Production of Neoplasms by Injection of Fractions of Mammalian Neoplasms

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The discovery that certain tumors may be induced in certain species by viruses led to attempts to transmit mammalian tumors by injection of cell-free preparations. Taylor and co-workers (7, 8) claimed success with such material, but others failed to corroborate their findings (2, 9). We are aware of no previous report of transmission of a mammalian neoplasm by injection of isolated cytoplasmic or nuclear components of tumor cells.1

MATERIALS AND METHODS

Two types of tumor were employed: (a) 2-acetaminofluorene-induced hepatomas in rats and (b) the Murphy rat lymphosarcoma.

AAF-INDUCED HEPATOMAS

Hepatomas were induced in male Wistar rats by feeding 2-acetaminofluorene (3). Tumor tissue was obtained either from single large hepatomas or from several pooled smaller tumors.

Preparation of mitochondria fraction.—Five to 8 gm. of tissue was homogenized in a refrigerated Waring Blendor in a cold-room (20—4°C.), under aseptic conditions, in either 0.85 per cent NaCl solution or 0.88 M sucrose solution (dilution 1:10). The material was fractionated in a refrigerated centrifuge by the differential centrifugation technique of Claude (4) as modified by Hogeboom et al.1

Cell remnants, nuclei, and erythrocytes were removed by centrifuging the homogenate at 600 g for 10 minutes. This procedure was repeated twice, the sediment being discarded and the supernatants pooled.

The mitochondria fraction was isolated by centrifuging the pooled supernatant at 24,000 g for 20 minutes; the sediment was resuspended in NaCl or sucrose solution and recentrifuged at 24,000 g for 20 minutes.

Smears and sections of paraffin-imbedded preparations were made from every sample of this fraction. These were stained with Janus green B and Mallory's phosphotungstic acid-hematoxylin stain, respectively.

One hundred gm. of normal liver was fractionated in the same manner, the mitochondria fraction was suspended in 0.85 per cent NaCl solution containing a little methylene blue, and the entire sample was examined carefully in a blood-counting chamber.

Preparation of chromatin fraction.—Under aseptic conditions, 20—30 gm. of tissue was ground with an equal weight of beach sand for 5 minutes, with 100 cc. of buffered (pH 7.4) 0.9 per cent NaCl solution added progressively. The triturated mixture was passed through a double layer of gauze and transferred to centrifuge tubes. The subsequent procedure was that of differential centrifugation described by Claude and Potter (5).

The following experimental procedures were employed, under aseptic conditions:

1. Saline suspensions of intact tumor cells (0.2—0.3 ml.) were injected into the liver in 23 normal adult Wistar rats at laparotomy under ether anesthesia. The surface opening was sealed with Oxyceol.

2. The mitochondria fraction was injected in the same manner, suspended in saline in 45, and in sucrose in 73, cases.

3. The chromatin fraction, suspended in 0.2—0.3 ml. saline, was injected in the same manner in fifteen animals.

4. Similar preparations of normal liver cells
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were injected in the same manner in 23 animals.

5. Crystals of 2-acetaminofluorene, approximately 0.5 mg., were suspended in rat plasma, which was subsequently coagulated and implanted in the liver in twelve rats.

All animals were sacrificed after 12–98 days.

MURPHY RAT LYMPHOSARCOMA(10)

Actively growing tumors were obtained from Dr. Murphy and were continued by transplantation in our animal colony (Wistar-Carworth rats).

Three types of preparation of this tumor were employed:

1. Suspensions in 1-ml. 0.85 per cent NaCl solution of intact cells representing approximately 250 mg. of tumor tissue.

2. Suspensions in 1- or 2-ml. NaCl solution of the mitochondria fraction obtained from 20–40 gm. of tissue.

3. Suspensions in 1- or 2-ml. NaCl solution of the chromatin fraction obtained from 20–40 gm. of tissue.

Cell suspensions and chromatin fractions were prepared also from several of the tumors which developed after subcutaneous injection of the chromatin fraction of the original tumor.

These preparations (1 ml.) were injected subcutaneously (groin), and into the spleen, bone marrow, and thymus, the number of animals used in each case being indicated in Table 2. Intact cell suspensions were also injected into the liver in three animals (not indicated in the table).

The following procedures were employed in an attempt to establish the "purity" of the chromatin fraction—especially to eliminate, in so far as was possible, the possibility of contamination with intact cells:

1. Smears were made of a portion of each injected chromatin fraction. These were stained with toluidine-blue, Wright's stain, and by the Feulgen technic.

2. Suspensions were made, in 1-ml. NaCl solution, of intact tumor cells, ranging in number from 500 to 6,000,000, counted in a blood-counting chamber. These were injected subcutaneously in an attempt to ascertain the approximate number of cells and the time required to produce a tumor of the size obtained with the fractionated material.

3. Eighty smears were made of the entire chromatin fraction of 45 gm. of tumor tissue. These were stained with Wright's stain and examined.

RESULTS

AAF-INDUCED HEPATOMA

The pertinent data are presented in Table 1.

We are greatly indebted to Dr. James B. Murphy, of the Rockefeller Institute, for these tumors.

No intact cells, nuclei, or recognizable cell fragments were observed in any of the stained smears or paraffin sections of the mitochondria fraction. Invariably, structures resembling mitochondria appeared to be the only particulate bodies encountered in this fraction. Their structure was particularly well preserved when the sucrose technic was employed (Fig. 1).

No cells or nuclei were seen in the entire mitochondria fraction of 100 gm. of normal liver, suspended in saline containing a little methylene blue and examined in a blood-counting chamber.

Hepatomas were found at the site of injection (liver) in 2 of 118 animals injected with the mitochondria fraction. One of these (Figs. 2–4) had been prepared by the saline (4) and the other (Figs. 5–7) by the sucrose (6) technic. The former was found 30 days and the latter 60 days after injection.

TABLE 1

<table>
<thead>
<tr>
<th>Material</th>
<th>Total Injections</th>
<th>Tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact Cells</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>Mitochondria (saline)</td>
<td>45</td>
<td>1</td>
</tr>
<tr>
<td>Mitochondria (sucrose)</td>
<td>75</td>
<td>1</td>
</tr>
<tr>
<td>Chromatin</td>
<td>15</td>
<td>0</td>
</tr>
</tbody>
</table>

Morphology of lesions.—

1. Rat 1914 (Wistar male, stock diet): The liver of this animal was inoculated with the mitochondria fraction of a hepatoma obtained from a Wistar rat after 232 days on a diet containing 0.08 per cent 2-acetaminofluorene. The recipient animal was killed 30 days later. At the site of injection there was a pale, gray-brown nodule, measuring 1.2 × 1.0 × 1.0 cm. (Fig. 2). Grossly, the liver was otherwise normal.

On microscopic examination, the nodule presented the appearance of a typical AAF-induced hepatoma (3) (Figs. 3 and 4). There was complete obliteration of the normal liver architecture. The lesion was composed of atypical epithelial cells, varying in size, shape, and nuclear chromatin content. The majority were oval or polyhedral, with basophilic cytoplasm and large nuclei, many of which were hyperchromatic. There were numerous typical and atypical mitotic forms. The cells were generally irregularly arranged, but occasionally showed a cordlike arrangement.

There were hemorrhagic and necrotic areas in the marginal portion of the nodule. Sections from other areas of the liver showed a moderate degree of parenchymatous and fatty change, with well preserved lobular architecture.
Fig. 1.—AAF-hepatoma mitochondria. ×1,000

Fig. 2.—Hepatoma induced by intrahepatic injection of AAF-hepatoma mitochondria 30 days previously.

Fig. 3.—Marginal portion of tumor in Figure 2. ×35

Fig. 4.—Higher magnification of section of tumor in Figure 2. ×460.
Fig. 5.—Hepatoma induced by intrahepatic injection of AAF-hepatoma mitochondria 60 days previously.

Fig. 6.—Marginal portion of tumor in Figure 2

Fig. 7.—Higher magnification of section of tumor in Figure 2. ×460.

Fig. 8.—Lymphosarcoma chromatin. ×600
2. Rat 2161 (Wistar male, stock diet): The liver of this animal was injected with the mitochondria fraction of several pooled hepatomas obtained from three rats after 240–280 days on a diet containing 0.03 per cent 2-acetaminofluorene. The recipient animal was killed 60 days later. At the site of injection there were several confluent yellow-gray nodules, measuring about 1.5 × 1.0 × 0.8 cm. (Fig. 5). On section, the cut surface was light gray with occasional areas of hemorrhage. The remainder of the liver appeared normal grossly.

On microscopic examination (Figs. 6 and 7), the nodules presented the characteristic appearance of certain AAF-induced hepatomas, with areas of necrosis and inflammatory reaction. One portion consisted of atypical epithelial cells arranged in irregular cords. The cells varied in size and shape, being polygonal, oval, or elongated. The cytoplasm was basophilic, and the nuclei showed variations in size, shape, and chromatin content. There were many large, hyperchromatic nuclei; multinucleated giant cells and abnormally large cells with deeply stained, lobulated nuclei were also seen. Few mitotic forms were noted. Large portions of the nodules were necrotic, the adjacent areas showing an inflammatory reaction with cirrhosis.

No tumors were observed after intrahepatic injection of suspensions of intact hepatoma cells, cell-residue fractions, chromatin fractions, or suspensions of 2-acetaminofluorene crystals in rat plasma.

MURPHY RAT LYMPHO SARCOMA

The pertinent data regarding the incidence of tumors and leukemia in animals which received injections of various preparations in different tissues are presented in Table 2. Tumors developed in two of three animals at the site of intrahepatic injection of a suspension of intact cells (not included in the table).

In the animals which received subcutaneous injections, tumors were usually palpable (in positive cases) in 3–4 days when intact cells were employed, in 3–7 days with the chromatin fraction, and in 7–18 days with the mitochondria fraction. The animals were killed after 10–30 days, the size of the tumors in those receiving intact cells and those receiving cell fractions being essentially the same.

It was found that a suspension of at least 3,000 intact cells in 1-ml. NaCl solution, injected subcutaneously, was required to produce a tumor that approximated the rate of growth of the slowest growing tumor after injection of chromatin fractions.

In the case of several of the tumors indicated in Table 2 as developing after subcutaneous injection of cell suspensions and of chromatin fractions, the injected material was derived from tumors that had developed after subcutaneous injection of chromatin fractions.

No intact cells or nuclei or recognizable cytoplasmic or nuclear fragments (other than chromatin threads) were seen in any of the smears of the injected chromatin fractions. The only recognizable particulate bodies were the chromatin threads (Fig. 8). The same statements apply to the 80 smears that represented the entire chromatin fraction of 45 gm. of tumor tissue.

Morphology of lesions.—These tumors presented a remarkably uniform appearance; there was no significant difference between those induced by injection of intact cell suspensions (Fig. 9) and those which followed injection of cell fractions (Figs. 10–12).

The tumors consisted chiefly of closely packed cells resembling medium-sized lymphocytes. The cells were round, oval, or polyhedral, with a rather scant, basophilic cytoplasm, often forming a narrow rim around the nucleus. The nuclei were large, hyperchromatic, and sometimes indented. The nuclear membranes were distinct, and there were many mitotic figures. Abnormally large cells were seen occasionally.

There were occasional areas of hemorrhage. The stroma was scant and consisted of a faintly outlined fibrillary network containing delicate blood vessels. There was frequent evidence of invasion of adjacent tissues (fat, muscle, bone).

DISCUSSION

The significance of these observations rests upon the establishment of two fundamental
Fig. 9.—Murphy rat lymphosarcoma induced by subcutaneous injection of suspension of intact tumor cells 11 days previously. ×400.

Fig. 10.—Murphy rat lymphosarcoma induced by subcutaneous injection of chromatin of lymphosarcoma cells 20 days previously.

Fig. 11.—Section of tumor in Figure 10. ×65

Fig. 12.—Higher magnification of section of tumor in Figure 10. ×400.
points: (a) the neoplastic nature of the induced lesions and (b) the absence of intact tumor cells in the injected mitochondria and chromatin fractions.

In the case of the AAF-induced hepatoma, proof of the neoplastic nature of the lesion is difficult. In an extensive experience with this tumor in the rat, we have not been able to transplant it successfully. Bielschowsky (1) reported its successful transplantation into the Wistar rat in three instances. Establishment of the neoplastic nature of the lesions which developed in two animals at the site of intrahepatic injection of mitochondria fractions must depend upon their cytological characteristics, which were identical with those of hepatomas produced by oral administration of 2-acetaminofluorene (3).

There can be no doubt of the neoplastic nature of the lesions that followed subcutaneous injection of chromatin and mitochondria fractions of the Murphy rat lymphosarcoma. Their gross appearance, rate of growth, and cytological features seem to be characteristic. Definite proof is afforded by the fact that identical lesions were produced by subcutaneous injection of both cell suspensions and chromatin fractions of the chromatin-induced tumors. These "third-generation" lesions were indistinguishable from the original tumors.

We know of no way in which contamination of the mitochondria and chromatin fractions with intact cells can be excluded with absolute certainty. Direct proof of the absence of cells in these fractions is impossible. We believe, however, that the following observations indicate the improbability of such contamination:

1. Not a single recognizable cell, nucleus, or cell fragment was seen in smears or paraffin sections of the 118 mitochondria and 15 chromatin fractions of hepatoma cells and the 58 mitochondria and 80 chromatin fractions of lymphosarcoma cells injected into the recipient animals in this study.

2. No cells or nuclei were seen in the entire mitochondria fraction of 100 gm. of normal liver, prepared in the same manner as the hepatoma mitochondria fraction.

3. No cells or nuclei were seen in the entire chromatin fraction of 45 gm. of lymphosarcoma tissue.

4. When intact lymphosarcoma cells were added to the suspended chromatin fraction and the mixture was recentrifuged at 1,500 g for 10 minutes, intact cells could be readily identified in smears. It seems unlikely that such cells would have consistently escaped detection in the examination of smears and paraffin sections made routinely from every chromatin fraction.

We feel that it is highly improbable that the chromatin and mitochondria fractions were contaminated with intact cells. The evidence appears to warrant the tentative conclusion that the tumors that appeared after injection of the chromatin fractions of lymphosarcoma cells were induced by this material, presumably by entrance of the chromatin strands or some component of these structures into lymphocytes of the recipient animals.

The fact that only 3 tumors developed in 58 animals which received injections of the mitochondria fraction of lymphosarcoma cells, in contrast to the high incidence in those receiving the chromatin fraction, raises a question as to the possibility of contamination of the mitochondria with chromatin fragments. Such contamination is indeed probable, inasmuch as the mitochondria fractions were prepared after preliminary homogenation of the tissue in a Waring Blender, a procedure which causes fragmentation of nuclei. Chromatin strands and fragments would then be incorporated into the mitochondria fraction sedimented at 24,000 g, inasmuch as they probably would not have been removed by the preceding centrifugation at 600 g. The positive results obtained with mitochondria fractions may therefore have been due to chromatin material. It is interesting in this connection, however, that tumors developed in 44 per cent of nine animals injected subcutaneously with a mixture of mitochondria and chromatin, as compared with 24 per cent of those receiving chromatin alone and 5 per cent of those receiving mitochondria alone. The striking difference in incidence of tumors induced by fractions of hepatoma and lymphosarcoma cells may be explained on the basis of the difference in transplantability of these tumors in the rat.

There was a rather striking difference in the incidence of leukemia (infiltration of liver and kidney) in animals that developed local tumors following subcutaneous injection of intact cells (11 per cent leukemia) and in those receiving cell fractions (chromatin, 70 per cent; mitochondria, 93 per cent; chromatin and mitochondria, 50 per cent). A difference of this magnitude and in this direction would not be anticipated if the development of the malignant lesions in the recipient animals were due to contamination of the cell fractions with intact cells.

If these observations are valid, they indicate that malignant potentialities of neoplastic cells reside in the chromatin material and may be transmitted to normal cells, probably by entrance of
the chromatin threads or some component of these structures into the cells. The significance of mitochondria in this connection is open to question.

Experiments are in progress in which attempts are being made to insure destruction of cells by various means without altering the structure or activity of chromatin and mitochondria.

SUMMARY

Lymphosarcomas developed in rats at the site of subcutaneous and intramedullary injection of chromatin and mitochondria fractions of the Murphy rat lymphosarcoma. Leukemia occurred in a high percentage of animals which developed local tumors following subcutaneous injection of lymphosarcoma cell fractions.

Hepatomas developed in two rats at the site of intrahepatic injection of the mitochondria fraction of AAF-induced rat hepatomas. This fraction may have been contaminated with chromatin fragments.

Direct proof of the absence of intact cells in these fractions is impossible, but data presented seem to indicate that such contamination is highly improbable.

REFERENCES

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