Ultrastructural and Histochemical Observations on a Transplantable Reticuloendothelial Tumor in Rats

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SUMMARY

Tumors induced in inbred Wistar rats by repeated injections of trypan blue occur in the liver and portal lymph nodes and can be transplanted. The ultrastructure and histochemistry of these tumors are described. Ultrastructural studies demonstrated that the cells in both primary and transplanted tumors were pleomorphic mesenchymal cells with numerous cytoplasmic pseudopodial processes and associated with electron-dense, nonperiodic fibrillar material in the intercellular ground substance. The enzymes nonspecific alkaline phosphatase and the specific phosphatases of adenosine mono- and triphosphate were localized round the tumor cells. Cystic spaces were seen in induced primary tumors but not in transplanted tumors or their metastases. The spaces contained histiocytic cells that were rarely associated with fibrillar material or with phosphatase enzymes. These cells were present, however, in the transplanted tumors in which many resembled the tumor cells that were associated with fibrils. Also they resembled morphologically the bi- and multinucleate cells seen in all the tumors. These results would be compatible with the tumor cells being histiocytes of endothelial origin.

INTRODUCTION

The induction of tumors in liver and lymph nodes of Wistar rats by repeated injections of trypan blue has been described by Gillman et al. (7, 8). These authors considered that the tumors should be classified as reticuloendothelial sarcomas that arose from endothelial histiocytes (6). The tumors were transplantable (14). The morphology of both primary and transplanted tumors is somewhat similar to that of Hodgkin's disease in man (9).

An ultrastructural and histochemical study of the primary tumors in liver and lymph nodes was made to demonstrate that the morphology and histochemistry of the tumor cells was compatible with their being of histiocytic origin. As the cells of the primary tumors are pleomorphic, ultrastructural and histochemical studies were made on transplanted tumors to see whether these tumors were composed of the same cell types as the primary tumors. The ultrastructure and histochemistry of these tumors have not been described previously and are reported in this paper.

MATERIALS AND METHODS

Twenty-five male Wistar rats were given repeated injections of trypan blue, and 7 of these rats were used in this study. The rats were from an inbred strain that had been maintained at the Brabraham Laboratories of the Agricultural Research Council for 6 years and had been originally purchased from the Medical Research Council Animal Laboratories, Carshalton, Surrey, England. The trypan blue was from the same batch of Grubler's dye as used in previous studies (7, 8). Each rat received s.c. injections of 10 mg of dye freshly dissolved in 1 ml of glass-distilled water. The injections were given once every other week and started when the rats were 9 to 10 weeks old and weighed approximately 220 g. Tumors developed in livers and lymph nodes after the rats had received 150 mg or more of dye, i.e., from about 39 to 40 weeks onwards.

A portal lymph node that had been replaced by tumor was removed from 1 rat that had received 160 mg of dye. The tumor was cut into small pieces and pressed through a stainless steel sieve (36 mesh) with a glass plunger. The cells were washed in Hanks' medium, and the larger pieces of tumor were allowed to settle. The suspended cells were removed, pelleted by centrifugation at 150 X g for 5 min, resuspended in Hanks' medium, and counted. One million cells were injected s.c. into 9-week-old Wistar rats of the same strain (14), and tumors subsequently developed at the site of injection. These were transplanted serially with 2 recipient animals at each stage. The tissue used in this study consisted of primary and transplanted tumors. The primary tumors were in the liver and portal lymph nodes of three 50-week-old Wistar rats that had received 200 mg trypan blue and weighed approximately 370 g. The transplanted tumors were from four 16-week-old Wistar rats, 2 of which had received s.c. inoculations 40 days previously of 3rd-passage and 2 of 4th-passage trypan blue-induced tumor tissue. They were situated either at or close to the inoculation site or were found as secondary tumors in the kidneys.

The rats were either anesthetized with halothane or killed by overdosage with CO2. For electron microscopy, tumor tissue was fixed in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, for 1 to 3 hr; washed continuously in the phosphate buffer overnight; and cut into 50-um slices. These were postfixed in 1% osmic acid in Veronal acetate buffer, dehydrated through graded alcohols and acetone, and embedded in Araldite. After polymerization at 70°, suitable histological areas were chosen with phase-contrast microscopy.
Sections 0.5 to 1 μm thick were cut from these areas on an LKB Ultratome 1 and stained with toluidine blue to check the tissue morphology. Ultrathin sections were cut from selected areas, stained in a solution of 20% uranyl acetate (analytical grade) in absolute methanol for 10 min, washed in deionized water, and restained in Reynolds' lead citrate for 5 min (20). The sections were examined in a Hitachi 7S electron microscope. Tissue was also fixed in 4% phosphate-buffered formaldehde, dehydrated through alcohols, and embedded in paraffin wax. Sections cut from these tissues were stained with hematoxylin and eosin to check that the areas selected for study were representative. For histochemical studies, tissues were quenched for 30 sec in dichlorodifluoromethane (Arcton12) cooled to −158° by liquid nitrogen, and then stored in closed ampuls over liquid nitrogen. Twenty-four to 30 hr later the tissue blocks were sectioned at 5 to 10 μm thickness in a Bright's cryostat at −20°. The following histochemical methods were used: nonspecific alkaline phosphatase (11), specific phosphatases of AMP, ADP, and ATP (10), nonspecific acid phosphatase (21), nonspecific esterase (3), and β-glucuronidase (13). For nonspecific alkaline phosphatase, sections were fixed for 10 min in formol-calcium at 4°, washed for 5 min in glass-distilled water, and then incubated for 60 min at 37° with sodium β-glycerophosphate as substrate. Control sections were fixed either in substrate-free medium or with the addition of sodium fluoride to give a medium concentration of 0.01 M. For specific phosphatases of AMP, ADP, and ATP, sections were unfixed and were incubated for 10 to 30 min at 37° at pH 7.2, with naphthol-AS-D-acetate (Sigma Chemical Co., St. Louis, Mo.) as substrate. Control sections were either incubated in substrate-free medium or with the addition of sodium fluoride to give a medium concentration of 0.002 M. For nonspecific acid phosphatase (21), nonspecific esterase (3), and β-glucuronidase (13), sections were fixed as for nonspecific alkaline phosphatase and then incubated for 10 to 30 min at 37° at pH 5.3 with sodium /3-glycerophosphate to give a medium concentration of 0.01 M. For specific phosphatases of AMP, ADP, and ATP, sections were unfixed and were incubated for 10 to 30 min at 37° at pH 7.3 with adenosine-5-mono-, di-, and triphosphoric acid (British Drug House, Poole, England) as substrate. Control sections were incubated in substrate-free medium. For nonspecific acid phosphatase, sections were fixed and washed as for nonspecific alkaline phosphatase and then incubated for 10 to 30 min at 37° at pH 5.3 with sodium β-glycerophosphate as substrate. Control sections were either incubated in substrate-free medium or with the addition of sodium fluoride to give a medium concentration of 0.01 M. For nonspecific esterase, sections were fixed and washed as for nonspecific alkaline phosphatase and then incubated for 10 min at 20° at pH 7.2, with naphthol-AS-D-acetate (Sigma Chemical Co., St. Louis, Mo.) as substrate. Control sections were either incubated in substrate-free medium or with the addition of sodium fluoride to give a medium concentration of 0.002 M. For β-glucuronidase, sections were fixed as for nonspecific alkaline phosphatase, washed for 30 min in hypertonpurygum sucrose, rinsed in glass-distilled water, and then incubated for 15 to 30 min at 37° at pH 5.4, with naphthol-AS-BI-glucuronic acid (Sigma) as substrate. Control sections were incubated in substrate-free medium. For ultrastructural histochemical studies, tissue was fixed for 15 min in 3% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.3, chopped into 50-μm-thick slices, and replaced in the fixative for a total fixation time of 30 min. The slices were washed for 30 min in the cacodylate buffer and then transferred to the incubation mixtures. The enzymes demonstrated were nonspecific alkaline phosphatase; the specific phosphatases of AMP, ADP, and ATP; and nonspecific acid phosphatase. The incubation and control mixtures were identical to those used for localization in the frozen sections. The incubation times varied between 10 and 30 min. They were kept as short as possible to ensure that the lead deposited at or close to the enzyme site neither diffused from that site nor obscured morphological detail. A 1% solution of lead nitrate (analytical grade) in 0.9% NaCl solution replaced the 2% cobalt acetate solution used in the standard Gomori nonspecific alkaline phosphatase method. At the completion of the histochemical reactions, all slices were rinsed in 0.9% NaCl solution, osmicated, and processed as for the ultrastructural studies. The ultrathin sections were examined both unstained and stained.

RESULTS

The macroscopic and histological appearances of both primary and transplanted tumors have been described previously and will be mentioned only briefly (7, 8).

Induced Primary Tumors

The results obtained from the 3 rats were similar. The animals were healthy, but at the time of laparotomy their skin had a blue tinge and their abdomens were distended. After the ascitic fluid was removed, the abdomen was opened. The liver, spleen, and portal lymph nodes were enlarged. The cut surface of the liver had a mottled appearance with a mixture of focal or coalescent pale areas among dark and congested liver tissue. The cut surface of the portal lymph nodes was uniformly pale blue.

The normal structure of the liver was replaced by a pleomorphic mesenchymal tumor that infiltrated the hepatic lobules. Mitoses were frequent in the tumor cells. Sections from resin-embedded tissue stained with toluidine blue showed that the tumor cells were arranged rather loosely in a fibrillar matrix. The cells were of variable size and shape with slightly granular cytoplasm and irregular heterochromatic nuclei; binucleate and multinucleate cells were also present (Fig. 1). Monocytes and occasional lymphocytes were scattered among the tumor cells. Where the tumor was densely cellular, more cells were seen in mitosis, fewer multinucleate cells were present, and the surrounding ground substance contained deeply basophilic fibrils.

The portal lymph nodes contained some normal lymphoid tissue but were mainly replaced by part cellular and part cystic tumor tissue (Fig. 2). The cellular tumor was similar histologically to the liver tumors.

Ultrastructural studies confirmed the pleomorphism of the tumor cells (Fig. 3). They were large cells of variable shape. The nuclei were also of irregular shape, and the cytoplasm contained few mitochondria, well-developed Golgi complexes, abundant ribosomes, rough and smooth endoplasmic reticulum, and vacuoles. Pseudopodial processes that contained occasional vacuoles and numerous filaments protruded all round the cell surface. The cells infiltrated between normal hepatocytes and around normal bile ducts. The ground substance between the tumor cells contained masses of finely granular or fibrillar electron-dense material which were often closely applied to the cell membranes of tumor cells (Fig. 4). Occasionally, the fibrils appeared to be
issuing from the tumor cells. There was no evidence that the cells were actively secreting, but their cytoplasm contained numerous vacuoles that contained granular material similar to that seen in the ground substance. The fibrils were often orientated to each other to form bundles but no periodic striation was ever seen in any of this fibrillar material (Fig. 5). Collagen fibers or bundles were present also but were not oriented toward the fibrillar material.

The tumor cells in the portal lymph nodes were less well differentiated and more densely packed than in similar cellular areas in the liver. Electron-dense fibrillar material was present, however, in many of the intercellular spaces. Binucleate and multinucleate tumour cells were seen and, apart from their increased size, both their nuclear morphology and their complement of cytoplasmic organelles was similar to those of the mononucleate cells that surrounded them. The tumor cells in the cystic areas resembled histiocytic cells (Fig. 6). The cytoplasm contained numerous vacuoles, some contained fat, and others enclosed numerous electron-dense microcrystals. Some of the cells appeared to be “attached” to the sinus walls and formed part of its double-layered endothelial lining. Extracellular fibrillar material was seen occasionally in these cystic areas. Occasionally, cross-band ing of 20.6 nm was seen, which suggested that some of this material was fibrin.

Transplanted Tumors

No differences were noted between tumors of different passage number so a single description will be given.

The tumors situated in s.c. tissue were unattached to skin but were attached to muscle, were smooth and lobulated, and were not necrotic. Their cut surface was uniformly pale and did not bleed excessively. Paraffin sections showed that the tumors were very cellular and well vascularized, and they infiltrated surrounding muscle. Sections from resin-embedded tissue blocks contained some microcrystalline material that destroyed both the knife and the sections. Ultrathin sections processed tissue blocks contained some microcrystalline material that destroyed both the knife and the sections. Therefore, the results described were obtained from the transplanted tumors and their secondaries. Ultrathin sections cut from slices incubated in control media usually showed small amounts of lead deposited in cell nuclei but rarely in cytoplasmic organelles or in the extracellular spaces. The size of lead phosphate crystals was larger in ultrathin sections that had been stained with uranium and lead, but the position of the crystals remained unchanged. Nonspecific alkaline phosphatase (Figs. 11 and 12) and adenosine mono- and triphosphatase activity was weak or not demonstrable. It was not clear whether the nonspecific alkaline phosphatase activity was localized extra- or intracellularly (Fig. 10).

Ultrastructural Localization. It was not possible to study this in primary tumors in the liver or lymph nodes, as the processed tissue blocks contained some microcrystalline material that destroyed both the knife and the sections. The histochemical results were variable, but nonspecific alkaline and specific adenosine mono- and triphosphatase activity was weak or not demonstrable. It was not clear whether the nonspecific alkaline phosphatase activity was localized extra- or intracellularly (Fig. 10).

Histology

Frozen Section Localization. The histochemical results were similar in all the tumors studied. The controls in which the enzyme substrate was absent were negative in nonspecific alkaline phosphatase, nonspecific esterase, and β-glucuronidase; but there was some deposition of lead in the nuclei in nonspecific acid phosphatase, and the specific phosphatase of AMP, ADP, and ATP. The controls in which enzyme-specific inhibitors were added were negative except in nonspecific esterase, which was not inhibited by 0.002 M fluoride. In the cellular areas, the tumor cells were strongly stained by nonspecific alkaline phosphatase, AMP, and ATP but only moderately or weakly stained by nonspecific acid phosphatase, β-glucuronidase, and nonspecific esterase (Table 1). In the cystic areas of lymph node and of liver, the histochemical results were variable, but nonspecific alkaline and specific adenosine mono- and triphosphatase activity was weak or not demonstrable. It was not clear whether the nonspecific alkaline phosphatase activity was localized extra- or intracellularly (Fig. 10).

DISCUSSION

Although both the induced primary tumors and the transplanted or metastatic tumors were composed of cells with an almost infinitely variable morphology, their cytological
relationship has been demonstrated. They were all tumors the cells of which were characterized by the pseudopodial cytoplasmic processes, their association with fibrillar material, and the localization of phosphatase enzymes on the cell membrane.

The pseudopodial processes and more particularly the ruffled border of the multinucleate cells suggested a histiocytic origin. The electron-dense, nonperiodic fibrillar material was similar, however, to that seen in both benign endothelial malformation (22), benign hemangiomas, and malignant hemangiopericytomias and hemangioendotheliomias (18). Such material is referred to as “basement membrane material” and is characteristic of endothelial tumors. In cases described by Murad (17) the tumor cells contained vacuoles of fibrillar material similar to that seen in the extracellular ground substance. These appearances were also seen in cells of the trypan blue-induced tumors. Neither the morphology nor biological behavior of the induced tumors was similar to that of hemangiomas. There was no real attempt to form blood vessels nor for the cells to be arranged in cellular clusters; therefore the induced tumors did not resemble angiosarcomas. The cellular relationships between lymph node endothelium and abnormal monocytic cells called “endothelioid histiocytes” was clearly demonstrated by Gillman and Gillman (6).

The histochemical results support a possible endothelial origin for the tumor cells. Alkaline phosphatase is localized to the endothelium of normal lymph nodes (1). The enzyme is also present in lymph nodes that are either invaded by epithelial cancers, particularly mammary cancer (5), or that are partly replaced by sclerotic areas of some lymphomas (2). The ultrastructural morphology of cells in trypan blue-induced tumors did not resemble that of either epithelial or fibroblastic cells and the fibrillar material did not resemble tropocollagen. The location of lead phosphate over this fibrillar material was related to the demonstration of alkaline phosphatase as it was absent in slices of tissue incubated in control media or in media to demonstrate other enzymes. Nevertheless, it was probably artifactual. Dische (4) has shown that the glycans of basement membrane and reticulin are similar but that reticulin is more fibrillar than basement membrane. Reticulin is specifically stained by metallic ions, and so the local concentration of metallophilic protein present in the fibrillar areas could attract lead during the conversion of calcium phosphate to lead phosphate that was part of the histochemical procedure. The presence of “microcrystalline” material in blocks of induced primary liver and lymph node tumors was due also to histochemical processing. It was not enzyme dependent because fixed tissue incubated in substrate-free media gave rise to the same defect. It occurred only in tumor tissue that contained trypan blue. Possibly, this formed with the lead a complex that subsequently caused destruction of the glass cutting edge. Nonspecific esterase and acid phosphatase are localized to histiocytic cells in normal and hyperplastic lymph nodes, but they may be either present or absent in histiocytic tumors (2, 15). Similar comparative histochemical studies have not been reported of normal, hyperplastic, and neoplastic proliferation of histiocyte proliferations in rat lymph nodes. However, at present there is no direct evidence that the abnormal cells in lymph nodes of rats treated with trypan blue arise in these nodes. Abnormal mononuclear cells are present in the blood (8) and these could colonize lymph nodes.

In previous papers Gillman et al. (6, 8, 9) suggested that a similarity existed between the morphology of changes seen in lymph nodes and spleen from cases of Hodgkin’s disease in humans and those seen in lymph nodes from rats that received trypan blue. The present results do not support this. Although tumorous lymph nodes from these rats contained malignant cells as well as inflammatory cells such as monocytes and lymphocytes, the latter were present only in small numbers, fibrosis was rarely seen, and the multinucleate cells did not resemble those seen in or characteristic of Hodgkin’s disease (16). Recently, Gillman and Kinns have discussed the similarity of the trypan blue-induced lesions to those seen in lymph nodes of human cases of histiocytic lymphoma (T. Gillman and A. M. Kinns, in preparation). These tumors are composed of histiocytes in various stages of maturation and differentiation, and in some cases the histiocytic cells are each surrounded by extracellular fibrillar material that resembles reticulin (19). The present paper supports Gillman and Kinns’ suggestion of morphological similarity between histiocytic lymphoma in humans and trypan blue-induced tumors in rats.

### Table 1

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<th>Enzymes</th>
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<td>$\beta$-Glucuronidase</td>
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*a* –, no reaction product; +, ++, ++++, increasing amount of reaction product.
ACKNOWLEDGMENTS

Dr. Hallowes gratefully acknowledges the help of members of the late Dr. Gillman's department in the preparation of this manuscript. Dr. Hallowes would also like to thank Mr. D. G. West for technical assistance, Miss S. Owen for typing the manuscript, and Mr. J. Leach for photographic assistance.

REFERENCES

Fig. 1. Primary tumor in the liver after 20 injections of 10 mg each of trypan blue. The liver is replaced by a cellular infiltration consisting of pleomorphic mononuclear cells with abundant cytoplasm (m), and small mononuclear cells. The cells are loosely arranged in fibril-containing ground substance. Toluidine blue, X 400.

Fig. 2. Primary tumor in portal lymph node after 20 injections of 10 mg each of trypan blue. Pleomorphic mono- and binucleate cells are similar to those seen in the liver. The cells are present in a lymph sinus (S) and have replaced the surrounding cortical lymphoid tissue by a part cellular, part cystic tumor. Toluidine blue, X 400.

Fig. 3. Liver tumor. Characteristic pleomorphic cells with numerous pseudopodial processes comprise the tumor. Collagen as well as a small amount of fibrillar material (arrow) is present in the intercellular spaces. X 4,000.

Fig. 4. Liver tumor. Pseudopodial process of a tumor cell. The cytoplasm of the cell body contains numerous profiles of endoplasmic reticulum, but only fine filaments are present in the process. Electron-dense fibrils are present in the surrounding ground substance. X 12,000.

Fig. 5. Liver tumor. A mixture of electron-dense fibrillar material and a few collagen bundles (C) in the ground substance adjacent to tumor cell. The fibrillar material shows no periodic staining and is not oriented to the collagen, which shows periodic staining. X 40,000.

Fig. 6. Lymph node tumor. The cortical wall of a sinus (S) is replaced by histiocytic cells, some with well-developed "ruffled borders" (R). The adjacent lymphoid tissue has been replaced by histiocytic cells that lie within a cystic space. X 4,000.

Fig. 7. A s.c. tumor removed 40 days after s.c. implantation of 3rd-passage, lymph node-derived cells. The tumor cells are arranged in a cellular nodule, and their cytoplasm is less granular than in the liver or lymph node tumors. The nodule is surrounded by a less cellular and more fibrillar area. Toluidine blue, X 400.

Fig. 8. Renal tumor removed 40 days after s.c. implantation of 3rd-passage, lymph node-derived cells. Two renal tubules (r) are surrounded by pleomorphic cells, one of which is in mitosis, in a fibrillar ground substance. Toluidine blue, X 400.

Fig. 9. Edge of infiltrating s.c. transplanted tumor. Large bi- and trinucleate cells are present among other tumor cells or their processes. The ground substance contains collagen and fibrils (arrow). X 4,000.

Fig. 10. Primary tumor in liver after 20 injections of 10 mg each of trypan blue. Nonspecific alkaline phosphatase, as localized by black grains, is present in the solid tumor (T), is scanty in the cystic area (C), and is absent in the adjacent liver (L). Methyl green counterstain, X 300.

Fig. 11. A s.c. transplant. Nonspecific alkaline phosphatase. Black grains of lead phosphate are localized along the cell membranes of tumor cells or their process. They are also present among the intercellular fibrillar material. X 20,000.

Fig. 12. Renal tumor. Nonspecific alkaline phosphatase. The lead phosphate (black grains) is localized round the cell membranes or cell processes of most of the poorly differentiated tumor cells. It is also present among the intercellular fibrillar material (arrow). X 4,000.
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