Role of Immune Mechanisms in Metastatic Patterns of Hemopoietic Tumors in Rats

Harry L. Ioachim, Antonia Pearse, and Steven E. Keller

SUMMARY

Two hemopoietic tumors induced in rats by Gross leukemia virus and dimethylbenz(a)anthracene, respectively, display distinctive and consistent patterns of metastases, the former in the thymus and lymph nodes, the latter in the liver and spleen. To investigate the role of circulatory anatomy in the localization of metastases, $^{51}$Cr-labeled cells were injected i.v., and their distribution was followed at various intervals. To explore the influence of immune mechanisms, Gross leukemia virus- and dimethylbenz(a)anthracene-induced leukemic cells as well as a line of antigenically modulated cells were administered to newborn, X-irradiated, and immunologically unresponsive recipients. The circulation of tumor cells through various organs was indiscriminate. The immune response of the host was operative in limiting the local and metastatic tumor growth but not in determining the site of secondary tumors. The conclusion of these experiments was that the selective organ distribution of tumor metastases was solely dependent on intrinsic cellular properties.

INTRODUCTION

The spread of primary tumors to secondary sites occurs according to mechanisms that have remained almost entirely obscure. Although of great practical importance, we do not know how to estimate the metastasizing potential of a tumor, and we cannot predict the organs most likely to be affected by its dissemination.

The peculiar distribution of tumor metastases is poorly understood, and the attempts to explain it by circulatory anatomy alone have been unsatisfactory. The reason some organs are so often the site of metastases (liver), while others endowed with a similarly rich blood supply (spleen, muscle) are not, is inapparent. Even more puzzling is the preference of some tumors for certain secondary sites illustrated by the frequency of bone metastases in prostatic carcinoma, of lung metastases in renal carcinoma, or of ovarian metastases in cancer of the breast.

In neoplasms of hemopoietic tissues the problem is compounded by the ubiquity of stem cells in which tumors of various types could originate, thereby obscuring the distinction between primary and secondary tumor. Consequently, until the recent introduction of lymphangiography and staging of lymphomas, the concept of spreading by metastases was not even considered applicable to hemopoietic tumors.

In this context, animal tumors displaying consistent patterns of metastasis would be particularly interesting for the study of possible surface determinants or other intrinsic cellular properties that could explain their behavior. Earlier observations in our laboratory (17, 25) have shown that 2 hemopoietic neoplasms of rats induced by viral and chemical carcinogens (26), respectively, spread in a different manner, establishing consistent and specific patterns of metastases. Viral-induced leukemic cells injected i.p. in syngeneic infant rats always formed tumors in the thymus (17, 25, 26) (Figs. 1b and 5), whereas chemically induced leukemic cells metastasized only to the liver and spleen (17, 25) (Figs. 1a and 4). This observation was of great interest, not only because the 2 types of leukemic cells displayed a remarkable metastatic selectivity but also because the organs to which each of them consistently metastasized were those in which their parent tumors had originated: the viral-induced leukemic cells in the thymus and the chemically induced leukemic cells in the liver and spleen (26).

To document the selectivity of metastatic spread in hemopoietic neoplasms and to explore its mechanisms, a series of experiments was devised in which the roles of leukemic cell types, routes of administration, and host immunity were separately investigated. In other experiments the spread of cells was monitored by using $^{51}$Cr-labeled leukemic cells under similar conditions. Finally, to investigate the possibility that immune mechanisms are involved in the tissue selectivity of metastatic leukemic cells, similar experiments were undertaken using, alternately, immunosuppressed hosts and antigenically modulated cells.

MATERIALS AND METHODS

Viral-induced Leukemic Cells. As in previously reported work (15, 16, 26), lymphomas were induced by injecting 0.1 ml (1:10) stock GLV$^{3}$ i.p. into 1- to 4-day-old W/Fu rats. Our stock GLV is a 20% cell-free filtrate of rat GLV-induced thymoma, suspended in Medium 199 and stored in liquid nitrogen at $-195^\circ$. Lymphomas developed in the thymus with or without additional involvement of lymph nodes, spleen, and liver, in close to 100% of the recipients after an

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3 The abbreviations used are: GLV, Gross leukemia virus; MuLV, murine leukemia virus; DMBA, 7,12-dimethylbenz(a)anthracene.
average latency period of 75 days (15, 16, 26). Morphologically, the leukemic cells displayed the features of poorly differentiated lymphocytes and exhibited C-type virus particles at the plasma membranes and in the extracellular spaces (18, 26). When fresh, nonfixed as well as acetone-fixed lymphoma cells were reacted with rat anti-MuLV antiserum in indirect immunofluorescence, both membrane- and cytoplasmic-specific antigens were revealed by strong specific staining, as previously described (22, 26). Cell-free filtrates of lymphoma cells consisted of suspensions of GLV particles that consistently induced lymphomas in newborn recipients.

Chemically Induced Leukemic Cells. According to the method of Huggins and Sugiyama (14), 6 i.v. injections of DMBA in a 15% fat emulsion were given at 2-week intervals to 1-month-old W/Fu rats in doses of 35 mg/kg body weight as previously described (26). Leukemia developed in 60% of rats surviving more than 120 days with an average latency period of 200 days. The tumors localized exclusively in the liver and spleen, without ever involving the thymus and only very rarely involving the lymph nodes. The leukemic cells had morphological features of poorly differentiated myeloblasts as previously established by ultramicroscopy and cytochemistry (23, 26). Virus particles were not present, and no reactivity to anti-MuLV antiserum was detected either with fresh leukemic cells or with long-term tissue cultures. Cell-free filtrates of leukemic organs were consistently negative on bioassays in newborn rats (23, 26).

Antigenically Modulated Leukemic Cells. We have previously reported the loss of antigenicity in GLV-induced lymphoma cells that had been serially transplanted through normal adult W/Fu rats after an initial passage through an X-irradiated recipient (20, 24). These cells (MuLV−), although morphologically indistinguishable from other lymphoma cells (MuLV+), do not carry virus particles and are totally negative for both membrane and cytoplasmic antigens when they are reacted with anti-MuLV antiserum in indirect immunofluorescence. In contrast to GLV-induced lymphoma cells carrying the Gross virus (MuLV+) that are consistently rejected when transplanted in normal W/Fu adult rats, the antigenically modulated cells (MuLV−) are always accepted and grow progressively into large tumors (21, 24).

51Cr-labeled Leukemic Cells. Leukemic cells to be transplanted were washed twice and suspended in Puck's medium with 10% fetal bovine serum, assayed for viability with the trypan blue dye test, and adjusted to 106 cells/ml. For labeling the leukemic cells, the following procedure adapted from current techniques (11, 13) was used. A 2 × 106/ml cell suspension was incubated with 1 mCi of Na251CrO4 (sodium chromate; specific activity, 10 μCi chromium per μg at a concentration of 10 μCi/ml) for 2 hr at 37°, under continuous agitation. Unbound chromium was removed by repeated washing of the cells in large amounts of media. The cell pellet was resuspended in Puck’s medium at a concentration of 10 × 10^6 cells/ml.

Adult rats received 0.5 ml (5 × 10^6) labeled cells i.v. An amount of radioactive chromium identical to that of a representative tumor cell pellet was injected i.v. into control rats to investigate the possible nonspecific retention of 51Cr in the organs to be studied. The recipients were sacrificed at various intervals between 30 min and 20 days after the injection of labeled cells. A sample of 0.5 ml blood was obtained before anesthesia; then, the rats were perfused with 500 ml lactated Ringer's solution with heparin to clear all organs of blood. The organs were removed, placed in Bovials, and counted in a Beckman gamma counter. Each organ was counted for 10 min, and results were calculated for each organ as a percentage of total counts per animal.

Recipients. W/Fu rats from our colony were used in all experiments. Adults were 2 to 3 months old and newborns were 2 to 7 days old at the time of injection. In addition to the normal recipients, other groups of adult rats received total-body X-irradiation with 400 R (Picker Vanguard, 300 kV, 2.0 mm Cu, 103 R/min in air), 24 hr before administration of leukemic cells. In still other groups of recipients a state of specific unresponsiveness was induced by neonatal i.p. injection of 0.1 ml of a soluble extract prepared from GLV-induced leukemic cells as previously reported (22). These rats later, in adult life were unresponsive to transplantation of the GLV-induced leukemic cells that they failed to reject (21).

Experimental Protocols. To compare the capacity of various leukemic cells to establish tumors both at the place of injection and at distant sites, the following variables were examined as detailed in Tables 1 and 2: (a) leukemia cells: GLV-induced (MuLV+), GLV-induced (MuLV−), DMBA-induced; (b) routes of administration: i.p., s.c., i.p.; (c) recipients: normal adults, X-irradiated adults, specifically unresponsive adults, newborns.

Newborns received injections of 100,000 cells i.p. or 500,000 cells s.c. Adults received injections of 5 × 106 cells i.p. or were grafted with 3-cm3 portions of tumor tissue s.c. All recipients were sacrificed when gravely ill or at 180 days and were examined grossly and microscopically. The experiments were duplicated with 51Cr-labeled leukemic cells of all 3 types.

RESULTS

Virus-induced, Antigenically Positive Leukemic Cells (MuLV+). GLV-induced leukemic cells which continued to replicate the Gross virus and to exhibit specific membrane antigenicity were almost always rejected when grafted either s.c. or i.p. in normal adult syngeneic rats and never produced any metastases. Only 2 of 94 recipients (2 of 82 s.c. and 0 of 12 i.p.) showed a tumor at the site of injection and none had metastasis (Tables 1 and 2). This was entirely consistent with our previous experience with virus-induced lymphomas (15, 19, 26). Microscopic sections of the graft site showed necrosis of the transplanted leukemic tissue with a minimal peripheral infiltrate of lymphocytes (Fig. 6).

When similar cells were administered to newborns (Tables 1 and 2), only rarely were tumors present at the site of injection (1 of 3 s.c. and 0 of 58 i.p.). Instead, considerable involvement of the thymus and parathymic lymph nodes was noted in all cases (3 of 3 s.c. and 58 of 58 i.p.). The tumor mass in the thymus was usually huge (Fig. 1b), constituting either the sole or the major tumor of these animals.
The involvement of the thymus was by cellular metastases and not by viral induction, although the injected cells carried virus to which newborn recipients are highly susceptible. The distinction was easily made, based on the latency period (19) which was on the average only 14 days (compared to 75 days for viral induction) and on the involvement of lymph node chains (axillary and parathymic for s.c. grafts; mesenteric, left lumbar-aortic, left paraspinal, and parathymic for i.p. grafts), indicating the route of metastasis (19).

When GLV-induced (MuLV+) cells were administered to X-irradiated adults by both s.c. and i.p. routes, large tumors developed at the site of injection (10 of 10 s.c. and 8 of 10 i.p.). In addition, numerous recipients (7 of 10 s.c. and 5 of 10 i.p.) also had tumors in the thymus and lymph nodes (Fig. 2) similar to those seen in newborns. When adults made specifically unresponsive to MuLV antigens received injections of leukemic GLV+ cells, huge local tumors were produced (9 of 10) without distant metastases. Examined in indirect immunofluorescence, the cells of thymic and lymph node metastases of all recipients were strongly positive for MuLV antigens.

**Virus-induced, Antigenically Negative Leukemic Cells (MuLV–).** Leukemic cells that did not express GLV-associated antigens grew into huge tumors at the site of injection (Fig. 3) in normal adult rats (74 of 74 s.c. and 42 of 42 i.p.) and never produced any metastasis (0 of 74 s.c. and 0 of 42 i.p.). Microscopic examination at the site of the graft showed that the local tumor was fairly well demarcated and grew by expansion without infiltrating the surrounding tissues (Fig. 7). When grafted in newborns and in X-irradiated adults (Tables 1 and 2), the MuLV– leukemic cells also formed large local tumors (8 of 8 and 4 of 4, respectively). However, in these recipients the leukemic cells produced metastases (7 of 8 in newborns and 3 of 4 in X-irradiated adults) which involved lymph nodes, the thymus, and, on occasion, the spleen. Examined in indirect immunofluorescence, the cells of all these tumors appeared negative when they were reacted with fluoroisothiocyanate-labeled anti-MuLV antiserum.

**DMBA-induced Leukemic Cells.** Injected i.p., the DMBA-induced leukemic cells did not form local tumors in any of 8 normal adults, 64 newborns, or 2 X-irradiated adults, but they colonized in large amounts in the liver and spleen in 6 of the 8 normal adults, in all 64 newborns (Fig. 1a) and in 2 X-irradiated adults (Tables 1 and 2). When the DMBA tumors were grafted s.c. in 11 normal adults, none had a palpable or visible tumor 6 months later at the dissection of the site of transplantation. However, 4 of 11 normal adults had large metastases in the liver and spleen. Four of 4 newborns had local s.c. tumors, and 2 of them also had huge involvement of liver and spleen, whereas the 2 X-irradiated adult recipients grafted s.c. had tumors in the liver and spleen but not at the site of the implant. The histological examination of the graft showed vigorous cellular invasion of the surrounding tissues (Fig. 8). In none of all 68 newborns, 19 normal

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**Table 1**

<table>
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**Table 2**

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adults, or 4 X-irradiated adults grafted with DMBA-induced leukemic cells were the thymus, lymph nodes, or other organs grossly or microscopically involved (Fig. 4). However, at the time of death, particularly in newborns, the liver and spleen had reached huge proportions (Fig. 1a).

**51Cr-labeled Leukemic Cells.** To estimate the temporal distribution of metastatic cells in different organs, 51Cr-labeled MuLV+, MuLV−, and DMBA-leukemic cells were injected i.v. into adult rats, and 3 recipients were sacrificed at intervals of 30 min, 24 hr, and 4, 11, and 20 days. At the latest date, tumors produced by injected cells were microscopically present. The figures in Table 3 indicate average percentages of total counts of at least 3 animals for each type of leukemic cell, for each different organ. They show the highest counts at 30 min to be in the lungs and the liver. However, although the counts in lungs and peripheral blood decreased sharply and permanently after the 1st hours, the numbers of labeled cells increased continuously in the liver, spleen, and kidneys. Counts in the thymus and skin were insignificant.

In the control group receiving i.v. injections of 51Cr alone, organs were sampled as for the experimental group. It was found that, after 30 min, only 50% of the injected 51Cr remained in the animal, most of which was located in the kidneys. After 24 hr this amount decreased to 15% and after 4 days decreased to only 5%. Where 51Cr-labeled cells were injected, 50% of the radioactive material was still present in the tissues sampled at 20 days. Since whole organs were sampled, our assay would have picked up any radioactivity retained in phagocytic cells.

The conclusion of this experiment appeared to be that, after i.v. injection, the early organ distribution is similar for all 3 types of leukemic cells with the highest counts in the lung and peripheral blood. However, a different organ distribution later seems to prevail, with lung and blood counts decreasing and liver and spleen counts increasing. These results are based on percentages of total counts for every organ. Each figure in Table 3 represents an average of 3 animals. The conclusions of these experiments are expressed as trends, each based on a total of 15 animals for each tissue and for each type of leukemic cell (3 animals per leukemic cell type, per organ, per time period).

**DISCUSSION**

The questions raised by what frequently appears as the "illogical" distribution of metastases are not new. Langenbeck, Bilroth, and Paget, the great surgeons of a century ago, were preoccupied by this problem and urged that an answer be found to the question, "What is it that decides what organ shall suffer in a case of disseminated cancer?" (32). In a paper in *Lancet* of 1889, Stephen Paget (32) was making the attempt to demonstrate that the distribution of secondary growths in cancer is not a matter of chance and advanced the concept of "the congenial soil" as the only place where the seed carried in all directions can finally live and grow.

A part of the answer must be related to the capacity of the host to respond to his tumor. Immune surveillance probably explains why in some cases metastases occur very early and in other cases they occur very late or never, and why some patients have a single secondary tumor, whereas others experience metastatic showers. Immunosuppression by cortisone (1, 2, 34), X-irradiation (4, 7, 27, 36), chemotherapeutic agents (30), and antilymphocytic serum (35, 10) consistently correlated with a substantial increase in the number of metastases. Similarly, tumors became more metastatic in thymectomized and/or splenectomized rats (28), and nonmetastatic tumors became highly metastatic in T-lymphocyte-deprived mice (37).

Finding the answer to the selectivity of tumor metastases appears to be more difficult. In humans, the tendency of some tumors to metastasize preponderantly in certain organs has long been noted (32, 38). In animal systems, numerous workers have observed selective organ metastases of spontaneous and experimental tumors. It has been reported that lymphoma cells injected i.v. into mice localized in the ovary or in the kidney according to strain (9); tumor cells injected into the left ventricle of rats metastasized in 85% of recipients in the kidneys and in 61% in the adrenals but not in large organs such as the liver and spleen (35). In the same study some peculiar predilections of secondary tumors were observed, cells of rat sarcoma R 39 that metastasized to the jaws in 7 of 10 graft recipients and cells of Flexner-Jobling carcinoma that metastasized to the anterior chamber of the eye in 8 of 18 transplanted rats; Walker tumor cells given by jugular injection were present in large amounts in the lungs and absent in the liver at the time of death, although the opposite picture had been recorded in the 1st 24 hr after administration (8); B 16 melanoma cells injected into C57BL/6J mice produced metastases only in the lungs (6); S-91 melanoma cells similarly metastasized to the lungs in DBA/1 mice (33); Ehrlich ascites tumor cells of a hypotetraploid clone produced mainly lung metastases in contrast to the hyperdiploid stock of the same tumor that metastasized mostly to the liver (29).

In the present work we have observed a striking dissimilarity in the metastatic patterns of 2 hematopoietic tumors. Injected in similar amounts, regardless of the route of administration, virus-induced leukemic cells metastasized to the lymph nodes and thymus, whereas chemically induced leukemic cells formed secondary tumors exclusively in the liver and spleen. Even when the grafted tumor cells were no longer present at the site of inoculation, large, lethal tumors were involving these characteristic places of metastasis. To explain the remarkable organ selectivity of these tumor cells, 3 hypotheses seem to be worthy of consideration.

1. Different circulatory pathways for the 2 types of leukemic cells, produced either by a difference in their ability to penetrate blood and lymphoid vessels or by different homing patterns. It has been shown that lymphocytes, after their original colonization in different sectors of the lymphoid system, are able to recirculate and home to the organs of origin (12, 31). However, the organ distribution of hematopoietic tumor cells in the present work did not seem to be determined by specific circulatory pathways. This possibility was disproven by our experiments with 51Cr-labeled leukemic cells, which indicated that the organ distribution, at least for the 1st hours, was not significantly different for the 3 types of leukemic cells and that only later do specific patterns of tumor localization begin to emerge.

2. Differences in organ receptivity for tumor cells, per-
Table 3

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* The abbreviations used are: GLV+, GLV-induced leukemic cells expressing MuLV antigens; GLV−, modulated GLV-induced leukemic cells not expressing MuLV antigens; DMBA, DMBA-induced leukemic cells.

* Trends: ↓, decreasing number of labeled tumor cells in a given tissue over 20 days; ↑, increasing number of labeled tumor cells in a given tissue over 20 days; →, insignificant changes in number of labeled tumor cells in a given tissue over 20 days. All 3 tumor types (GLV+, GLV−, DMBA) exhibited similar trends in distribution for a given tissue, during the time period studied.
haps involving mechanisms related to cellular recognition and immune surveillance. To test this hypothesis, we used immunoincompetent recipients, newborns, and total-body X-irradiated adults for the injection, by various routes, of similar numbers of leukemic cells of the 2 types to be compared. These experiments consistently showed that virus-induced leukemic cells localized in the thymus and lymph nodes, whereas chemically induced leukemic cells metastasized to the liver and spleen (Tables 1 and 2). For the virus-induced leukemic cells there was no comparable picture in normal adults since, in the vast majority, immuno-

competent recipients rejected the grafted tumors (80 of 82). However, the only ones (2 of 82) that did accept the graft showed the same thymus-lymph node pattern of tumor spread. In the chemically induced leukemic cells, the immu

noincompetent hosts presented a metastatic pattern identi

cal to that of their normal adult counterparts.

These findings were rather surprising since we expected, based on general knowledge, that immunoincompetent re-
cipients such as newborns and X-irradiated rats would offer no resistance to the spread of tumors and would present a picture of generalized, randomly distributed metastases. This was not the case, and the specific patterns of metas-
tases remained remarkably consistent, although the immu

nological deficiency of these recipients was proven by the tumors growing at the site of injection even with the GLV-
positive leukemic cells, which are usually rejected by nor-
mal adults. The same GLV-positive leukemic cells injected into adult rats rendered specifically unresponsive to GLV-
antigens, as well as GLV-negative leukemic cells injected in normal adults, were not rejected but formed huge tumors at the injection site. In addition, when GLV-negative leukemic cells were given to immunologically deficient recipients such as newborns and X-irradiated rats, they too produced metastases, again localized in the thymus and lymph nodes.

All these experiments, alternately using antigenic and nonantigenic leukemic cells in immune-competent and in immune-incompetent hosts, showed that immunodeficiency may promote the formation of metastases but does not influence their selectivity. Thus, it seemed justified to conclude that, although the acceptance or rejection of a tumor graft as well as its metastatic potential are immune dependent, the site of metastases may not be conditioned by immunological factors.

3. Differences in intrinsic cellular properties that may determine their preferential growth in various organs. Tu-
mor cells may circulate in similar amounts through different organs but may be unable to survive in unfavorable environments. Circulating tumor cells may colonize and proliferate in those tissues to which they were conditioned by origin and that represent their optimal microenvironment. Some tumor cells known to metastasize more widely, such as melanoma cells, may have simpler requirements to fulfill for their survival. Similarly, the loss of metastatic selectivity shown by some cancers during their evolution may represent the selection of cellular clones that are more self-sufficient, less dependent on special environments, and, therefore, of enhanced aggressiveness and malignancy. This view is in accordance with experiments using labeled B 16 melanoma cells, in which no more than 1% of cells survived 24 hr after i.v. injection into mice and yet 400 melanoma cells still present in the lungs after 14 days were sufficient to produce an average of 78 metastases (6).

Hemopoietic tumors in humans and animals more than any other neoplasms show a metastatic preference for or-
gans related to their tissues of origin. The 2 hemopoietic tumors described in this paper display a remarkable organ specificity for metastases which is maintained over many generations of serial transplantations as well as under conditions in which both specific and nonspecific immunity are abolished. The comparative analysis of these 2 tumor cell lines appears particularly promising for the study of those intrinsic cellular features that determine the preferential growth of tumors in various organs.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the excellent assistance of Marianela Villaverde, Rosemary Spitalieri, and Tove Sambenger for technical, photog

raphic, and secretarial help.

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AUGUST 1976


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