effects of Intratracheally Instilled Nickel Subsulfide on B6C3F$_1$ Mice

G. L. FISHER,* C. E. CHRISP,† AND D. A. MCNEILL*

Battelle Columbus Division, Toxicology and Health Sciences Section, 505 King Avenue, Columbus, Ohio 43201, and †Unit for Laboratory Animal Medicine, Medical School, University of Michigan, Ann Arbor, Michigan 48109

Received October 1, 1984

A nickel subsulfide (Ni_3S_2) lung tumor model for the study of metal carcinogenesis was evaluated using intratracheally dosed B6C3F$_1$ mice. A preliminary study of the survival of mice 14 days after a single intratracheal dose of Ni_3S_2 displayed an LD_{50} of 4 mg/kg. A lifetime study was then initiated using five graded doses of Ni_3S_2 or saline alone, administered once a week for 4 weeks. Animals which survived more than 60 days after the final dose were evaluated by histopathology. The study was terminated 27 months after initiation when ~50% of the control animals had died. There was no increase in neoplastic or nonneoplastic lesions observed in animals treated with Ni_3S_2 nor was there evidence of damage to the organs of the respiratory tract from this treatment. The lack of significant biological response appears to be the result of relatively low tolerated dose, efficient lung clearance, and repair of early lung lesions.

INTRODUCTION

Nickel has been the subject of many reviews of metal carcinogens IARC, 1976/7; Flessel *et al.*, 1979; Furst, 1977; Sunderman, 1973, 1976, 1978). In the past, increased incidences of lung and nasal sinus cancers have been associated with certain nickel refining operations (Cuckle *et al.*, 1981). The agent(s) responsible for these cancers has not been identified but most of the high risk operations involved exposures to high concentrations of nickel subsulfide (Ni_3S_2). Animal studies have demonstrated nickel subsulfide to be a most potent form of nickel, which has produced cancers in laboratory animals using a variety of exposure routes, treatment regimens, and animal species (IARC, 1976/7; Flessel *et al.*, 1979; Furst, 1977; Sunderman, 1973). Most commonly, intramuscular injection has been utilized to produce a high frequency of sarcomas with relatively short latency periods. The latency period is reported to be a function of the administered dose. Sunderman (1973) reports that intramuscular or subcutaneous injection of nickel subsulfide dust varying from 3 to 40 mg generally produced high incidences of local sarcomas (rhabdomyosarcomas and fibrosarcomas) with animal survival times of 30 weeks or greater. Although nickel carcinogenesis has been well studied in laboratory animals by intramuscular, intrarenal, or other injection route, few studies have evaluated the effects on the respiratory tract of nickel subsulfide.

The IARC monograph (1976/7) on nickel and nickel compounds indicates that intratracheal instillation of nickel powder, nickel subsulfide powder (mean particle diameter 10 μm), or nickel oxide did not produce a significant increase in

0013-9351/86 $3.00

tumor incidence in mice, rats, or hamsters, respectively. Inhalation studies with relatively low concentrations of nickel subsulfide performed by Ottolenghi *et al.* (1974) demonstrated a significant increase in tumors of the lung, particularly adenomas, adenocarcinomas, and squamous cell carcinomas. In contrast, lifetime inhalation studies with Syrian golden hamsters exposed to green nickel oxide did not produce significant increases in tumor incidence (Wehner *et al.*, 1975). The difference between the Ottolenghi study (Ottolenghi *et al.*, 1974) and the Wehner study (Wehner *et al.*, 1975) appears to be due to the difference in potency between the two chemical forms of nickel. Because other investigators have been successful at producing a highly reproducible incidence of tumors at the site of injection with nickel subsulfide exposure, we investigated the possibility of developing a nickel subsulfide lung tumor model for the study of mechanisms in metal carcinogenesis. The intratracheal route of exposure was chosen because of its simplicity, cost effectiveness, and reproducibility.

MATERIALS AND METHODS

Particle Preparation

Ni_3S_2 was kindly supplied by INCO Limited. Analysis of the material by light microscopy showed that the samples were homogeneous on an individual particle basis with respect to both morphology and color. Particle size of all samples was too coarse for experimental use. Because of the importance of particle size in pulmonary response to particulate insults, size classification was employed to achieve a respirable size of nickel subsulfide.

Powders were prepared for settling by repeated grinding in a carbon steel grinding chamber of a SPEX Model 8000 Mixer-mill until a major portion of the particles were less than the targeted 2-μm cutoff diameter. The powders were then suspended in pure grade ethanol and settled in an Andreasan pipet for 5 hr 41 min, the calculated time required to remove all particles greater than the cut-off size from the sampling zone. Fines were gently drawn off, centrifuged to remove excess solvent, and dried in an oven below the boiling point of the solvent. Particle size and morphological purity were evaluated by light microscopy. X-Ray diffraction of the Ni_3S_2 before and after size separation by sedimentation confirmed that no other detectable contaminants or species were present. Particle size was determined by light microscopic evaluation with subsequent conversion to volume distribution by assuming all particles were spheres and multiplying the count by the cube of the geometric mean of the class boundaries.

Delivery System

For intratracheal instillations, a Gilman Pipetman 20-μl micropipet was calibrated using Fe_2O_3 particles (0.4 to 3.7 μm) in phosphate-buffered saline (PBS). A 1 in. × 23-gauge sterile blunt needle was attached to the pipet and a 20-μl sample of the Fe_2O_3 suspension withdrawn. Pipet delivery showed excellent precision and accuracy (<3% error).

Animals

$B6C3F_1$ mice were selected for study because of the extensive historical data available on neoplastic and nonneoplastic lesions in untreated control animals

(Ward *et al.*, 1979). These animals have a very low incidence of spontaneous lung sarcomas (0.03% for males); bronchiolar adenoma and carcinomas were also low (7.7 and 5.0%, respectively).

$B6C3F_1$ male mice, 5 weeks of age, were obtained from the National Cancer Institute as there were no commercial vendors available for this strain. Mice were housed five per cage in polycarbonate cages containing Absorb-dri bedding and filter tops. Purina Certified Lab Chow and water from the public water supply were available *ad libitum*. After viral screening and 3 weeks of quarantine, animals were determined to be in good health and free from disease.

Intratracheal Instillations

$B6C3F_1$ male mice, 8 weeks of age, were anesthetized with 60 mg/kg sodium pentobarbitol and placed vertically on a specially fabricated holder (Ho and Furst, 1973). A fiberoptic light was used to illuminate the buccal cavity and the tracheal opening was visualized. A Gilman Pipetman with a sterile blunt 23-gauge needle affixed to it was inserted down the trachea to a point just anterior to the primary bifurcation and the dosing material slowly instilled. All dose volumes were 20 μl. Mice were maintained on the holder in a vertical position until normal breathing resumed.

Statistical Evaluation

Statistical analysis of the tumor incidence data (Table 4) was done using a 2×2 contingency design. χ^2 and Cochran–Armitage (linear trend) values were calculated (Snedecor and Cochran, 1967). Linear regressions were also done.

Experimental Design

A preliminary lethality study was performed to aid in dose selection. Eight-week-old male $B6C3F_1$ mice were randomly assigned by body weight to treatment groups (10 mice per group) and were acutely exposed to a single graded dose of nickel subsulfide. Survival was quantified 14 days after treatment.

For the chronic toxicity study, male mice (8 weeks of age) were randomly assigned by body weight to six groups of 20 animals per group after viral screening and a 3-week quarantine. The mice were intratracheally instilled once weekly for 4 weeks with either graded doses of Ni_3S_2 suspended in saline or saline alone. The surviving animals were maintained for 27 months at which point the vehicle control population was reduced to 50% of the animals which survived 60 days after the initial dose administration.

RESULTS

Preliminary Study

Eight-week-old male $B6C3F_1$ mice were intratracheally instilled with single graded doses of nickel subsulfide. Only one vehicle control animal died during dose administration. At the end of the study (Day 14) 50% of the animals were alive at the 4 mg/kg dose (Table 1). Because of the nature of the dose response and the design of the study, this dose was considered the LD_{50} and was used to approximate the highest dose for the toxicity study.

TABLE 1
SURVIVAL OF B6C3F$_1$ MICE AFTER A SINGLE INTRATRACHEAL INSTILLATION OF Ni_3S_2 OR SALINE

Day postdosing	Ni_3S_2 dose (mg/kg)			
	0	4	20	100
1	9/10[a]	10/10	10/10	10/10
2	9/10	10/10	10/10	4/10
5	9/10	8/10	6/10	1/10
6	9/10	8/10	0/10	1/10
7	9/10	6/10	0/10	1/10
12	9/10	5/10	0/10	1/10
14	9/10	5/10	0/10	1/10

[a] Number of survivors/number treated.

Lifetime Study

Animals were randomized by body weight into six groups of 20 male mice after 3 weeks of quarantine and negative titers to the following viruses: pneumonia virus of mice, reovirus type 3, mouse encephalomyelitis (GD VII), Kilham virus, polyoma, Sendai, minute virus of mice, ectro, mouse adenovirus, murine hepatitis, and lymphocyte choriomengites. The mice were intratracheally instilled once weekly for the 4 weeks with the following doses of nickel subsulfide in saline: 0, 0.024, 0.056, 0.156, 0.412, or 1.1 mg/kg.

Animals not surviving 60 days after final dosing were simply tabulated in the survival data for toxicity of the nickel. No final body weight, necropsy, or histopathology were studied for the animals that died during the first 60 days. Table 2 lists the number of survivors at the 60-day period, and at 21 and 27 months, as well as the average body weights for each group at 27 months.

Gross and histopathologic evaluation were made on organs and tissues from all

TABLE 2
SURVIVAL OF B6C3F$_1$ MICE AFTER INTRATRACHEAL ADMINISTRATION OF Ni_3S_2 OR SALINE ONCE A WEEK FOR 4 WEEKS

Months after last dose	Ni_3S_2 Dose (mg/kg)					
	0	0.024	0.056	0.156	0.412	1.1
2	18/20[a]	15/20	20/20	13/20	17/20	9/20
21	17/20	14/20	19/20	12/20	16/20	7/20
27	9/20	7/20	14/20	9/20	14/20	6/20
Body weight (g)						
$\bar{x}$	37.2[b]	37.7	39.1	36.4	37.9	39.1
SD	5.4	1.9	4.8	5.3	3.3	4.8
n	9	7	14	9	14	6

[a] Number of survivors/number treated.
[b] Average body weight of animals terminated at 27 months.

animals surviving the 60-day toxicity period with two exceptions. The tissues from one animal in the lowest dose group (0.024 mg/kg) and one animal in the 0.412 mg/kg dose group were badly cannibalized at necropsy and were discarded. Control mice and those from the five dose groups were examined. Gross lesions were recorded and all tissues showing gross lesions were examined microscopically. In addition, the following tissues were examined histopathologically: nasal turbinates (cross section), salivary gland, mandibular lymph node, trachea, esophagus, thyroid, parathyroid, lung, thymus, stomach, jejunum, colon, caecum, rectum, heart, liver, kidneys, and adrenals.

Table 3 shows the neoplastic lesions found in each dose group and the controls. Table 4 summarizes more detailed information on the incidence of total tumors, benign tumors, and malignant tumors in all dose groups and controls.

There was no evidence of a dose-related increase in tumor incidence in treated mice when compared to controls. Similarly, there was no dose-related increase in nonneoplastic lesions in treated animals versus controls. Particularly, there was no damage evident to organs of the respiratory tract. The nonneoplastic lesions either occurred in small numbers or in numbers normally expected in aged male $B6C3F_1$ mice.

Tumor data from Table 4 were statistically analyzed using the following assumption: Animal deaths that occurred during the first 60 days after dosing were an acute toxic effect of the dose and the deaths were independent of tumor for-

TABLE 3
TUMOR INCIDENCE IN $B6C3F_1$ MICE AFTER INTRATRACHEAL ADMINISTRATION OF Ni_3S_2 ONCE A WEEK FOR 4 WEEKS

Tumor type	Ni_3S_2 Dose (mg/kg)					
	0	0.024	0.056	0.156	0.412	1.1
Malignant lymphoma	5	1	5	5	5	1
Hepatocellular carcinoma	5	2	2	3	2	—
Hepatocellular adenoma	3	5	9	7	8	4
Subcutaneous undifferentiated sarcoma	1	—	—	—	—	—
Subcutaneous fibrosarcoma	—	2	4	1	—	—
Alveolar/bronchiolar carcinoma	1	1	2	—	—	—
Alveolar/bronchiolar adenoma	—	3	3	—	1	2
Hemangiosarcoma	—	1	—	—	—	2
Dermal neurofibrosarcoma	1	—	—	—	—	—
Renal adenocarcinoma	—	—	—	—	—	1
Renal adenoma	—	—	—	—	1	—
Gastric squamous cell papilloma	1	—	—	—	—	—
Thyroid adenoma	2	—	—	—	—	1
Pheochromocytoma	2	—	2	1	—	—
Adrenal capsular adenoma	—	1	—	—	—	—
Subcutaneous fibroma	—	1	—	—	—	—
Harderian gland adenoma	2	—	1	—	1	—
Total tumors	23	17	28	17	18	11

TABLE 4
SUMMARY OF COMBINED TUMOR INCIDENCE IN B6C3F$_1$ MICE AFTER INTRATRACHEAL ADMINISTRATION OF Ni_3S_2 ONCE A WEEK FOR 4 WEEKS

	Ni_3S_2 Dose (mg/kg)					
	0	0.024	0.056	0.156	0.412	1.1
Total animals with primary tumors/ total primary tumors	17/23	14/17	17/28	12/17	13/18	7/11
Total animals with benign tumors/ total benign tumors	8/10	5/10	10/15	6/8	10/11	5/7
Total animals with malignant tumors/ total malignant tumors	12/13	5/7	12/13	9/9	6/7	3/4
Total animals with metastatic tumors[a]/ total metastatic tumors	9/10	1/1	6/6	4/4	3/4	2/2
Total animals/dose group	18	14	20	13	16	9

[a] Malignant lymphoma in more than one tissue was considered metastatic.

mation. An analysis of the tumor incidence in the animals was done using standard χ^2 and Armitage tests (Snedecor and Cochran, 1967). There were no significant differences found between the tumor incidence and dose. Linear regressions for the data were also not significant.

DISCUSSION

In our studies of the carcinogenicity of intratracheally instilled nickel subsulfide in B6C3F$_1$ mice, we were unable to demonstrate an increase in lung or other tumor incidence. Furthermore, lung morphology appeared normal in animals exposed to four once-weekly intratracheal instillations of nickel subsulfide beginning at 11 weeks of age. For the period beginning 60 days after administration of the final dosage of nickel subsulfide, survival was excellent, indicating minimal chronic toxicity by this route of exposure. Furthermore, observations of the animals throughout their lifetimes, body weight data, and light microscopic morphological evaluations all indicated a complete recovery of animals surviving the initial stages of dosage administration. Tumor incidence data for all treated and control groups are consistent with our laboratory historical control data for B6C3F$_1$ mice. It is clear that in our study we achieved dosages beyond the maximum tolerated dose since 1.1 mg/kg appeared to be the $LD_{50,60}$. The study was terminated when vehicle controls achieved a mortality of approximately 50%. This occurred at 27 months of age. At this time, the average body weights between survivors within dosage groups were similar.

It is interesting to contrast the results of this intratracheal administration with previous studies using other routes of administration. The intramuscular route is most efficient in producing tumors with a single injection, oftentimes eliciting well over a 50% tumor incidence at the site of injection (Sunderman, 1973). Dosages used with this route of exposure tend to range from 3 to 10 mg/animal in either mice or rats. The highest achievable dose in our study was 4.4 mg/kg, or 0.11 mg/mouse. Similarly, intrarenal studies have produced approximately a 50%

incidence at the site of injection utilizing approximately 5 mg nickel subsulfide administered in a single injection in rats (Ho and Furst, 1973). Only one study has successfully produced neoplastic changes in lungs of rats exposed to nickel subsulfide (Ottolenghi *et al.*, 1974).

Ottolenghi *et al.* (1974) exposed male and female Fischer 344 rats to approximately 1 mg/m^3 of fine nickel subsulfide particles. Animals were exposed for approximately 80 weeks at 6 hr/day, 5 days/week. Nine of 110 males and 5 of 98 females had malignant tumors of the lung. In contrast, an additional 8 males and 7 females had benign adenomas of the lung. These authors also report a high incidence of nonmalignant lung changes, including hyperplasia of approximately 65% incidence compared to approximately 20% in controls, atypical hyperplasia of 50% compared to approximately 15% in controls, and squamous metaplasia in approximately 20% compared to 5% in controls.

The dose to the lung in the Ottolenghi study (Ottolenghi, *et al.*, 1974) may be calculated using assumptions relative to the deposition of the inhaled aerosol and the minute volume of the animals. The deposition fraction is determined by the aerodynamic diameter of the test aerosol. The authors indicate that the aerosol had 75% of the particles less than 1 μm in size. If we assume that the count median diameter of this aerosol was indeed 0.8 μm, and further assume a geometric deviation of approximately 1.8 (a dispersion typical of laboratory generated aerosols), we may calculate the aerodynamic diameter assuming the density of nickel subsulfide at 5.8 g/cm^3. The resulting aerodynamic diameter is approximately 5.5 μm. Raabe *et al.* (1976) reported lung (pulmonary and bronchial) deposition of approximately 10% for slightly smaller monodisperse aerosols (3 μm) in laboratory rats. Assuming a minute volume of 0.10 liter per minute, the cumulative lung deposition is estimated to have been approximately 1.5 mg/rat, or approximately 7 mg/kg. Our maximum dosages of approximately 0.1 mg/animal, or 4 mg/kg, are substantially less than the calculated cumulative lung dose in the rat inhalation study. Furthermore, if the intratracheal dose regimen were to achieve a tumor incidence similar to that of the inhalation study, it is likely that we would not observe a significant increase in tumors because of the relatively small numbers of animals at the highest dosage.

In contrast to the reports by Ottolenghi *et al.* (1974), we did not observe a significant increase in hyperplasia or metaplasia in the lungs of the intratracheally treated animals. We have performed studies of the pulmonary clearance of radiolabeled nickel subsulfide from Strain A/J mice (Valentine and Fisher, 1984). Lung clearance was observed to be biphasic with initial and final phase biological half-lives of 1.2 and 12.4 days, respectively. In previous unpublished studies, we have observed hyperplastic and fibrotic changes in mice 1–2 weeks after intratracheal administration of nickel subsulfide. Thus, it appears that the lack of significant pathology at 27 months is the result of rather efficient clearance mechanisms which remove most of the nickel subsulfide within 2 months after intratracheal exposure. Subsequently, respiratory tract lesions appear to be repaired after removal of the primary insult.

In summary, we have performed studies to evaluate the utility of nickel subsulfide intratracheal instillation in B6C3F$_1$ mice as a model system for metal carci-

nogenesis of the lung. Our findings indicate that intratracheal administration once a week for 4 weeks of nickel subsulfide at concentrations as high as the LD_{50} does not produce significant late effects. In particular, neither malignant or benign tumors, nor other pathological changes in the lungs were observed in mice maintained until 27 months of age. The lack of significant biological response appears to be the result of two primary factors: (1) efficient clearance of nickel subsulfide after intratracheal instillation and (2) repair of early lung lesions.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the assistance of Ms. K. L. McNeill in compiling data and manuscript preparation. The study was supported by the Electric Power Research Institute (RP 1639-2) and the Nickel Producers Environmental Research Association.

REFERENCES

Cuckle, H., Doll, R., and Morgan, L. G. (1981). Mortality study of men working with soluble nickel compounds. *In* "Nickel Toxicology" (S. S. Brown and F. W. Sunderman, Eds.), pp. 1–34. Academic Press, New York.

Flessel, C. P., Furst, A., and Radding, S. B. (1979). A comparison of carcinogenic metals. *In* "Metal Ions in Biological Systems" (Sigel, Ed.), pp. 1–32. Dekker, New York.

Furst, A. (1977). Inorganic agents as carcinogens. *In* "Advances in Modern Toxicology" (Kraybill, H. F. and Mehlman, M. A. Eds.), pp. 209–229. Halsted Press, Washington, D.C.

Ho, W., and Furst, A. (1973). *Oncology* **27**, 385–393.

IARC (1976/7). Nickel and nickel compounds IARC Monogr. Eval. Carcinog. Risk Chem. Man, 9–11, pp. 75–112.

Ottolenghi, A. D., Haseman, J. K., Payne, W. W., Falk, H. L., and MacFarland, H. N. (1974). Inhalation studies of nickel sulfide in pulmonary carcinogenesis of rats. *J. Natl. Cancer Inst.* **54**, 1165–1170.

Raabe, O. G., Yeh, H. C., Newton, J., Phalen, R. F., and Velasquez, D. J. (1976). Deposition of inhaled monodiperse aerosols in small rodents. *In* "Inhaled Particles IV," pp. 1–18. Pergamon-Press, Elmsford, N.Y.

Snedecor, G. W., and Cochran, W. G. (1967). "Statistical Methods." Iowa State Univ. Press, Ames.

Sunderman, F. W., Jr. (1973). The current status of nickel carcinogenesis. *Ann. Clin. Lab. Sci.* **3**, 156–180.

Sunderman, F. W., Jr. (1976). A review of the carcinogenicities of nickel, chromium, and arsenic compounds in man and animals. *Prev. Med.* **5**, 279–294.

Sunderman, F. W., Jr. (1978). Carcinogenic effects of metals. *Fed. Proc.* **37**, 40–46.

Sunderman, F. W., Jr., Maenza, R. M., Hopfer, S. M., Mitchell, J. M., Allpass, P. R., and Damjamove, I. (1979). Induction of renal cancers in rats by intrarenal injection of nickel subsulfide. *J. Environ. Pathol. Toxicol.* **2**, 1511–1527.

Valentine, R., and Fisher G. F. (1984). Pulmonary clearance of intratracheally administered $^{63}Ni_3S_2$ in strain A/J mice. *Environ. Res.* **34**, 328–334.

Ward, J. M., Goodman, D. G., Squire, R. A., Chu, K. C., and Linhart, M. S. (1979). Neoplastic and non-neoplastic lesions in aging (C57BL/6N × C3H/HeN)F_1 (B6C3F_1) mice. *J. Natl. Cancer Inst.* **63**, 849–854.

Wehner, A. P., Busch, R. H., Olson, R. J., and Craig, D. K. (1975). Chronic inhalation of nickel oxide and cigarette smoke by hamsters. *Amer. Ind. Hyg. Assoc. J.* **36**, 801–810.