Induction of Testicular Sarcomas in Fischer Rats by Intratesticular Injection of Nickel Subsulfide

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ABSTRACT

Nickel subsulfide (Ni₃S₂) was injected in various amounts into the testis of adult Fischer rats for the study of the acute and chronic effects of Ni₃S₂ on testicular cells. Rats given injections of 0.6 to 10 mg of Ni₃S₂ developed an immediate inflammatory response at the site of injection, followed by a delayed, slowly evolving coagulation necrosis of seminiferous tubules and interstitial cells. The extent of testicular necrosis was dose dependent, but at doses of 5 or 10 mg of Ni₃S₂, the rats invariably developed subtotal destruction of the testes. The testis became atrophic, without regeneration of seminiferous tubules. No damage was seen in the other testis, and no systemic effects were noted. Malignant testicular neoplasms developed in 16 of 19 rats within 20 months after an injection of 10 mg of Ni₃S₂. These neoplasms were classified by light and electron microscopy as fibrosarcomas, malignant fibrous histiocytomas, and rhabdomyosarcomas. None of the testicular neoplasms was derived from germ cells or genital cord cells. The occurrence of rhabdomyosarcomas in the testis, an organ normally devoid of striated muscle, suggests that Ni₃S₂ induces malignant transformation of undifferentiated, pluripotential mesenchymal cells.

INTRODUCTION

The carcinogenic properties of nickel compounds have been reviewed in several recent publications (8, 18, 22, 23). Nickel subsulfide (Ni₃S₂) is the most potent carcinogen of the numerous nickel compounds that have been tested in experimental animals (4, 8, 25). Pulmonary carcinomas have developed in rats that were chronically exposed to inhalation of Ni₃S₂ (19), and renal carcinomas have developed in rats that received an i.r. injection of Ni₃S₂ (10). Diverse sarcomas have developed in rats, mice, and hamsters following an i.m. injection of Ni₃S₂ (4, 8, 23, 24, 26). In this study, Ni₃S₂ was administered to rats by i.t. injection in an endeavor to induce malignant tumors of germ cell origin. As will be described in this paper, no germ cell tumors were observed. However, i.t. injection of Ni₃S₂ proved to be an efficient method for induction of sarcomas of the testis, including rhabdomyosarcomas. The occurrence of testicular rhabdomyosarcomas was unexpected, since the testis does not normally contain striated muscle cells.

MATERIALS AND METHODS

Test Compounds. Nickel subsulfide (αNi₃S₂, median particle diameter 1.4 μm) was provided by INCO Ltd., Toronto, Ontario, Canada. The Ni₃S₂ dust was analyzed for aluminum, cobalt, chromium, copper, iron, and manganese by emission spectroscopy (performed by Dr. Stuart Warner, J. R. Gordon Research Laboratory, INCO Ltd., Clarkson, Ontario, Canada), and contamination by each of these metals was found to be less than 0.01% by weight. X-ray diffractometry of the Ni₃S₂ dust (performed by Dr. Edward Kostiner, Institute of Materials Science, University of Connecticut, Storrs, Conn.) yielded an X-ray diffraction pattern that was identical with that previously reported for αNi₃S₂ by Kullerud and Yund (14). Iron dust (iron >99.9% by weight; median particle diameter, <2 μm) and ZnCl₂ (reagent grade) were purchased from Alpha Inorganics Division, Ventrion Corporation, Beverly, Mass. Immediately before injection, the Ni₃S₂ and iron dusts were suspended in sterile 145 mM NaCl in concentrations of 10 mg of Ni₃S₂ or iron per 0.3 ml of vehicle, and ZnCl₂ was dissolved in distilled water to yield a solution that contained 2 mg of Zn(II) per 0.3 ml.

Injections i.t. The experimental animals were 88 male albino Fischer 344 rats (Charles River Breeding Laboratory, Wilmington, Mass.). The rats were housed in stainless steel cages, 2 or 3 rats per cage, and were fed laboratory rat chow (Ralston-Purina Co., St. Louis, Mo.) and tap water ad libitum. The rats were 8 weeks old, and the body weights averaged 155 g (range, 135 to 170 g) at the time of the i.t. injection. Each rat was lightly anesthetized with diethyl ether, and the right testis was gently pushed into the scrotum. By use of a 1-ml tuberculin syringe with a 20-gauge needle, 0.3 ml of the vehicle containing the test compound was slowly injected into the center of the testis. Under the experimental conditions there was very little if any spillage of the injected material into the scrotum. The increased testicular volume, the testis could not be retracted into the abdominal cavity during the first day after the injection, but the testis invariably resumed its normal mobility as soon as the initial swelling had subsided. Physical examinations of the rats were performed daily during the first week after the i.t. injection and thereafter at weekly intervals.

Acute Dose-Response Study. Eighteen rats were ran-
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which the following amounts of Ni$_3$S$_2$ dust were suspended:

domly divided into 6 groups of 3 rats that were given i.t. injections of 0.3-ml volumes of the 145 mM NaCl vehicle in which the following amounts of Ni$_3$S$_2$ dust were suspended: 0, 0.6, 1.2, 2.5, 5, and 10 mg, respectively. All of the rats were killed 1 week after the injection. Both testes were excised, fixed in formalin, sectioned at 4 µm, stained with hematoxylin and eosin, and examined by light microscopy.

Serious Sacrifice Study. Eighteen rats were given single i.t. injections of 10 mg of Ni$_3$S$_2$ in 0.3-ml volumes of the 145 mM NaCl vehicle. Six control rats were given i.t. injections of 0.3 ml of the 145 mM NaCl vehicle. Three rats that received Ni$_3$S$_2$ and 1 control rat were killed at each of the following intervals after the injection: 8 hr, 24 hr, 2 days, 5 days, 2 weeks, and 6 weeks. Both testes of each rat were examined by light microscopy. Portions of the injected testis were diced in 4% cacodylate-buffered glutaraldehyde at 0°C, and postfixed in 1% cacodylate-buffered 2% osmium tetroxide. The tissues were embedded in epoxy resin for ultrathin sectioning. The sections were stained with lead citrate and uranyl acetate. Electron microscopy was performed by use of a Phillips 300 electron microscope at 60 kV.

Carcinogenesis Study. Forty-six rats were randomly divided into 2 major groups, A and B, that contained 18 and 19 rats, respectively, and 2 minor groups, C and D, that each contained 4 rats. Rats in Group A were given a single injection of 0.3 ml of the 145 mM NaCl vehicle into the right testis. Rats in Group B were given an i.t. injection of 10 mg of Ni$_3$S$_2$ dust suspended in 0.3 ml of 145 mM NaCl vehicle. Rats in Group C received an injection of 10 mg of iron dust suspended in 0.3 ml of 145 mM NaCl vehicle, and rats in Group D received an injection of 2 mg of Zn(II) (as ZnCl$_2$) dissolved in 0.3 ml of water. During the study, the rats either died spontaneously or were killed when tumors became so large that the rats could not move about their cages and hence could not obtain food or water. All rats in Group B died within 20 months after the injection, and the surviving rats in Groups A, C, and D were killed at 20 months after the injection (i.e., at =22 months of age). All of the rats were completely autopsied, and their tissues and organs were examined by light microscopy. Each testicular tumor was examined by electron microscopy. In 3 rats that died spontaneously, the testicular tumors were mildly autolyzed. However, the autolysis was never so severe that electron microscopic examinations could not be performed.

RESULTS

Acute Dose-Response Study. Necrosis of testicular tissue was seen in all rats that were killed at 1 week after i.t. injection of Ni$_3$S$_2$ at dosages of 0.6, 1.2, 2.5, 5, and 10 mg. At the 2 higher dosages (5 and 10 mg of Ni$_3$S$_2$), the entire testis was black on gross examination, and the testicular parenchyma was massively necrotic on histological examination. The centrally located testicular components were completely disintegrated, and only a narrow band of subcapsular seminiferous tubules was preserved. The necrotic testicular tissue was heavily infiltrated with mononuclear cells and polymorphonuclear leukocytes. At the 3 lower dosages (0.6, 1.2, and 2.5 mg of Ni$_3$S$_2$), there were localized areas of testicular necrosis. Surrounding these well-demarcated foci of necrosis, the testicular parenchyma appeared to be intact. No pathological changes were seen in the testes of the control rats that were killed at 1 week after an i.t. injection of the 145 mM NaCl vehicle.

Serial Sacrifice Study. In testes of rats that were killed at 8 hr after an i.t. injection of 10 mg of Ni$_3$S$_2$, practically all of the seminiferous tubules were intact. Particles of Ni$_3$S$_2$ were mostly located in the interstitial spaces, although a few particles of Ni$_3$S$_2$ were seen in the cytoplasm of mononuclear cells. At 24 hr after Ni$_3$S$_2$ injection (Fig. 1), numerous macrophages and polymorphonuclear leukocytes were seen in the testes. Many macrophages contained cytoplasmic particles of Ni$_3$S$_2$, but most of the Ni$_3$S$_2$ particles were still extracellular. At 2 days after Ni$_3$S$_2$ injection, the infiltration of macrophages and polymorphonuclear leukocytes was most intense, and the Ni$_3$S$_2$ particles were predominantly intracellular. Although the outlines of the seminiferous tubules were preserved, the tubular lumens contained numerous detached Sertoli cells and germ cells. No active spermatogenesis was evident. The seminiferous tubules were surrounded by nuclear fragments and other cellular debris, as well as by Ni$_3$S$_2$ particles. At 5 days after Ni$_3$S$_2$ injection (Fig. 2), most seminiferous tubules had completely lost their internal structure. In some locations, the tubular outlines were poorly discernible, and the tubules were nearly replaced by masses of inflammatory cells. At 2 weeks after Ni$_3$S$_2$ injection (Fig. 3), the seminiferous tubules in the center of the testes had been transformed into an amorphous mass. At the periphery of the testes, in juxtaposition to the testicular tunica, there were eosinophilic tubular "ghosts" situated within dense granulation tissue. Most of the seminiferous tubules that could still be discerned were devoid of germ cells and were lined only by Sertoli cells. At 6 weeks after Ni$_3$S$_2$ injection (Fig. 4), the testes were small, hyalinized, and fibrosed. Ni$_3$S$_2$ particles, as well as some hyalinized, acellular tubules, could be seen in the connective tissue. In testes of the control rats that received i.t. injection of 145 mM NaCl vehicle, the only pathological changes that were seen in the serial sacrifice study were edema at 8 hr and mild inflammatory cell infiltration at 24 hr and 2 days after the injection.

Carcinogenesis Study. Sarcomas of the testes were found in 16 of 19 rats (84%) of Group B that had received an i.t. injection of 10 mg of Ni$_3$S$_2$ (Table 1; Fig. 5). The sarcomas all developed in the testes that had received the injection, and the noninjected testes were normal. The cumulative incidence of testicular sarcomas in rats of Group B is illustrated in Chart 1. The first testicular sarcoma was detected by palpation as a pea-sized nodule that was "hard as a rock" at 7 months after the i.t. injection of Ni$_3$S$_2$. The median interval between initial palpation of the 16 sarcomas and death of the rats was 4 weeks (range, 2 to 7 weeks). Cumulative mortality curves for rats in Groups A and B are illustrated in Chart 1. On the basis of light microscopic examinations, the testicular tumors were classified as fibrosarcomas (10 of 16; 63%) (Fig. 6), malignant fibrous histiocytomas (soft-tissue giant cell sarcomas) (3 of 16; 19%) (Fig. 7), and rhabdomyosarcomas (3 of 16; 19%) (Fig. 8). Striated muscle cells were identified in each of the 3 rhabdomyosarcomas. The testicular capsule was tret-
Table 1

Induction of testicular sarcomas in Fischer rats

The test substances were administered to Fischer rats by a single i.t. injection in a volume of 0.3 ml. The experiment was terminated at 20 months after the injection upon the death of the last surviving Ni₃S₂-treated rat (Group B).

<table>
<thead>
<tr>
<th>Group</th>
<th>Test substance</th>
<th>No. of rats</th>
<th>Mortality by 20 mos.</th>
<th>Testicular sarcomas</th>
<th>Classification of testicular sarcomas</th>
<th>Tumor metastases</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>Vehicle</td>
<td>18</td>
<td>10</td>
<td>0</td>
<td>Fibrosarcomas (11)</td>
<td>2/10⁴⁺⁺⁺⁺⁻</td>
</tr>
<tr>
<td>B</td>
<td>Ni₃S₂ (10 mg)</td>
<td>19</td>
<td>19</td>
<td>16</td>
<td>Malignant fibrous histiocytomas (4)</td>
<td>1/3⁴⁺⁺⁺⁻</td>
</tr>
<tr>
<td>C</td>
<td>Iron (10 mg)</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>Rhabdomyosarcomas (4)</td>
<td>1/3</td>
</tr>
<tr>
<td>D</td>
<td>Zn(II) (2 mg)</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a NaCl solution, 145 mM, which served as the vehicle for the injections of Ni₃S₂ and iron dusts.
b p < 0.025 versus Group A, computed by x² test.
c p < 0.001 versus Group A, computed by x² test.
d Metastases to lung and kidney in one rat and retroperitoneal metastases in the other rat.
e Metastases to lung.
f Metastases to kidney and spleen.

![Chart 1. Incidence of testicular sarcomas and mortality data for rats that received an i.t. injection of the 145 mM NaCl vehicle (Group A) or 10 mg of Ni₃S₂ dust (Group B). No testicular sarcomas occurred in the control rats (Group A). O, cumulative incidence of Ni₃S₂-treated rats (Group B). C, cumulative mortality of Ni₃S₂-treated rats (Group B). C, cumulative mortality of the control rats (Group A).](image)

passed by cancer cells in all of the testicular tumors. Metastases to lung, kidney, and/or abdominal lymph nodes were found in 4 of the 16 tumor-bearing rats (Table 1). Other pathological changes in the tumor-bearing rats included emaciation and pneumonia, which were the presumed causes of death in animals without metastases.

Electron microscopic examinations of the 16 testicular sarcomas confirmed the light microscopic diagnoses in all cases. The electron microscopic classification of these sarcomas was based upon the morphology of the most differentiated tumor cells. The fibrosarcomas (Fig. 9) were generally homogeneous tumors composed of bundles of elongated cells surrounded by collagen fibers. There was little variation in the morphology of the fibrosarcomas. The cytoplasm of the elongated cells invariably contained cisterns of granular endoplasmic reticulum and sparse mitochondria. The malignant fibrous histiocytomas (soft-tissue giant cell sarcomas) (Fig. 10) were composed of populations of large and small cells, admixed without any discernible pattern. Multinucleated cells were frequent. The large and small cells all contained well-developed cytoplasm, with relatively abundant mitochondria, endoplasmic reticulum, and lysosome-like vacuoles. In some histiocytic cells, the cytoplasm was foamy and appeared to contain lipid. In addition to histiocytic cells, the malignant fibrous histiocytomas all contained fibroblasts of typical morphology. The rhabdomyosarcomas (Figs. 11 and 12) contained a wide spectrum of cells with all the features of differentiating rhabdomyoblasts that have been described by Bruni and Rust (3) in Ni₃S₂-induced rhabdomyosarcomas of skeletal muscle in Fischer rats. At one end of the spectrum, there were small undifferentiated cells with bean-shaped nuclei and sparse cytoplasm that contained a few short cisterns of granular endoplasmic reticulum and scanty mitochondria. The hyaloplasm was crowded with free ribosomes. No myofibrils were seen in these cells. At the other end of the spectrum, there were large cells with irregularly shaped nuclei and abundant cytoplasm that contained numerous organelles. The cytoplasmic organelles included (a) numerous mitochondria, (b) prominent dilated cisterns of smooth and granular endoplasmic reticulum, and (c) myofibrils (with or without Z-bands) in association with networks of branching, irregularly-shaped myotubules and glycogen granules. In each of the 3 rhabdomyosarcomas, myofibrils containing Z-bands were identified as 1 component of the spectrum of cells.

No primary malignant tumors were found at any location except the testis in rats of Group B. No malignant tumors were found in any rats of Group A, C, and D which received i.t. injections of the 145 mM NaCl vehicle, iron dust, or zinc chloride solution, respectively. Benign Leydig cell tumors were seen by light microscopic examination in the testes of 13 of the 18 control rats in Group A. Such Leydig cell tumors are commonly found in testes of senescent Fischer rats (9, 15, 20). At the sites of i.t. injection of iron dust in rats of Group C, there was focal fibrosis with replacement of seminiferous tubules. These foci of connective tissue contained abundant brown pigment. At the sites of i.t. injection of zinc chloride solution in rats of Group D, there was extensive fibrosis with calcification of hyalinized semi-
niferous tubules. Other pathological changes in rats in Groups A, B, C, and D included pneumonia as a common finding.

DISCUSSION

Previous workers have shown that administration of soluble nickel salts to rats by p.o. (16, 27), dermal (17), s.c. (7, 12), and i.t. (11) routes produces testicular damage with degeneration of seminiferous tubules and/or arrest of spermatogenesis. There have not been any previous investigations of the carcinogenicity of nickel compounds following i.t. injection in experimental animals. We undertook this study in an attempt to develop an experimental method for induction of testicular seminomas, teratomas, or embryonal carcinomas. This endeavor was unsuccessful, for no tumors of germ cell origin developed in the Ni₂S₂-treated rats. However, we discovered that i.t. administration of Ni₂S₂ is an extremely effective technique for induction of testicular sarcomas. In comparison to the numerous sarcomas that were observed in this study, Guthrie (5) induced fibrosarcomas in only 2 of 15 rats that received an i.t. injection of 1 mg of methylcholanthrene. The Ni₂S₂-induced sarcomas of the testis that were observed in this study were histologically identical with the sarcomas that were induced previously in Fischer rats by injection of Ni₃S₂ into skeletal muscle (3, 24). Moreover, the incidence and latent periods for Ni₂S₂ induction of testicular sarcomas were practically the same as were previously reported for Ni₃S₂ induction of sarcomas in the hind-leg musculature (24, 25).

Primitive, pluripotent mesenchymal cells would seem likely to be the common progenitors of the histologically and biologically identical sarcomas that are induced by Ni₃S₂ in testis and muscle of Fischer rats. On the basis of this study, it is impossible to determine whether the progenitor cells of the sarcomas are normally present in the testicular interstitium or whether the progenitor cells are constituents of the granulation tissue that infiltrates the testis in reaction to Ni₃S₂. In the opinion of the authors, the second speculation appears to be plausible, since primitive mesenchymal cells in granulation tissue are generally considered to be progenitors of diverse specialized and differentiated cells, such as (a) contractile myofibroblasts, (b) fibroblasts that produce collagen, and (c) angioblasts that produce capillary basement membrane. The authors cannot entirely exclude the possibility that the rhabdomyosarcomas might have arisen from striated muscle cells derived from the cremaster muscle. However, this possibility seems unlikely, since these tumors were first identified by palpation as firm nodules in the center of the injected testes and since no pathological reactions of the cremaster muscles were observed in any of the rats that received i.t. injections of Ni₃S₂ in the serial sacrifice study.

The control rats that were studied in this research included vehicle controls (Group A) and 2 additional groups (C and D) that were given i.t. injections of iron dust and ZnCl₂ solution, respectively. These compounds were selected since the iron dust closely resembled the Ni₃S₂ dust in its particle size and relative insolubility in aqueous media and since ZnCl₂ solution had previously been reported to induce tumors of germ cell origin following i.t. injection in Wistar rats (21) and Syrian hamsters (6). In this study, no malignant tumors of the testis (or indeed of any other organs) were found in the control rats in Groups A, C, or D. The absence of testicular sarcomas in these control rats tends to negate the possibility that the induction of testicular sarcomas in Ni₃S₂-treated rats might simply be a consequence of: (a) trauma owing to the volume of injected fluid; (b) a foreign body reaction to the presence of metal dust; or (c) disintegration, fibrosis, and hyalinization of seminiferous tubules.

Sunderman et al. (24) reported that Ni₃S₂ particles were predominantly extracellular during the first 2 days after i.m. injection in Fischer rats. Beginning at 1 week after i.m. injection and continuing throughout the subsequent 7 weeks, Sunderman et al. (24) observed that Ni₃S₂ particles were localized primarily within macrophages and fibroblasts at the injection site. On the basis of radionuclide studies with ⁶³Ni, Sunderman et al. (24) and Kasprzak and Sunderman (13) showed that ⁶³Ni(II) gradually becomes released from the insoluble ⁶³Ni₂S₂ particles. ⁶³Ni(II) is transported from the injection site in the form of ⁶³Ni complexed with albumin and ultrafilterable ligands. Most of the ⁶³Ni is excreted in urine and bile within 2 months after an i.m. injection of ⁶³Ni₂S₂ (24). Consistent with these earlier findings, we have observed in this study that many of the Ni₃S₂ particles in the testicular interstitium become ingested by macrophages and polymorphonuclear leukocytes, and most of the Ni₃S₂ particles gradually disappear from the injection site. A small proportion of the Ni₃S₂ particles is trapped within acellular collagen deposits and remains quasipermanently deposited at the injection site. However, no metallic particles were discerned in the testicular sarcomas that developed in Ni₃S₂-treated rats. Studies of the metabolism and excretion of ⁶³Ni in rats that have received i.t. injection of ⁶³Ni₂S₂ are currently being initiated in our laboratory.

Injection i.t. appears to have 3 practical advantages over i.m. injection as an experimental system with which to investigate nickel carcinogenesis. First, the injection site is localized and encapsulated, and it can be excised completely at any time during the latent period. Second, the testis is easily palpated, and tiny tumors can be detected more readily in the testis than in the leg musculature. This leads to more accurate quantification of the latent period. Third, the testis does not normally contain striated muscle cells. Therefore, it should be possible to study the differentiation of rhabdomyoblasts from undifferentiated mesenchymal cells more easily in testis than in muscle, where satellite cells normally constitute a minor proportion of the cellular population (2).

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