Immunochemical Differentiation of Rhabdomyosarcomas*

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SUMMARY

When six rhabdomyosarcomas were stained with antisera by the fluorescein label technic, three (classified as embryonal and usually difficult to diagnose as rhabdomyosarcoma) appeared to contain myosin, since they reacted with antimyosin antibody, whereas the remaining three, which were easier to classify as typical rhabdomyosarcoma histologically, appeared to contain no myosin. Anticonnective tissue serum gave different results in that the embryonal cells did not contain connective tissue antigenic components, whereas the typical rhabdomyosarcoma cells did (except for one of the adult rhabdomyosarcomas where only partial staining was seen). The question arises whether the adult muscle tumors are of muscle origin or of stromal origin. If these tumors come from skeletal muscle cells, they must have lost the ability to synthesize myosin in amounts detectable by the fluorescent antibody technic.

A problem of major importance in tumor pathology is the determination of the tissue of origin of a tumor. It would appear that a tumor is most certainly derived from a particular tissue or cell type if a substance found to be present in the tumor is known to be a constituent only of the cells of that particular tissue. However, the absence of such a unique substance in the tumor in the face of other positive evidence does not necessarily rule out the possibility that the tumor was derived from that tissue; the tumor may have lost the capacity to produce it at a high enough concentration for detection. The fact that antibody to malignant melanoma stains neurilemma but not epidermis was taken as evidence that this tumor is of a neuroectodermal source rather than derived from epidermal cells (7). Similarly, the possibility was raised that a transplanted "liver cell" line of tissue culture cells is actually of connective tissue origin (4).

We have now carried out a study of rhabdomyosarcomas. These tumors are derived from muscle which contains an easily separable protein, myosin. This protein is known to occur only in skeletal muscle, heart muscle, and, to a lesser extent, in smooth muscle. Evidence has been presented that the myosin is concentrated in the muscle striations (2). More recently, others have strengthened this conclusion by demonstrating myosin in these striations using the fluorescent antibody technic of Coons (3, 9). We have investigated several specimens of tumors classified as rhabdomyosarcomas to determine whether these contain myosin, and the results are reported here. Of those tested only the embryonal rhabdomyosarcoma (three out of three tested) contained myosin, whereas three other rhabdomyosarcomas were found to be free of myosin. This observation may be of particular importance in the identification of embryonal rhabdomyosarcomas which are difficult to characterize.

MATERIALS AND METHODS

Human myosin.—Myosin from human skeletal muscle (fresh autopsy material) was isolated and purified by the method outlined by Hawk et al. for isolation of rabbit myosin (5). Briefly, 300 gm. of fresh muscle was ground in the cold and extracted for 1 hour with 1 l. of solution of 3 M KCl and 0.15 M potassium phosphate (pH 6.5). Four liters of cold water was added (pH 6.5), and the mixture was strained through several layers of gauze. The solution was diluted to a final volume of 12 l. and allowed to stand in the cold for 3 hours. The supernate was removed and the precipitate collected by centrifugation. The protein was dissolved in 60 ml. of 3 M KCl and 30 ml. of a solution containing 0.25 M K,HPO4 and
The solution was clarified by centrifugation and filtration; to this 240 ml. H_{2}O was added and the actomyosin precipitate removed by centrifugation. To the supernate, 1 l. of cold water was added, precipitating the myosin. The steps for removal of actomyosin and precipitation of myosin were repeated. The myosin was then dissolved in 0.5 M KCl and dialyzed.

Antisera.—The horse antirabbit γ-globulin serum (7), rabbit antiserum prepared against human melanoma and known to react with human connective tissue (4), and rabbit antihuman γ-globulin serum (4) have been described previously. Antimyosin serum was made by giving three rabbits injections of human myosin and Freund adjuvants. A solution of myosin (7.7 mg protein/ml) was mixed with an equal volume of complete adjuvant (8). Each rabbit was given injections subcutaneously at six sites with a total of 1.5 ml. of the mixture. They were then given injections intraperitoneally weekly for 3 weeks of 0.5 ml., 1.0 ml., and 2.0 ml. of the same myosin solution. Rabbits were bled 4 days after the last injection. Weekly immunization and bleedings were continued, and the sera were pooled.

Fluorescent antibody staining techniques.—These were carried out as described previously (8). Numerous sections of each specimen were treated with the appropriate rabbit serum, washed, and then treated with fluorescein-labeled horse antirabbit globulin to detect any adsorbed antibody. All antisera were absorbed with rat liver sediments or human liver sediments as described elsewhere (8).

Tissues.—Normal tissue and tumor specimens were obtained at autopsy and frozen immediately.

RESULTS

Staining of muscle.—The antimyosin serum used was treated with human liver to remove antibodies capable of staining connective tissue. The antisemum so absorbed failed to stain normal liver, spleen, or kidney. It gave good staining of specimens of skeletal muscle and cardiac muscle (some muscle specimens were obtained from the individuals supplying the rhabdomyosarcomas). Staining in most cases was most intense in the region of the sarcolemma and in the striations. Striations were not always observed throughout the section by the fluorescein label technic, although H. and E. staining of similarly cut frozen sections (4–5 μ) always showed striations. The muscle cell nuclei were unstained. Only faint staining of smooth muscle was obtained.

Muscle specimens were also treated with antisemum known to give good staining of connective tissue (4). After this serum was treated with rat liver sediment to remove nonspecific staining elements, it still gave intense staining of stroma in all organs. This capacity to stain connective tissue can be removed readily by absorption with human liver sediment.

The contrast between staining of striated muscle by antimyosin and by connective tissue-reacting antibodies is strikingly shown in Figures 1 and 2. Connective tissue-reacting serum absorbed with rat liver sediment stained the stroma of skeletal and cardiac muscles. In the skeletal muscles where the endomysial sheaths were fairly thick (areas of blood vessels), strong staining of reticular connective tissue was seen. There was intense staining (Fig. 2) between groups of fibers, indicating that there were thin strands of connective tissue between these larger bundles of muscle fiber. No staining of the striations which contain myosin or of the nuclei was seen. Some similar staining was obtained with antimyosin serum, but this must have been due to a component other than connective tissue, since all connective tissue activity had been removed by absorption with human liver sediments. The staining may be due either to antimyosin or to antibodies against other muscle components, e.g., sarcolemma substances.

The only staining seen with rabbit antibody prepared against human γ-globulin was a weak staining in the stromal areas.

Staining of rhabdomyosarcomas.—Rhabdomyosarcomas from six individuals were stained with antimyosin antiserum and with the antibody reagent staining connective tissue. Three of these rhabdomyosarcomas were classed as embryonal rhabdomyosarcomas and showed definite staining with antimyosin serum. The staining was very intense in the cytoplasm of the round carcinomatous cells, particularly around their periphery, and involved large nests as well as scattered individual cells. The stroma surrounding these cells did not stain with this serum (Figs. 3, 5, 7). When the reagent staining connective tissue was employed on the sections of the three embryonal rhabdomyosarcomas none of the nests of tumor cells stained. (The cell outlines in Figs. 4 and 6 were due to blue autofluorescence.) Intense fluorescence of stromal elements surrounding the numerous cells was seen (Figs. 4, 6).

The other three rhabdomyosarcomas studied were not of the embryonal type and were all negative with antimyosin serum. Only residual normal muscle elements stained for myosin (Figs. 8, 10). With connective tissue-reactive antisera,
tumors from two individuals (Fig. 9) stained strongly. Tumor from the third individual showed staining with connective tissue-reactive antibody in one area (Fig. 11), but sections from another area did not show staining with this serum (Fig. 12). The cells staining with connective tissue-reactive antibody in all three individuals were similar, being fibroblastic in appearance, more typical of rhabdomyosarcoma. The cells (from the third individual) which did not stain with connective tissue-reactive antiserum were large and connective tissue-reactive antibody in one area (Fig. 11), but sections from another reactive antibody in all three individuals were similar, being fibroblastic in appearance, more typical of rhabdomyosarcoma. The cells (from the third individual) which did not stain with connective tissue-reactive antiserum were large and carcinomatous in appearance, although they were part of the same tumor mass in which the fibroblastlike cells were found to stain.

All the above tissues were also treated with antihuman γ-globulin antibody but showed no significant staining, indicating that none of the staining observed with other sera would be due to γ-globulin in the sections.

DISCUSSION

The identification of rhabdomyosarcoma is difficult (10, 11), because the cells are without differentiating features and may appear like poorly differentiated fibroblasts. They may be confused with liposarcomas or fibrosarcomas. At present, identification is based on morphological characteristics such as “racket-shaped cells,” granular cytoplasm, cross striations or longitudinal myofibrils, giant cells, cytoplasmic arrangement of chondriosomes, etc. One of the most important features is the observation of striations indicating the presence of myosin. Occasionally, cross striations may be seen only in the metastasis and not in the primary tumor; also, the altered muscle fibers of invaded normal muscles may be confused as tumor elements and may serve as a source of error for diagnosis of an otherwise extremely undifferentiated tumor which may not have arisen from muscle tissue (1).

The use of a specific stain for a component unique for muscle would be of value in aiding the diagnosis in some of these cases. Fluorescein-labeled antibody techniques involving antimyosin antibodies constitute such a specific stain. Even without striations myosin can be identified by the staining reactions. Three of the rhabdomyosarcomas studied were classified as embryonal and contained myosin; thus, they must have been derived from muscle. The fact that embryonal rhabdomyosarcomas readily reacted with antimyosin serum but not with connective tissue antibodies parallels the results obtained for normal skeletal muscle and indicates the presence of myosin in these tissues. The results suggest that active synthesis of myosin must be taking place in the embryonal rhabdomyosarcoma cells even when no visible striations or myofibers can be seen. In tissue culture, myosin can be detected in the fetal muscle cells, but no striations are visible (unpublished observation). The same lack of striation formation exists with the embryonal rhabdomyosarcoma.

The remaining rhabdomyosarcomas were easier to classify as typical rhabdomyosarcomas histologically but did not contain enough myosin to give a positive test. Two of the adult rhabdomyosarcomas and part of a third tumor reacted with the connective tissue-reactive antibodies, indicating the presence of these antigens in their cytoplasm. Since these antigens were not detected in normal skeletal muscle, there is a question whether or not the rhabdomyosarcomas arose from muscle. If so, they must have lost the capacity to synthesize myosin and gained the ability to synthesize connective tissue substances. It is also interesting that part of the third tumor contains some tumor cells unreactive with both connective tissue and antimyosin antibodies, indicating that two types of cells compose this tumor mass. The possibility exists that both cell types may be different, neither being a rhabdomyosarcoma. There also remains the possibility that both types are derived from muscle, one having lost the capacity to produce connective tissue antigens at a concentration high enough for detection. These problems are still under investigation.

It is interesting that there was staining of the sarcolemma in normal muscle by the antimyosin serum. Such staining has been observed by Klatzo et al. (9), who claimed it to be nonspecific staining. However, this must be some type of specific staining, since anticonnective tissue antibody or normal rabbit serum appears not to give such staining. The staining of sarcolemma may be due to the presence of myosin or the presence of another muscle protein in the myosin preparation which is a sarcolemma substance. This point is under further investigation. If the sarcolemma substance is different from myosin and the staining is due to a different antibody, the lack of staining of the rhabdomyosarcomas by the antimyosin antibody rules out the presence of any such specific substance in the tumor.

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Fig. 1.—Normal skeletal muscle treated with rabbit anti-myosin serum and subsequently stained with fluorescein-labeled horse antirabbit globulin. Staining is seen in the sarcolemma as well as the muscle striations. The nuclei of the muscle cells were unstained. X200.

Fig. 2.—Normal skeletal muscle treated with rabbit anti-connective tissue serum and stained as above. The muscles are unstained, the endomysial connective tissue separating the muscle stained strongly. X200.

Fig. 3.—Embryonal rhabdomyosarcoma (Rhe) treated as in Figure 1. Nests of round, carcinomatous-appearing cells scattered throughout the fibrous stroma were staining strongly in the cytoplasm, presumably due to the presence of myosin within these cells. The cell nuclei are negative. X5

Fig. 4.—Embryonal rhabdomyosarcoma (Rhe) treated as in Figure 2. Staining is confined to stromal elements; the round tumor cell nests are not stained. X500.
FIG. 5.—Embryonal rhabdomyosarcoma (Leb) treated as in Figure 1. The results here are identical with those seen for the previous case. X200.

FIG. 6.—Embryonal rhabdomyosarcoma treated as in Figure 3. The dense fibrous stroma stained intensely with the anticonnective tissue serum; large nests of tumor cells were unstained. X200.

FIG. 7.—Embryonal rhabdomyosarcoma (Bow) treated as in Figure 1. Staining was again confined to the tumor elements. Many of the cells ranged from round to spindle shape in appearance. The staining for myosin was quite intense in the cytoplasm of these cells. X200.

FIG. 8.—Rhabdomyosarcoma (Mor) (metastasis to the heart) treated as in Figure 1. Staining is confined to residual cardiac muscle tissue. The infiltrating tumor elements between and around these fibers are unstained. X200.
FIG. 9.—Rhabdomyosarcoma (Mor) (metastasis to the heart) treated as in Figure 2. The residual cardiac muscle fibers are unstained, but the tumor cell masses are fluorescing brightly with the anticonnective tissue serum. The nuclei of the cells are not stained. ×200.

FIG. 10.—Rhabdomyosarcoma (Ros) treated as in Figure 1. Negative staining of tumor cells; positive staining of degenerating skeletal muscles which have not been completely replaced by the tumor. ×200.

FIG. 11.—Rhabdomyosarcoma tumor I (Ros) treated as in Figure 2. Part of the tumor mass when treated with the connective tissue reagent showed the following: In areas where the cells were histologically more fibroblastic in appearance, akin to those seen in the previous tumor Figure 9, the staining involved both the cellular tumor and the stroma. These tumor cells did not stain with antimyosin serum. ×200.

FIG. 12.—Rhabdomyosarcoma II (Ros) treated as in Figure 2. Part of the same tumor mass as above showed cells that were large and roundish in appearance. In this area staining is confined only to stromal elements and not the round carcinomatous-appearing cells. These cells did not stain with antimyosin serum. ×200.
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