Rapid Induction of Sarcomas in Rats by Combination of Nickel Sulfide and 3,4-Benzpyrene

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SUMMARY

The interval between administration of carcinogen and development of sarcomas was significantly shorter in male Fischer rats that received bilateral injections into thigh muscles of a combination of 10 mg of Ni$_3$S$_2$ and 5 mg of 3,4-benzpyrene (Group C) than in rats that received only 10 mg of Ni$_3$S$_2$ (Group A) or only 5 mg of 3,4-benzpyrene (Group B). No sarcomas developed in control rats (Group D), which received only the injection vehicle (penicillin suspension). The period between injection of carcinogens and death from sarcoma averaged 24 ± 3 weeks in Group C versus 26 ± 5 weeks in Group A and 31 ± 10 weeks in Group B (p < 0.001). The period between injection of carcinogens and initial palpation of tumors at the injection sites averaged 18 ± 3 weeks in Group C versus 26 ± 5 weeks in Group A and 31 ± 10 weeks in Group B (p < 0.001). The proportion of primary sarcomas which were classified as rhabdomyosarcomas was 91% in Group C versus 81% in Group A and 13% in group B. This study furnishes (a) additional support for speculations concerning carcinogenic interaction between nickel compounds and polycyclic aromatic hydrocarbons and (b) a rapid and convenient experimental technique for inducing rhabdomyosarcomas in rats. This technique is well adapted for use as a laboratory exercise in neoplasia for pathology students.

INTRODUCTION

In 1962, Gilman (6) reported that i.m. administration of nickel sulfide, Ni$_3$S$_2$, to rats results in the development of rhabdomyosarcomas. Subsequent studies have confirmed Gilman's observation and have elucidated the effects of (a) the dosage of Ni$_3$S$_2$ (7, 13, 17); (b) the physical form of Ni$_3$S$_2$ (8); (c) the age, sex, and strain of the rats (2, 15); and (d) various endocrine factors (14, 16, 18) upon the latent period and the incidence of the sarcomas. The biological characteristics of rhabdomyosarcomas which are induced in rats by injection of Ni$_3$S$_2$ have been reviewed in previous papers from our laboratory (25, 26). This study has been performed to ascertain whether or not the latent period for development of rat sarcomas can be shortened by combined administration of Ni$_3$S$_2$ together with 3,4-benzpyrene, a carcinogen which generally produces fibrosarcomas in rats following i.m. or s.c. administration (1, 3, 5, 10-12, 20, 21).

MATERIALS AND METHODS

The experimental animals were male rats of the Fischer strain (Charles River Breeding Laboratories, North Wilmington, Mass.), maintained on Purina laboratory rat chow. The rats were 8 to 9 weeks old at time of injection. The carcinogens were nickel sulfide powder, Ni$_3$S$_2$ (supplied by J. P. W. Gilman, University of Guelph, Guelph, Ontario, Canada), and 3,4-benzpyrene powder (Mann Research Laboratories, Inc., New York, N. Y.). The mean particle diameters of the Ni$_3$S$_2$ and 3,4-benzpyrene powders were approximately 2 and 20 μ, respectively, as estimated by electron microscopy. The Ni$_3$S$_2$ powder was free from detectable contamination with other metals, as verified by emission spectrographic analysis. In a test for purity, the 3,4-benzpyrene powder was dissolved in hexane:acetone (3:1, v/v), and was subjected to thin-layer chromatography on Silica Gel G, with a solvent system of hexane saturated with N,N'-dimethylformamide. The 3,4-benzpyrene migrated as a single fluorescent spot. The injection vehicle was penicillin G procaine suspension, 300,000 units/ml (Wycillin, Wyeth Laboratories, Inc., Philadelphia, Pa.). Suspensions of the carcinogens were prepared as follows: Suspension a, 20 mg of Ni$_3$S$_2$/ml of vehicle; Suspension b, 10 mg of 3,4-benzpyrene per ml of vehicle; and Suspension c, 20 mg of Ni$_3$S$_2$ + 10 mg of 3,4-benzpyrene per ml of vehicle. Thirty rats in each of 3 experimental groups (A, B, and C) were given bilateral injections into the thigh muscles of Suspensions a, b, and c, respectively; 30 rats in a control group (D) were given bilateral injections of the vehicle, alone. The 120 rats all received the injections on a single day. The injections were all made deep into the extensor musculature at the midlength of the thighs (0.5 ml/injection site). No acute mortality, morbidity, or severe local inflammatory reactions occurred in any of the rats.

Physical examinations of the rats were performed each week by an animal caretaker who is skilled in detecting muscle tumors and in gauging the progression of tumor growth. Rats in experimental Groups A, B, and C either died spontaneously or were killed when their tumors became so large that the rats...
could not move about their cages and hence could not obtain food or water. Rats in the control group, D, were all killed at 104 weeks after injection (ages, 112 to 113 weeks). All rats were autopsied, and the tissues were examined by light microscopy. The classification of sarcomas was based upon the histological criteria of Stout and Lattes (23).

Electron microscopy was performed upon sarcomas from 4 or 5 rats in each of the experimental groups. Portions of the tumors were diced in cacodylate-buffered p-formaldehyde at 0 to 4° and postfixed in s-collidine-buffered 2% Os04. The tissues were embedded, sectioned, and stained as described by Goldblatt et al. (9). The grids were examined by use of a Phillips 300 electron microscope at 60 kV.

Transplantations were performed with sarcomas from 3 rats in each of the experimental groups. Portions from the margin of each tumor (approximately 0.5 g) were diced in 5 ml of sterile 0.85% NaCl solution (0.85 g/dl), and the tissue was fragmented by repeated forceful aspiration and expulsion with a 5-ml syringe. The cell suspensions were rapidly adjusted to contain approximately 1 X 10^8 cells/ml, and 0.5 ml of each suspension was injected unilaterally into the thigh musculature of 2 or 3 male Fischer rats, age 8 weeks. The 23 rats that received tumor implants were examined 3 times each week. These rats either died spontaneously or they were killed when their tumors became so large that they were unable to move. The rats were autopsied, and the tissues were examined by light microscopy.

RESULTS

The results of the study are given in Tables 1 and 2. The rats in the experimental groups (A, B, and C) all died with either rhabdomyosarcomas or fibrosarcomas which developed at the sites of the injections. The longest survival was 61 weeks after the injection of the vehicle. Two benign tumors were found in these control rats: an adrenal adenoma in 1 rat and a s.c. lipoma in the flank of another rat. No malignant tumors were found in any of the control rats.

In their histological and biological characteristics, the sarcomas which developed in Group C (NiS2 + 3,4-benzpyrene) closely resembled the sarcomas in Group A (NiS2 alone) and generally differed from the sarcomas in Group B (3,4-benzpyrene alone). Thus, 42 of 46 sarcomas (91%) in Group C were classified histologically as rhabdomyosarcomas compared with 34 of 42 (81%) in Group A and only 5 of 39 (13%) in Group B. Metastases of the sarcomas to lungs or mediastinal lymph nodes were present in 16 of 30 rats (53%) in Group C, compared with 17 of 30 rats (57%) in Group A and only 6 of 30 rats (20%) in Group B (Fig. 5). In general, the presence of distant metastases was associated with sarcomas which were classified histologically as rhabdomyosarcomas, although 2 typical fibrosarcomas in Group B were metastatic to the lung.

The mean latent period (interval between injection of carcinogens and initial palpation of a tumor at an injection site) was significantly shorter in Group C (18 ± 3 weeks) than in either Groups A or B (26 ± 5 and 31 ± 10 weeks, respectively, p < 0.001). The mean survival time (interval between injection of carcinogens and death) was significantly shorter in Group C (24 ± 5 weeks) than in Groups A or B (33 ± 5 and 41 ± 11 weeks, respectively, p < 0.001). The mean interval between initial palpation of tumor and death was 6 ± 3 weeks in Group C, compared with 7 ± 3 in Group A (p < 0.05) and 10 ± 5 in Group B (p < 0.001).

Seven rhabdomyosarcomas and 2 fibrosarcomas were transplanted into a total of 23 recipients. Pleomorphic sarcomas similar to that shown in Fig. 6 developed in all of the recipients at the sites of injection. The transplanted tumors grew much more rapidly than the primary tumors. The transplanted tumors were first palpated at an average of 7 days after implantation (range, 5 to 9 days), and the recipient rats were all killed at 104 weeks after the injection of the vehicle.

Table 1

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Carcinogen</th>
<th>Rats with sarcomas (%)</th>
<th>Rats with rhabdomyosarcomas (%)</th>
<th>Rats with bilateral primary sarcomas (%)</th>
<th>Proportion of primary sarcomas classified as rhabdomyosarcomas</th>
<th>Rats with distant metastases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>NiS2a</td>
<td>100 (30/30)</td>
<td>80 (24/30)</td>
<td>40 (12/30)</td>
<td>81 (16/30)</td>
<td>57 (17/30)</td>
</tr>
<tr>
<td>B</td>
<td>3,4-BPb</td>
<td>100 (30/30)</td>
<td>13 (4/30)</td>
<td>30 (9/30)</td>
<td>p &lt; 0.001 vs. Ae</td>
<td>13 (5/39)</td>
</tr>
<tr>
<td>C</td>
<td>NiS2 + 3,4-BPc</td>
<td>100 (30/30)</td>
<td>93 (28/30)</td>
<td>53 (16/30)</td>
<td>p &lt; 0.001 vs. A</td>
<td>91 (42/46)</td>
</tr>
<tr>
<td>D</td>
<td>None d</td>
<td>0 (0/30)</td>
<td>0 (0/30)</td>
<td>0 (0/30)</td>
<td>p, N.S. vs. A</td>
<td>p, N.S. vs. A</td>
</tr>
</tbody>
</table>

a NiS2 (10 mg/injection site) administered i.m. into both thighs.
b 3,4-Benzpyrene (5 mg/injection site) administered i.m. into both thighs.
c Combination of NiS2 (10 mg/injection site) and 3,4-benzpyrene (5 mg/injection site) administered i.m. into both thighs.
d Vehicle (pencillin G procaine suspension, 150,000 units/0.5 ml) administered i.m. into both thighs.
e Probability based upon null hypothesis for differences between groups, computed by Yates’ x^2 test; N.S., not significant.
**Ni$_3$S$_2$-3,4-Benzpyrene Induction of Rat Sarcomas**

Table 2

*Latent periods for development of sarcomas and survival times for rats treated with Ni$_3$S$_2$ and 3,4-benzpyrene, singly and in combination*

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Carcinogen</th>
<th>All sarcomas</th>
<th>Rhabdomyosarcomas</th>
<th>Fibrosarcomas</th>
<th>Weeks between injection of carcinogen and death</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Ni$_3$S$_2$</td>
<td>26 ± 5</td>
<td>27 ± 6</td>
<td>26 ± 3</td>
<td>33 ± 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>27 (19-40)</td>
<td>(19-40)</td>
<td>(22-31)</td>
<td>(24-47)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N = 42</td>
<td>N = 34</td>
<td>N = 8</td>
<td>N = 30</td>
</tr>
<tr>
<td></td>
<td>31 ± 10</td>
<td>23 ± 3</td>
<td>32 ± 10</td>
<td>41 ± 11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 (17-54)</td>
<td>(18-27)</td>
<td>(17-54)</td>
<td>40 (21-61)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N = 39</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>3,4-BP</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>p &lt; 0.05 vs. A</td>
<td>p &lt; 0.05 vs. A</td>
<td>p &lt; 0.01 vs. A</td>
<td>p &lt; 0.01 vs. A</td>
</tr>
<tr>
<td></td>
<td>18 ± 3</td>
<td>19 ± 3</td>
<td>17 ± 2</td>
<td>24 ± 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18 (15-30)</td>
<td>(15-30)</td>
<td>(15-20)</td>
<td>23 (18-36)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N = 46</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Ni$_3$S$_2$ + 3,4-BP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>p &lt; 0.001 vs. A</td>
<td>p &lt; 0.001 vs. A</td>
<td>p &lt; 0.001 vs. A</td>
<td>p &lt; 0.001 vs. A</td>
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<tr>
<td></td>
<td>18 ± 3</td>
<td>19 ± 3</td>
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<td>24 ± 5</td>
<td></td>
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<tr>
<td></td>
<td>18 (15-30)</td>
<td>(15-30)</td>
<td>(15-20)</td>
<td>23 (18-36)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N = 46</td>
<td></td>
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<td></td>
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</tbody>
</table>

A The values on the 1st line of each entry are the mean ± S.D.; the values on the 2nd line are the median and range; the number of items in each category is indicated on the 3rd line of each entry.

b Ni$_3$S$_2$ (10 mg/injection site) administered i.m. into both thighs.

c 3,4-Benzyrene (5 mg/injection site) administered i.m. into both thighs.

d Combination of Ni$_3$S$_2$ (10 mg/injection site) and 3,4-benzyrene (5 mg/injection site) administered i.m. into both thighs.

e Probability based upon null hypothesis for differences between means, computed by Student's t test.

died at an average of 19 days after implantation (range 9 to 28 days). Pulmonary metastases were present in 10 of the 23 recipients (43%). The number of transplanted tumors was too small to permit any conclusions regarding differences in the transplantation characteristics of sarcomas from experimental groups A, B, or C.

**DISCUSSION**

This study has shown that sarcomas induced in Fischer rats by bilateral i.m. injections of 5 mg of 3,4-benzyrene (Group B) are predominantly fibrosarcomas and that sarcomas induced under identical conditions by 10 mg of Ni$_3$S$_2$ (Group A) or by the combination of 10 mg of Ni$_3$S$_2$ and 5 mg of 3,4-benzyrene (Group C) are predominantly rhabdomyosarcomas. The mean latent period of the sarcomas produced by the combination of Ni$_3$S$_2$ and 3,4-benzyrene (4.5 months) was 30% shorter than that observed with Ni$_3$S$_2$ (6.5 months). Such shortening of the latent period cannot be accomplished merely by increasing the dosage of Ni$_3$S$_2$. Thus, Gilman (7) has shown that the minimum latent period for induction of sarcomas by i.m. administration of Ni$_3$S$_2$ is achieved at a dosage of 2 mg of Ni$_3$S$_2$ per injection site and that no further diminution of latent period is achieved with dosages of Ni$_3$S$_2$, up to 20 mg per injection site.

From a theoretical viewpoint, this study furnishes support for speculations regarding carcinogenic interaction between nickel compounds and polycyclic aromatic hydrocarbons (4, 19, 22, 24, 25). Similar observations have been reported by Toda (27), who found that 5 of 30 rats (17%) that received intratracheal injection of nickel oxide in combination with 20-methylcholanthrene developed pulmonary neoplasms (squamous cell carcinomas), compared with no pulmonary neoplasms in rats that received only 20-methylcholanthrene.

For a practical viewpoint, this study furnishes an exceptionally rapid and convenient method for inducing sarcomas in rats. Based upon its use during the past 2 years in the curriculum for medical and dental students at the University of Connecticut, this technique is well suited as a laboratory exercise in pathology courses, as a means of demonstrating the induction of cancer. The particular advantages of this experimental method are as follows. (a) The technique is safe for student use, provided simple precautions are observed, such as use of disposable gloves, syringes, and needles. Accidental spillage and potential contamination with carcinogenic agents can be readily monitored by use of UV light to detect the fluorescence of 3,4-benzpyrene. (b) The carcinogens are administered parenterally in a single laboratory session, and no expensive laboratory equipment is needed. The rats can be housed in general animal quarters, without isolation precautions or special handling of excreta. (c) The mean latent period of 4.5 months permits a laboratory exercise in carcinogenesis to be initiated and the resulting tumors to be observed within the span of a single academic semester. (d) The relatively high incidence of distant tumor metastases permits the laboratory demonstration of an important facet of neoplastic disease to pathology students. (e) The sarcomas are readily transplantable, providing a source of neoplastic tissue for didactic and investigational purposes.

**ACKNOWLEDGMENTS**

We are grateful to Dr. John P. W. Gilman, Dr. Parvathi K. Basrur, Dr. Arthur Furst, and Dr. Marcus M. Mason for valuable advice and assistance; to Dr. Lewis J. Cralley for measurements of the mean particle diameters of the Ni$_3$S$_2$ and 3,4-benzyrene powders and for emission spectrographic analysis of the Ni$_3$S$_2$; to Dr. Svend W. Nielsen, Dr. Peter B. Hukill, and Dr. Leonard Robinson for consultations regarding histological classification of the sarcomas; to Dr. Peter J.
Goldblatt and Dr. Jenő E. Szakacs for assistance with electron microscopy; and to Mr. Richard Morang for skilful animal care.

REFERENCES


Fig. 1. Gross photograph of transected thigh from a rat in Group C (Ni3S2 + 3,4-benzpyrene). The bulk of the thigh musculature and femur have been displaced to the lower right by a pale lobulated tumor which contains several necrotic regions. Microscopy showed this tumor to be a rhabdomyosarcoma.

Fig. 2. Photomicrograph of a spindle cell fibrosarcoma from a rat in Group A (Ni3S2 alone). Electron microscopy of the tumor failed to demonstrate any myomatous elements. The histological appearance of this tumor is typical of the fibrosarcomas which occurred in all of the experimental groups. H & E, × 100; inset, × 400.

Fig. 3. Photomicrograph of a rhabdomyosarcoma from a rat in Group C (Ni3S2 + 3,4-benzpyrene). There are many large, myomatous cells with abundant cytoplasm and numerous multinucleate cells. The histological appearance of this tumor is typical of the rhabdomyosarcomas which occurred in all of the experimental groups, although some rhabdomyosarcomas were less differentiated. H & E, × 40.

Fig. 4. Electron micrograph of the rhabdomyosarcoma shown in Fig. 3, to demonstrate Z-band substance (Z) and thick and thin myofilaments (M) within the cytoplasm. The Z-band substance is scattered and disorganized, consistent with the malignant nature of this cell. Lead citrate and uranyl acetate stain, × 13,000.

Fig. 5. Photomicrograph of a poorly differentiated pulmonary metastasis of a rhabdomyosarcoma from a rat in Group C (Ni3S2 + 3,4-benzpyrene). The original tumor was classified as a rhabdomyosarcoma. H & E, × 250.
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