Characterization of a Transplantable, Canine, Immature Mast Cell Tumor


INTRODUCTION

Unequivocal evidence that a virus is the causative agent of cancer in dogs has not been demonstrated, despite extensive efforts in recent years. Most of this work has centered around studies of transplantable malignant lymphoreticular tissue, although transplants of nonlymphoid tumors have been reported also (1, 11, 15).

Lymphosarcomas have been successfully transplanted with cellular preparations through as many as 15 serial passages in irradiated, neonatal dogs (3, 6, 8, 9). Some of these lymphosarcomas (3, 6, 9) were thought to be transmitted in early passage, but neoplasms could not be induced by cell-free preparations of the tumor material and no evidence of virus particles was shown by electron microscopy.

In a preliminary report (2), we described a transplantable, canine, immature mast cell tumor which was carried through 10 in vivo passages without irradiation of the recipient dogs.

SUMMARY

A transplantable canine tumor has been established and carried through 10 serial passages in newborn beagles without immunosuppression of the recipient dogs. Histopathologically, the tumor appeared as a reticulum cell sarcoma, but ultrastructural studies by electron microscopy have shown the tumor to be an immature mast cell sarcoma. Histamine was demonstrated in the tumor cell extracts. Tumors were induced in dogs, no more than 10 days old, with 10^6 cells. A tissue culture-propagated cell line was established from a second in vivo passage dog, and this has been found to induce tumors after over 1 year of in vitro cultivation. Attempts to pass the tumor in vivo with cell-free extracts have not been successful, and no type C or other identifiable virus particles have been observed in the tumor cells by electron microscopy.

MATERIALS AND METHODS

Spontaneous Tumor Donor. The original donor was a 7-year-old neutered female purebred beagle with an immature mast cell neoplasm. The disease was characterized by generalized weakness, anorexia, mild anemia, and enlargement of both superficial inguinal lymph nodes and the right popliteal lymph node. The white blood cell count was 13,900/mm³. Neoplastic cells were not observed in the peripheral blood smear. The superficial inguinal lymph node nodes were surgically removed and prepared for inoculation. Eleven days after surgery, the dog was killed and necropsied.

The anterior mediastinal lymph nodes, deep inguinal lymph nodes, and spleen were markedly enlarged. The thymus was normal and skin lesions were not observed. A small perforated ulcer, loosely covered by the omentum, was present in the proximal duodenum.

Experimental Animals. Newborn dogs used for subpassage study were obtained from a randomly bred colony of purebred beagles (Hazleton Research Animals, Cumberland, Va.). The pups were derived by cesarean section and hand raised in sterile isolators except for a few that were whelped naturally and raised by the bitch. Pups in isolators were deprived of colostrum by feeding of a sterile commercial liquid diet (Orphlac; Riviana Foods, Inc., Topeka, Kan.) during the first 6 weeks after birth. The pups were inoculated i.p., usually within 72 hr after birth, and examined at least twice weekly for evidence of tumor formation. Immunosuppression or irradiation generally was not used.

Dogs with tumors were killed in the terminal stage of the disease, and selected tissue was removed for subsequent passage and pathological evaluation. Those dogs that did not develop tumors were removed from the isolators between 5 and 6 weeks of age and placed in standard dog cages in an isolated area within the facility. They were maintained in this area without vaccination for up to 12 months.

Inocula Preparations. The inguinal lymph node of the donor dog was minced, washed with balanced salt solution, and trypsinized according to standard procedures. Cells were suspended in Eagle's minimum essential medium with penicillin (100 units/ml) and streptomycin (100 μg/ml) so that the final solution contained approximately 2X 10^6 cells/ml.
Cells derived from ascitic fluid of dogs with transplanted tumor or from floating cells propagated in tissue culture were washed with balanced salt solution and resuspended in Earle's base minimal essential medium. Cell-free inocula consisted of 10 or 20% extracts of tumor tissue or tumor concentrates prepared by the method of Moloney (10).

**Histological Methods.** Necropsy examination was performed on all animals. Tissues were fixed in 10% neutral buffered formalin or Zenker's solution and were prepared for microscopic examination by embedding in paraffin. Sections were cut at 3 to 6 μm routinely and stained with hematoxylin and eosin. Selected tissues were stained with May-Grunwald-Giemsa, 0.5% toluidine blue 0 or Luna's stain. Tissues 1 to 2 cm in size were processed as described elsewhere (13). Ascitic fluid and tissue culture cells were initially pelleted by low-speed centrifugation, and the pellets were processed similar to the solid tissues. Sections were cut on a Porter-Blum MT-1 ultramicrotome and were double stained with alcoholic uranyl acetate, then with lead citrate. Sections were examined in a Philips 200 electron microscope.

**Electron Microscopy.** Tumor nodules and ascitic fluid cells for electron microscopy were obtained by biopsy or at necropsy. Cells from the various subpassage levels of tissue culture cell line 32043 were also processed.

**Pharmacodynamic Study.** For determination of the pharmacological activity of the tumor cells, 2 adult beagles (each weighing about 10 kg) were anesthetized by i.v. injection of pentobarbital sodium, 30 mg/kg. The dogs were prepared for measurement of respiration by endotracheal cannulation and for arterial blood pressure by a cannulated femoral artery. Both values were recorded on a Sanborn polygraph.

Responses to appropriate doses of epinephrine (1 μg/kg), acetylcholine (5 μg/kg), norepinephrine (1.5 μg/kg), and histamine (2 μg/kg), as standard reference agents, were obtained. The standard reference agents were prepared in 0.9% NaCl solution and administered i.v. in total volumes less than 1.0 ml. Diphenhydramine hydrochloride was used as an antagonist of histamine.

**RESULTS**

**Transplant Studies**

**Transplanted Tumor Dogs.** Summarized in Table 1 are the results of 10 serial passages representing 36 of 47 dogs inoculated by the i.p. or i.m. routes with whole-cell preparations. The table also shows the cell inocula, latent periods, and the times to death of pups at various passage levels. These in vivo passages are designated as Series A. A separate in vivo passage designated Series B, which is described later, was initiated with tissue culture cells from a 2nd-passage dog (32043) of Series A.

Latent period and death time of early-passage, Series A dogs ranged from 1 to 2 months, and neoplasms were characterized by massive tumor growth. In later passages, the induction and death time were greatly reduced, and less extensive gross
lesions were noted. Tumor regressions did not occur in dogs inoculated i.p., but 1 of 5 dogs inoculated i.m. regressed. Tumor induction in newborns was accomplished without immunosuppression. Older dogs in Passages 4 and 8 required treatment with anti-dog thymocyte serum to achieve tumor transplant (Table 1).

Tumors in most early-passage dogs were first detected as elevated s.c. masses at the site of i.p. inoculation. The abdomen became greatly distended and the dogs were often dyspeptic. Within 1 to 2 weeks, the dogs became terminal and died or were sacrificed.

**Gross Appearance of Tumor.** At necropsy of early-passage dogs, the abdominal cavity was found to contain massive quantities of cell-rich ascitic fluid. In addition, fluid was frequently found in the thoracic cavity. Numerous tumor nodules were observed on all serosal surfaces of both the abdominal and thoracic cavity, and discrete tumor masses were found in the parenchyma of most organs and in the s.c. tissues. The thymus and mediastinal lymph nodes were grossly enlarged and occupied one-third to one-half of the thoracic cavity (Fig. 1). The omentum was thickened, and nodules in the mesentery were common (Fig. 2). A perforated ulcer was seen in the proximal duodenum of 3 of the 36 dogs with induced tumors (8.4%). The cysterna chyli node complex often reached sizes up to 10 cm while the peripheral lymph nodes were generally not enlarged.

After several serial passages, the volume of ascitic and pleural fluid was substantially reduced and, although the thymus was enlarged, it never reached the size seen in early-passage dogs. Similarly, the number and size of tumor nodules were reduced, and seldom were discrete nodules noted in the parenchyma of any organ.

Dogs inoculated by the i.m. route developed tumors which were hard with an abundance of collagen and followed the fascia of the muscle fiber. Metastasized tumors in the peritoneal cavity and various organs were generally soft and discrete. Accumulation of ascitic fluid was rare in these dogs.

**Histopathology**

**Donor Animal.** Histopathological examination of the original donor dog revealed several lymph nodes (right popliteal, superficial inguinal, anterior mediastinal, and deep inguinal) that were affected with a diffuse infiltration of undifferentiated round cells resembling reticulum cells. The liver had diffuse neoplastic involvement with peripoled nodules and intrasinusoidal clusters of similar neoplastic cells. Nodular collections of neoplastic cells were present in the spleen. Reticular and collagen fibers were sparse and irregular in distribution. The application of special stains to detect mast cell granules was negative.

**Experimental Animals.** Tumors induced by i.p. inoculations occurred as unencapsulated masses at the site of inoculation or as nodules on the mesentery, omentum, or pleural cavity, or on the serosal surface of visceral organs. The tumor nodules usually consisted of poorly differentiated tumor cells similar to those seen in the donor dog. Reticulum and collagen fibers varied in amount and distribution.

Tumor masses that developed at sites of i.m. inoculation had an abundance of fibrovascular proliferation and inflammatory cells in addition to the tumor cells. Numerous organs such as liver, lung, pancreas, thymus, and heart were also involved with the neoplastic process in a focal or diffuse manner. Lymph nodes draining the sites of inoculation were often infiltrated with tumor cells.

**Cell Morphology.** The morphology of the transplanted tumor cells did not differ significantly from the original spontaneous tumor (Figs. 3 and 4). The cells varied in shape, tending to be round, oval, or pyriform. Spindle-shaped cells were less commonly observed. Neoplastic cells were generally large with a variable but abundant cytoplasm which stained eosinophilic or amphophilic. The cytoplasm was often vacuolated, fibrillar, or granular. In the donor and early-passage dogs, the cells existed as a syncytium with indistinct cell borders (Fig. 3). In other cases, particularly later passages, the tumor cells appeared more discrete in their outline, especially those tumor cells at the periphery of transplanted tumor nodules (Fig. 4). Nuclei were large and round, oval, or elongated. Nuclei had a vesicular appearance with a thick nuclear membrane and coarse chromatin particles. One to two prominent nucleoli were generally observed. Often, the strands of chromatin appeared to connect the nuclear membrane with the nucleolus. By light microscopic criteria, the poorly differentiated neoplasm bore a resemblance to a malignant lymphoma, histiocytic type (reticulum cell sarcoma). By the use of special stains, it was not possible to demonstrate unequivocal metachromatic granules in the cytoplasm of neoplastic cells of the original donor or in any of the 10 serial passages of the transplanted tumor. Subsequent ultrastructural examination with the electron microscope revealed additional features of the tumor cell which were characteristic of mast cells.

**Electron Microscopy**

**Cell Ultrastructure.** Electron microscopic examination of tumor-involved lymphatic tissues from the donor as well as from dogs bearing induced tumors revealed cell types of both normal lymph nodes and cells clearly identifiable by the presence of numerous cytoplasmic granules (Fig. 5). These “marker” granules facilitated the identification of these cells in ascitic fluid and tissues from normal-appearing nonlymphoid organs such as the liver and kidney (Fig. 6).

In sections, the cells were generally round or oval with abundant cytoplasm and an eccentrically located nucleus. The distribution of some of the cytoplasmic organelles such as mitochondria presented bipolar orientation to the cells.

The nuclei of the cells were round or oval and contained one or more prominent nucleoli. Aggregates of nuclear chromatinic substances were dispersed throughout the nucleoplasm with marked deposition along the nuclear membrane.

Numerous small oval and round profiles of mitochondria were distributed in the uniform and finely granular cytoplasmic matrix. The mitochondria were found generally on that portion of the cytoplasm containing the Golgi region complex. The ultrastructure of the mitochondria was striking in its clear internal matrix with very few cristae, which was in contrast to those observed in adjacent parenchymal cells (Fig. 6).
The Golgi region of the cells with the marker granules was prominent and well developed. The cells contained very little rough endoplasmic reticular membranes, although they were very rich in free ribosomal granules which were distributed throughout the cytoplasm.

The cell membranes were characterized by numerous microvillar projections, their number being greater in the samples obtained from ascitic fluid (Fig. 7).

In samples of the early in vitro passages, the general ultrastructural characteristics of the floating cells were similar to those of cells from ascitic fluid from tumor-bearing dogs. In later passages, however, many of the tissue culture cells were found to contain well-developed stacks of annulate lamellae, which were never observed in the in vivo tumor tissue samples.

**Cytoplasmic Granules.** The cytoplasmic granules were striking in their ultrastructural morphology (Fig. 8). The 100 to 500 nm granules were round or oval in profile with a well-demarcated double membrane. Some contained electron-dense particulate material, 30 to 40 nm in diameter, arranged in complex, whorl-like patterns. In others, only a single, large, electron-dense condensation was present. Following repeated in vivo or in vitro passage, the frequency of the granules with a single large electron-dense condensation increased markedly, although they remained in the same size range and could not be compared with granules seen in mature mast cell tumors (7).

In all cases, granules were found throughout the cytoplasm with greater accumulation in the Golgi region and near the cell membrane. In tissue culture cells, the granules appeared to be developing in the Golgi region canaliculi (Fig. 9).

The general ultrastructure of the cell and the appearance of the cytoplasmic granules are similar to descriptions of other mature and immature mast cells of normal and malignant origin (4, 14, 16, 17). After correlation of the histopathological observations with the electron microscopic findings, a diagnosis of immature mast cell tumor was established.

**Pharmacological Studies**

Since the electron microscopic examination of these tumor cells indicated that they were similar to mast cells, it was of interest to determine whether they contained histamine. A pharmacodynamic study of tumor tissue was made with 20% extracts of cells from the ascitic fluid of a 2nd- and 5th-passage dog. This material was tested for activity on the cardiovascular system of an anesthetized dog.

The cell extracts injected in graded amounts (0.16 to 1.6 ml) induced a dose-related vasodepressor response of brief duration; the response was not tachyphylactic upon repeated administration of high doses of the extract. Responses to acetylcholine and histamine were not changed by these doses of extract. Boiled extract samples induced vasodepressor responses comparable in magnitude to responses elicited by unheated samples.

An attempt was made to antagonize the vasodepressive response. A dose of diphenhydramine (20 mg/kg) was administered just prior to the injection of a reference standard dose of histamine or of the tumor cell extract. The vasodepressive response of histamine and extract was blocked to the same extent by the antihistamine, confirming the presence of histamine in the tumor cell extracts.

**Tissue Culture**

A floating culture cell line was established from tumor material of a 2nd-passage dog (32043). The cells have been continuously propagated for over 1 year without loss of tumorigenicity. The cells varied in size and grew singly or in multicellular clumps with a doubling time of about 20 hr. Examination of the cells in long-term culture when stained with toluidine blue O did not reveal metachromatic granules.

Dose range and inoculation route studies were conducted in newborn pups with the use of high-passage tissue culture cells. Dogs inoculated i.p. with 10^6 or more cells developed tumors as early as Day 17, with no regressions (Table 2). Eight of 16 dogs inoculated i.m. with 10^5 or more cells developed tumors, but 4 eventually regressed.

Age range studies were conducted in 21 dogs that were 1 to 72 days old and that were inoculated i.p. Dogs 10 days of age or less developed tumors and died, but none of the dogs inoculated when older than 10 days have developed neoplasms 5 months postinoculation, regardless of inoculum size.

Dogs inoculated i.p. with tissue culture cells developed abundant ascitic fluid with few tumor cells, while solid tumor development was less marked. Of 17 dogs inoculated with various passage levels of cultured cells, 6 (35%) developed duodenal ulcers.

**Table 2**

<table>
<thead>
<tr>
<th>No. of cells inoculated</th>
<th>Route</th>
<th>Resultsa</th>
<th>No. dying with tumor</th>
<th>No. regressing</th>
<th>Mean time (days) to</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Induction</td>
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<tr>
<td>10^6</td>
<td>i.p.</td>
<td>0/2</td>
<td>0</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td>10^6</td>
<td>i.p.</td>
<td>2/2</td>
<td>2</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td>10^5</td>
<td>i.p.</td>
<td>2/2</td>
<td>2</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td>10^4</td>
<td>i.m.</td>
<td>2/5</td>
<td>1</td>
<td>4</td>
<td>19</td>
</tr>
<tr>
<td>10^4</td>
<td>i.m.</td>
<td>4/4</td>
<td>2</td>
<td>2</td>
<td>15</td>
</tr>
</tbody>
</table>

a No. of dogs developing tumors/no. inoculated.
designated Series B. A total of 7 in vivo passages were achieved, representing 22 of 30 dogs inoculated (Table 3). The clinical appearance of the tumors in various passage dogs was similar to that seen in Series A-passage dogs, including the frequency of ulcers, i.e., 2 of 22 (9%). Tumor induction time was slightly less throughout various passages of Series B while death time was approximately the same as seen in Series A dogs.

Cell-free Inoculations

In our attempts to determine whether the tumor was induced by a viral agent, 16 feti or newborn dogs were inoculated with cell-free preparations of 1st-, 2nd-, 3rd-, or 8th-passage tumor material. No tumors have developed in dogs inoculated when newborn or in utero at 40 to 45 days of gestation after as much as 18 months of observation.

DISCUSSION

Weiss et al. (17) have discussed the problems associated with the diagnosis of immature mast cell tumors. Because of the absence of visible metachromatnic granules, such tumors can be mistaken for histiocytomas, Sticker tumors, or reticulum cell sarcomas. These problems can be readily resolved, however, by the use of the electron microscope. Whereas mature mast cells are characterized by the presence of electron-dense, round to oval granules that are approximately 600 to 700 nm in diameter, immature mast cells contain cytoplasmic granules that are smaller in size, with a wide variety of internal structures. Various other cytoplasmic and nuclear ultrastructural characteristics can also be used to distinguish between these 2 cell types.

The tumor described in this report was subject to the same difficulties of identification described by the above authors (17), and we arrived at a diagnosis of immature mast cell tumor only after a thorough comparison of the histochemical and electron microscopic data.

Although the various histochemical reactions used to demonstrate metachromasia in the original and transplanted tumor cells failed to reveal granules, a variety of other special stains such as the Alcian blue-safranin reaction used by Combs et al. (5) to classify embryonic rat mast cells could be used.

Table 3

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Mean time (days) to</th>
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<tbody>
<tr>
<td></td>
<td>Source Route Results</td>
</tr>
<tr>
<td>1 4.0 x 10^8 TC^b</td>
<td>i.p. 5/7 13</td>
</tr>
<tr>
<td>2 6.0 x 10^8 Ascites</td>
<td>i.p. 5/6 21</td>
</tr>
<tr>
<td>3 1.0 x 10^9 Ascites</td>
<td>i.m. 2/4 7</td>
</tr>
<tr>
<td>4 8.2 x 10^7 Leg tumor</td>
<td>i.p. 5/7 11</td>
</tr>
<tr>
<td>5 1.0 x 10^8 Ascites</td>
<td>i.p. 2/2 6</td>
</tr>
<tr>
<td>6 1.0 x 10^9 Ascites</td>
<td>i.p. 2/2 14</td>
</tr>
<tr>
<td>7 2.0 x 10^8 Ascites</td>
<td>i.p. 1/2 6</td>
</tr>
</tbody>
</table>

^a No. of dogs developing tumors/no. inoculated.
^b TC, tissue culture Passage 13 from 2nd-passage Dog 32043 of Series A.

The appearance of tumor nodules scattered throughout the body, the enlarged thymus, and the induction time of the immature mast cell tumor were similar to findings of Kakuk et al. (6) and Cohen et al. (3) with transplantable lymphosarcomas. The tumor also shows some of the characteristics of the induced canine mastocytomas reported by Lombard et al. (7) and Post et al. (12), including tumor induction in nonirradiated dogs, development of duodenal ulcers and ascitic fluid in transplant dogs, and the presence of histamine in tumor cell extracts. After passage of the tumor cells in tissue culture, the transplant tumors are characterized by a higher percentage of dogs that develop duodenal ulcers, 33% compared with 8% in the in vivo passage tumors. Possibly, this is related to the ultrastructural changes which were noted in the cytoplasmic granules after tissue culture passage, but whether this represents a developmental phase in the maturation of the tumor cells is not known. Various electron microscopic studies (4, 14, 16, 17) have suggested that the complex granules represent different developmental stages leading to the formation of the mature mast cell granule. The absence of mature granules and metachromasia in all tissue culture and in vivo passage of the tumor cells shows that these cells do not develop into a mature mast cell but that a tumor line of immature mast cells has been established.

The ease with which this tumor has been transplanted without irradiation or immunosuppression is of interest and may be related to the biological properties of mast cells. This is supported by the reports of Lombard et al. (7) and Post et al. (12) in which mature mast cell tumors were serially passed in vivo without irradiation. An added benefit in transplanting this immature mast cell may have been the use of the same breed of animal for recipients as was the donor, although transplantation of the mature mast cell tumors was accomplished in breeds other than the donor breed. Further, serial transplantation of canine lymphosarcomas has required irradiation of the recipient animals regardless of whether the animals were the same as (3) or a breed different from (6, 9) that of the donor.

Lombard and Post both report cell-free transmission of their mastocytomas, thus suggesting a viral etiology. No evidence for a virus has been found in the immature mast cell tumors studied in this laboratory. Cell-free transmission attempts have been unsuccessful, and electron microscopic examination of both tumor tissues and the tissue culture cell line has not revealed the presence of recognizable virus particles. The
presence of RNA-containing particles has been demonstrated by radioactive uridine labeling in density gradient-separated extracts of tissue culture cells of this tumor line (2), but no biological activity has been attributed to these particles as yet. Further studies are required to determine their nature.

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