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Histopathological and Immunohistochemical Studies on Nickel Sulfide-induced Tumors in F344 Rats

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Twenty-five tumors induced in F344 male rats were examined histologically and immunohistochemically using antibodies against myoglobin, myosin, desmin and cathepsin B. Eight were from rats which had been given intramuscular (im) injection and 17 were from rats which had been given subcutaneous (sc) injection of 5 mg of Ni_3S_2. Among 10 rhabdomyosarcomas, myoglobin was detectable in 3, myosin in 8, and desmin in all, but cathepsin B was present in none. Out of 8 malignant fibrous histiocytomas, cathepsin B was detectable in all, but the other antigens were absent. In a leiomyosarcoma, only desmin was detected. In two fibrosarcomas, none of the markers were detected. In four undetermined tumors, one reacted only with anti-desmin antibody, two with only anti-cathepsin B antibody, and one with none of the antibodies. Of the three myogenic markers utilized in this study, anti-desmin antibody appeared to be the most sensitive. Cathepsin B was found mainly in the histiocytic cells of malignant fibrous histiocytoma. Thus, desmin appears to be particularly valuable in distinguishing immature myogenic tumors from other primitive tumors, while cathepsin B is useful in distinguishing malignant fibrous histiocytoma from other plemorphic mesenchymal tumors.

Key words: Nickel sulfide — Desmin — Myosin — Myoglobin — Cathepsin B

Nickel sulfide (Ni_3S_2) is known to be a highly potent carcinogen in experimental animals. Gilman[1] reported a high incidence of tumor induction by single im injection of Ni_3S_2 in rats. Thereafter many investigators have employed this simple method for the induction and morphological, biological and biochemical examination of soft tissue tumors.[2–4] Most of these studies have been performed by im injection of Ni_3S_2 and the induced tumors have been categorized mainly as rhabdomyosarcoma, fibrosarcoma and unclassified sarcoma. In some rat studies, the induction of fibrosarcoma, rhabdomyosarcoma and immature mesenchymal tumor by sc injection of Ni_3S_2 has been reported.[3, 4]

Soft tissue tumors, especially rhabdomyosarcoma, may manifest variable cellular morphology in accordance with their stage of differentiation. Pleomorphic tumors are not always easy to differentiate from malignant fibrous histiocytomas. It may not be possible to ascertain an exact origin in tumors exhibiting immature cell morphology. Despite the many descriptions of the morphology of the tumors induced by Ni_3S_2, only a few have mentioned malignant fibrous histiocytoma, which is the commonest sarcoma in human soft tissues, and even then the immunohistochemical studies were restricted. We describe here the histological types of tumors induced in rats by Ni_3S_2 given by different routes of administration, im and sc, and present immunohistochemical results on the tumor tissue to obtain an accurate histogenesis.

MATERIALS AND METHODS

Tumor Tissues Tumor tissues used in this study were obtained from two different experiments (groups A and B). Eight tumors were from 8 F344 male rats injected with 5 mg of Ni_3S_2 in their right gastrocnemius muscle (group A). They developed between weeks 26 and 33 in the gastrocnemius muscle after the injection of Ni_3S_2 and ranged from 6 to 10 mm in their greatest dimension. Seventeen tumors were from 17 F344 male rats injected with 5 mg of Ni_3S_2 in the subcutaneous tissue of the back (group B). The tumors occurred between weeks 26 and 42 in the subcutaneous tissue after the injection of Ni_3S_2 and ranged from 4 to 78 mm in their largest diameter. Details of these experiments on tumorigenesis by Ni_3S_2 will be presented elsewhere (M. Shibata, unpublished).

Sections of all tumor tissues were routinely stained with hematoxylin and eosin (H-E), and prepared for immuno-stainings. Some selected sections were stained with phosphotungstic acid hematoxylin, periodic acid-Schiff and Naoumenko-Fegin's method for reticulin when necessary.

Antibodies Anti-myoglobin antibody was raised in a rabbit against myoglobin purified from gastrocnemius muscle of Fischer rats by a modification of the method of Miyoshi *et al.*[5] Anti-myosin antibody was raised in a rabbit against myosin purified from tensor fascia muscle of guinea pigs by the methods of Libera *et al.*[6]; the antibody reacted equally with type I and type II muscle fibers. Anti-desmin antibody was raised in rabbits against desmin purified from chicken gizzard by a modification of the method of Huiatt *et al.*[7] Anti-myosin and anti-desmin antibodies were kindly given by Dr. Jun-ichi Abe (The First Department of Pathology, School of Medicine, The University of Tokushiam). Anti-cathepsin B antibody was raised in a rabbit against cathepsin B purified from rat liver. This antibody against cathepsin B was kindly given by Dr. Eiki Kominami (Institute for Enzyme Research, The University of Tokushima). The specificities of all of the antibodies were confirmed by immunodiffusion or immunoblotting.

Immunohistochemistry Myoglobin, myosin and desmin were examined by the immunoperoxidase method using ABC kits (Vectastain, Vector, Burlingame, Calif.). Cathepsin B was examined by the direct immunoperoxidase method using F-ab′ fragment of rabbit IgG conjugated with horseradish peroxidase. After visualizing the positive reaction with 3,3′-diaminobenzidine (0.05%) and hydrogen peroxide (0.01%), the immunostained sections were counterstained with hematoxylin or methyl green.

Results

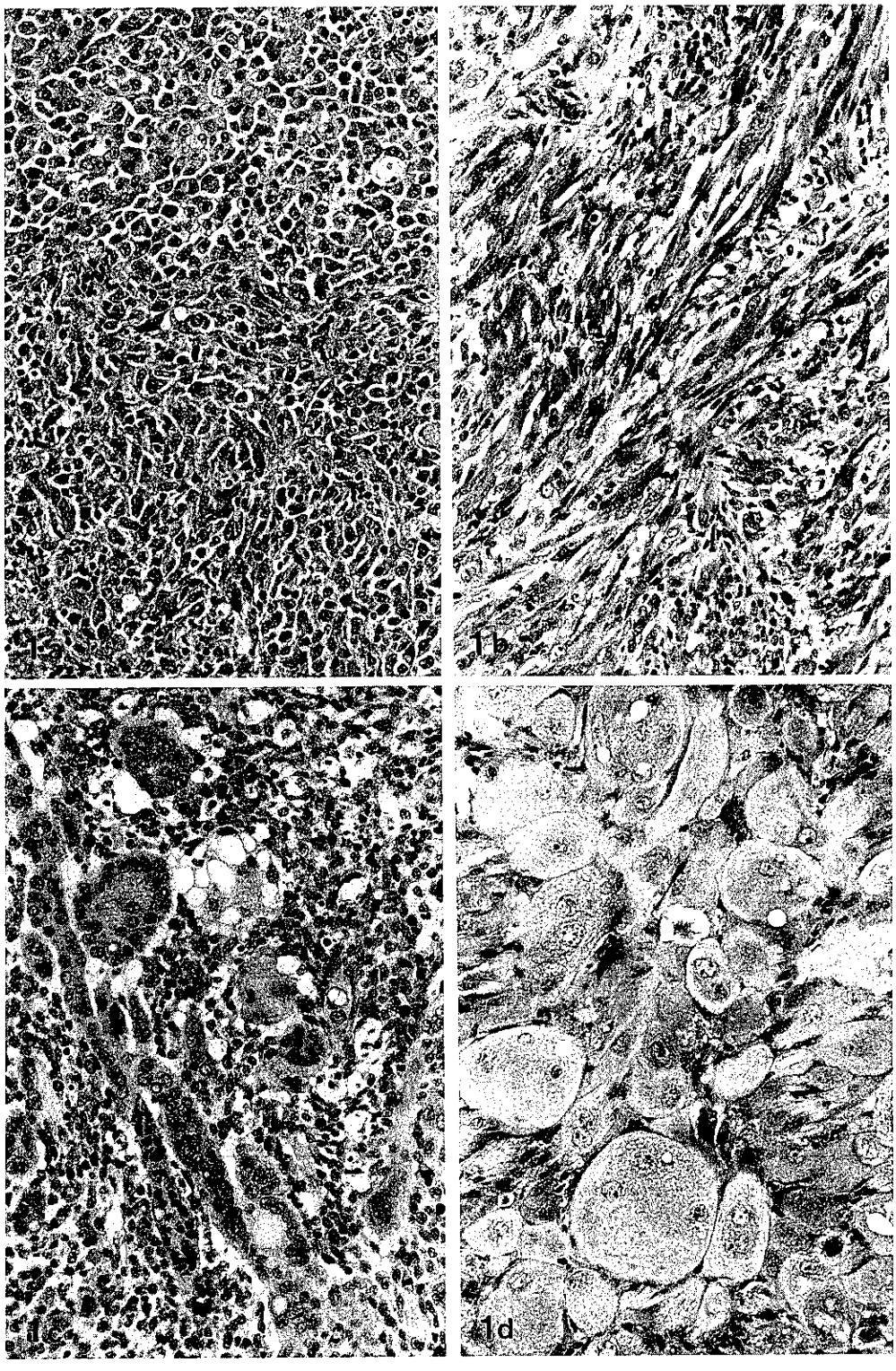
The 25 tumors examined in this study were categorized as 10 rhabdomyosarcomas (5 in group A, 5 in group B), 8 malignant fibrous histiocytomas (all in group B), 1 leiomyosarcoma (in group A), 2 fibrosarcomas (1 each in groups A and B) and 4 undetermined tumors (1 in group A, 3 in group B) by conventional histologic examination. We could not reach final histological diagnoses of the 4 "undetermined" tumors due to their immature morphology. We had great difficulty in deciding whether these tumors with complex and pleomorphic features should be diagnosed as malignant fibrous histiocytoma or rhabdomyosarcoma by the observation of conventionally stained sections. Histological types of the tumors and immunoreactivity of tumor cells are summarized in Table I.

Rhabdomyosarcomas contained a wide spectrum of cellular differentiation (Figs. 1, a–d). At one end of the spectrum, there were

Table I. Histological Types of Ni_3S_2 Induced Tumors and Their Immunohistochemical Characters

Histological type of tumors	No. of tumors	No. of tumors reacted with antibody against			
		Desmin	Myosin	Myoglobin	Cathepsin B
Group A					
Rhabdomyosarcoma	5	5	5	1	0
Leiomyosarcoma	1	1	0	0	0
Fibrosarcoma	1	0	0	0	0
Undetermined (UD 1)	1	1	0	0	0
Group B					
Rhabdomyosarcoma	5	5	3	2	0
MFH	8	0	0	0	8
Fibrosarcoma	1	0	0	0	0
Undetermined	3	0	0	0	2
(UD 2)		−	−	−	+
(UD 3)		−	−	−	+
(UD 4)		−	−	−	−

MFH: malignant fibrous histiocytoma.
Immunohistochemical study indicated that UD 1 might be classified as rhabdomyosarcoma, and UD 2 and UD 3 as malignant fibrous histiocytomas, but UD 4 still remained undetermined.


1a
1b
1c
1d

small undifferentiated, mostly round or polygonal cells with sparse cytoplasm (Fig. 1a). In the second group, the cells were elongated. With an increase of eosinophilic cytoplasm, there were distinctly larger cells than the round ones described earlier (Fig. 1b). At the other end of the spectrum, there were differentiated large cells with multinucleation resembling myotubes (Fig. 1c). In the most differentiated areas, there were multinucleated rhabdomyoblastic cells with large eosinophilic cytoplasms (Fig. 1d) in which cross and longitudinal striations were demonstrated.

Among these 10 rhabdomyosarcomas, myoglobin was detected in 3, myosin in 8 and desmin in all. Myoglobin was detectable in large multinucleated cells and mononucleated round cells with rich and deeply eosinophilic cytoplasms (Fig. 2). Myosin was positive in myoblastic cells and spindle cells with rather less differentiated cytoplasm (Fig. 3). Desmin-positivity was observed in all of 10 rhabdomyosarcomas regardless of cellular differentiation and morphology of the tumors (Figs. 4, a–c). When anti-desmin antibody was applied to rhabdomyosarcomas, not only

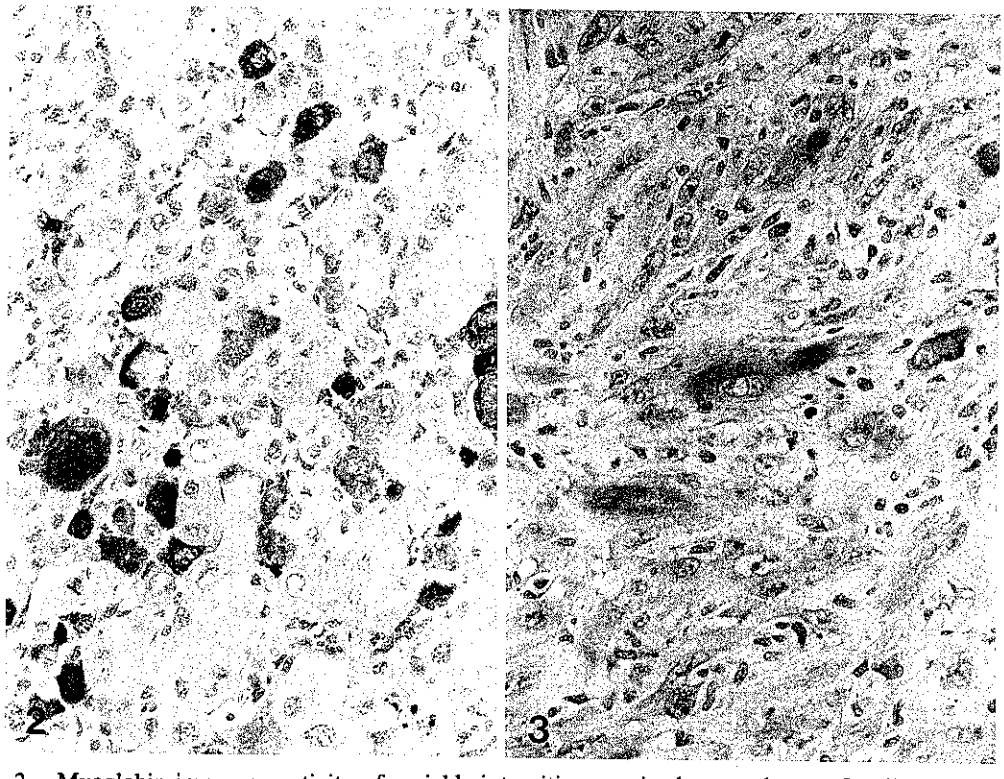


Fig. 2. Myoglobin immunoreactivity of variable intensities seen in the cytoplasms of well differentiated rhabdomyoblasts. ABC method for myoglobin, hematoxylin counterstain, ×160.

Fig. 3. Myosin immunoreactivity of elongated cells in a rhabdomyosarcoma. ABC method for myosin, hematoxylin counterstain, ×420.

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Fig. 1. Ni_3S_2-induced rhabdomyosarcomas showing a wide spectrum of histological appearance. For comparison, all 4 photomicrographs were taken at the same magnification. H-E, ×210. a, A tumor consisting of small round or polygonal cells. b, A tumor consisting of elongated cells with spindle nuclei and fibrillar eosinophilic cytoplasms. c, Multinucleated strap-shaped cells resembling myotubes. d, Multinucleated myoblastic cells with large eosinophilic cytoplasms.

rhabdomyoblastic cells, but also about one-third of small tumor cells which were negative for anti-myoglobin antibody showed positive reactions of various intensities. In the same way, desmin-positive cells were detected more frequently than myosin-reactive cells.

In one animal with rhabdomyosarcoma in group B, metastatic foci to the lungs were observed. The primary lesion was composed of immature small cells, but the metastatic lesions showed an appearance of moderately differentiated rhabdomyosarcoma. Tumor cells with positive reaction only to anti-desmin antibody were scattered in both primary and metastatic lesions.

All of 8 malignant fibrous histiocytomas showed a combination of storiform and pleomorphic areas (Figs. 5, a and b). The pleomorphic area contained plump fibroblasts, anaplastic mesenchymal cells and round histiocytic cells arranged without any particular orientation. Immunohistochemically, myoglobin, myosin or desmin was not detectable in any of the tumor cells. All 8 tumors showed cathepsin B immunoreactivity. Their positive reactions were seen as cytoplasmic granular aggregates predominantly in histiocytic cells and some fibrocytic cells (Fig. 5c), and occasionally in a few giant cells and immature mesenchymal cells.

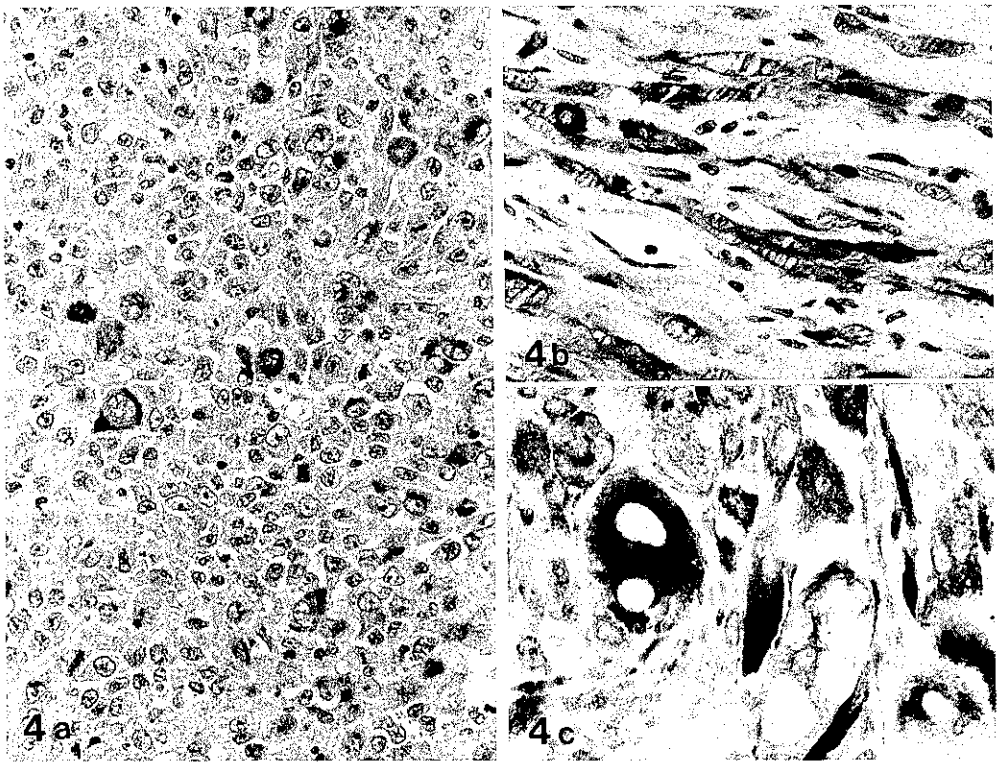
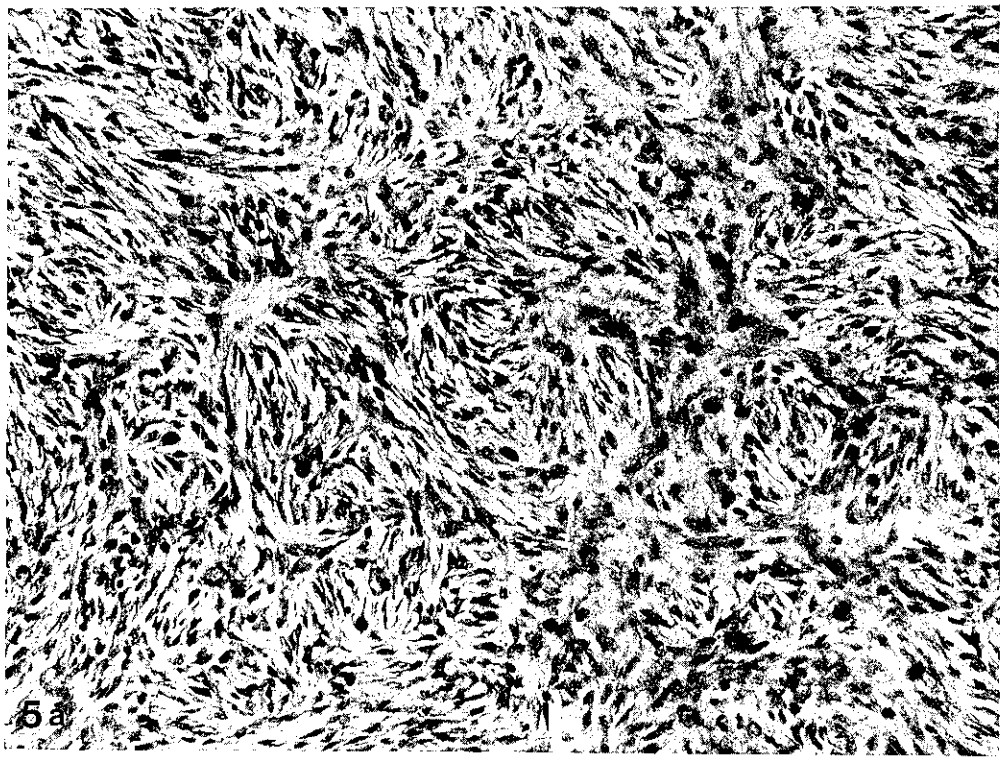


Fig. 4. Desmin immunoreactivity of rhabdomyosarcoma cells. Positive reaction is seen regardless of cellular morphology or differentiation. Not only the large rhabdomyoblasts (c) but also small immature cells (a) and elongated cells (b) reacted with antibody to desmin. These pictures were taken at the same magnification. ABC method for desmin, hematoxylin counterstain, ×320.

Fig. 5. MFH in group B. a, Typical storiform pattern composed of fibroblastic cells, H-E. ×160. b, Pleomorphic area with scattered histiocytic cells. H-E. ×210. c, Cathepsin B immunoreactivity seen in histiocytic cells. Direct immunoperoxidase method for cathepsin B, methyl green counterstain, ×420.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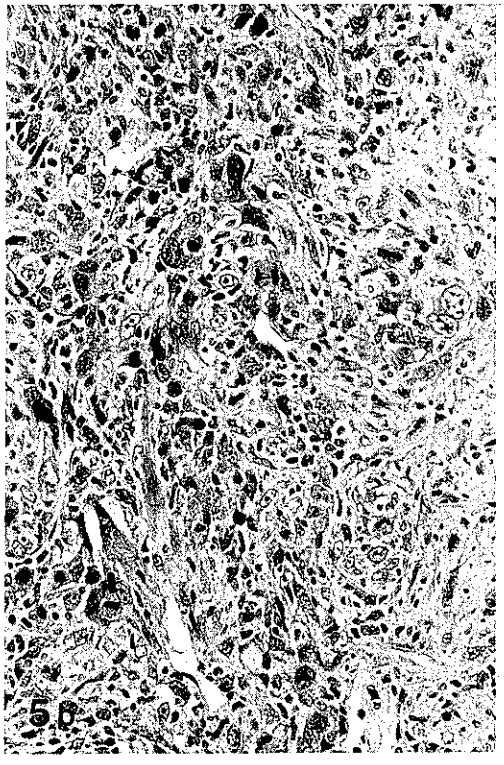
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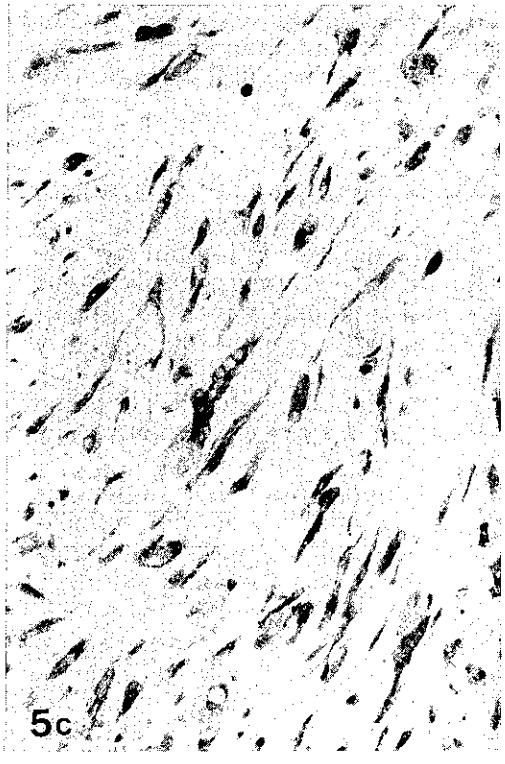
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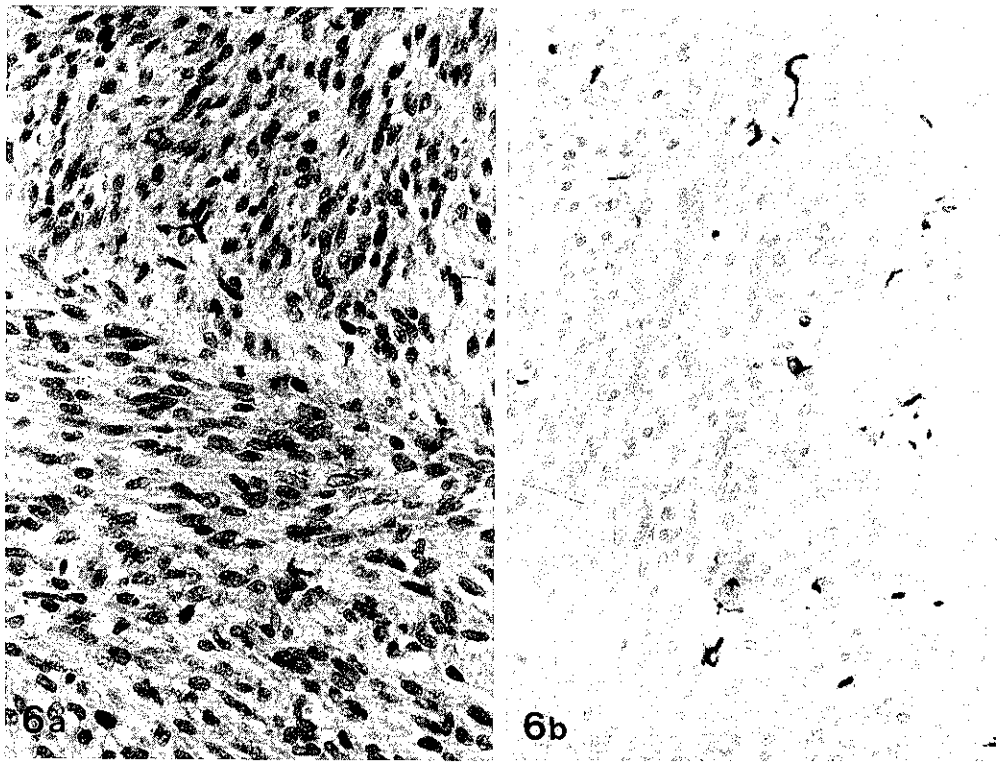

Fig. 6. Leiomyosarcoma in group A. a, The tumor is composed of uniform cells with ill-defined boundaries and blunt-ended nuclei, H-E. ×360. b, Some tumor cells reacted with anti-desmin antibody. ABC method for desmin, methyl green counterstan, ×320.

Leiomyosarcoma consisted of elongated cells with regular blunt-ended nuclei and eosinophilic cytoplasms. In most areas, tumor cells arranged in intertwining fascicles and well-oriented myofibrils were demonstrated by PTAH staining. Desmin-positive cells were scattered in the tumor, and the reactions were seen in perinuclear areas. However, the reaction was not so strong as that seen in rhabdomyosarcomas (Figs. 6, a and b).

Fibrosarcomas in two rats had a uniform interlacing growth pattern with spindle-shaped or fusiform cells with little plemorphism. A herring-bone pattern or interlacing fascicles were also observed in some areas. The cells in all parts of the tumors reacted with none of the antibodies used in this study.

Four "undetermined" (UD) tumors included one pleomorphic tumor (UD 2) and three immature small round cell tumors (UD 1, 3 and 4). UD 1 consisted of immature round cells with sparse eosinophilic cytoplasm and centrally located nuclei. The tumor cells reacted only with anti-desmin antibody, suggesting a myogenic origin of the tumor. UD 2 contained giant cells with large eosinophilic cytoplasm which resembled rhabdomyoblasts. However, the tumor cells reacted only with anti-cathepsin B antibody but not with any of the antibodies against myogenic markers. UD 3 was also composed of round cells, but their cytoplasms were not so eosinophilic as seen in tumor cells in UD 1, and a few cells reacted with only anti-cathepsin B antibody. UD 2 and 3 may be classified as malignant fibrous histiocytomas on the basis of these immunohistochemical findings. UD 4 consisted of small cells like UD 3, but the tumor cells reacted neither with myogenic markers nor with anti-cathepsin B antibody, so the

tumor still remained "undetermined" after immunohistochemical study.

Discussion

Many investigators have described the morphological appearance of tumors induced by im injection of Ni_3S_2. Among these reports, Yamashiro *et al.*[8,9] gave the most precise details on the tumors by means of light and electron microscopic studies. They classified the tumors into rhabdomyosarcoma, mesenchymal tumor, leiomyosarcoma-like tumor and fibrosarcoma-like tumor. Our findings are basically compatible with theirs, since some of their "mesenchymal" tumors had a tendency to differentiate into rhabdomyoblastic tumors at metastatic sites.

The predominant histological types of induced tumors were rhabdomyosarcoma in group A and malignant fibrous histiocytoma in group B. However, 5 rhabdomyosarcomas were also induced in group B and a few other sarcomas in group A. The facts suggest that muscle cells (group A) and fibrohistiocytic cells (group B) are highly susceptible to the given Ni_3S_2 at the site of injection and also immature pluripotential mesenchymal cells in subcutaneous or intramuscular connective tissue may give rise to rhabdomyosarcomas in group B, and a leiomyosarcoma and a fibrosarcoma in group A. Damjanov *et al.*[10] reported induction of rhabdomyosarcoma, malignant fibrous histiocytoma and fibrosarcoma by intratesticular injection of Ni_3S_2 and assumed that common progenitor cells in the testis were the origin of these tumors.

Among 4 "undetermined" tumors, possible myogenic differentiation of tumor cells in one (UD 1) of group A and histiocytic character of tumor cells in two (UD 2 and 3) of group B were suggested by the immunohistochemical observations. However, the remaining one (UD 4) is composed of cells so immature that its further direction of differentiation could not be revealed by the present study.

Although tumors induced by sc injection of Ni_3S_2 had been described as rhabdomyosarcoma, fibrosarcoma or immature mesenchymal sarcoma,[3,4] we observed that malignant fibrous histiocytoma was the commonest in this study. Induction of malignant fibrous histiocytoma by Ni_3S_2 was observed on im[11] or intratesticular[10] injection after the establishment of the general concept of the tumor. In recent years, there have been many reports of malignant fibrous histiocytoma induced by sc treatment of other carcinogens such as 4-hydroxyaminoquinoline 1-oxide,[12] 1,6-dinitropyrene[13] or dinitrofluoranthene.[14]

Myoglobin, an oxygen-binding protein distributed in striated muscle, has been found at a high incidence (up to 87%) in human rhabdomyosarcomas, mainly in the cells with differentiated eosinophilic cytoplasms.[15–17] However, in our study, myoglobin-positive cells were found in only 3 (30%) of 10 rhabdomyosarcomas. Lower incidence of the immunoreactivity, compared to that of human, may be attributed to the lower myoglobin content in rodent muscle fibers than human,[18] or the lower content of myoglobin in type II skeletal muscle fibers, which are the main component of rat gastrocnemius muscle, than in type I.[19]

Myosin is a contractile protein distributed in myogenic cells. It was detectable immunohistochemically in 87% of human rhabdomyosarcomas regardless of its histological features, and negative cases are limited to the tumors composed of undifferentiated small round cells lacking eosinophilic cytoplasms.[15,17] In this study, myosin-positive cells were found in 8 of 10 (80%) rhabdomyosarcomas, and 2 negative tumors were composed of immature round or spindle cells. Myosin appears earlier than myoglobin in normal muscle cytogenesis,[20] so it might be detectable even in immature tumor cells.

Desmin is one of the intermediate filaments found in muscle cells. Most human rhabdomyosarcomas contain desmin irrespective of their morphology or cellular differentiation.[21–23] Altmannsberger *et al.*[23] reported that 95% or more tumor cells showed desmin positivity, in rhabdomyosarcomas induced by im injection of 20 mg of Ni_3S_2 in Wistar rats. In our study, desmin was detectable in all of 10 rhabdomyosarcomas and one leiomyosarcoma even when the tumor cells showed rather immature morphology. Desmin appears to be particularly valuable to detect "myogenic" differentiation of tumor cells both in humans and animals, as Altmannsberger stated. In the present study, desmin positivity seen in UD 1 showed its myogenic character.

Cathepsin B is one of the lysosomal enzymes that are rich in macrophages as shown biochemically by Kominami *et al.*[24] Crocker *et al.*[25] detected it immunohistochemically in macrophages of benign and malignant lymphoid tissues. We detected cathepsin B in tumor cells in all of 10 malignant fibrous histiocytomas. The facts indicate that experimentally induced malignant fibrous histiocytomas have histiocytic character from an enzymological viewpoint and practical application of the anti-cathepsin B antibody would be of value in distinguishing malignant fibrous histiocytomas from other pleomorphic mesenchymal tumors. In fact, UD 2 and 3 could be classified as malignant fibrous histiocytomas from the results of the immunohistochemical study.

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