The Inhibitory Influence of a Transplanted Hamster Lymphoma on Metastasis

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SUMMARY

The investigation reported was undertaken to determine the factors concerned in the low metastatic rate of a transplantable lymphoblastic lymphoma in the hamster. Tumor cells were found to be present in the bloodstream on the 7th day after transplantation, but the great majority of animals lived for another month without the development of metastases. However, removal of the primary tumor on the 13th day of growth was followed by metastasis in all the animals so treated. Investigation indicated that the inhibitory influence exerted by the primary tumor was not directed against growth of the tumor cells but rather against their stromatization.

The natural history of the transplantable hamster tumors under study in this laboratory is characterized by death from metastasis in the great majority of cases. A noteworthy exception, however, relates to a lymphoblastic lymphoma. This tumor grows progressively to death, but metastases are present in less than 10 per cent of the animals. The investigation to be reported was undertaken in an attempt to determine the factors concerned in the low metastatic rate.

MATERIALS AND METHODS

The tumor concerned originated in the retroperitoneal space of a 2-year-old Golden Syrian hamster and has been maintained by serial subcutaneous transfer since March, 1955. Takes usually occur in all inoculated animals, and the tumor grows rapidly to form a mass 3 cm. in diameter by the 3d week. The majority of animals die between the 4th and 6th weeks with very large, ulcerated tumors, and, in most instances, death appears to result from hemorrhage. In any case, metastatic growths are found in less than 10 per cent at autopsy. It is significant that the metastatic rate does not increase with the survival time of the animal but is highest in animals dying during the 3d and 4th weeks.

Transfer is effected with tumor fragments placed in the axillary region by means of a trocar.

The axillary node of the homolateral side is usually enlarged at autopsy and has been found to contain centers of tumor growth as early as the 10th day. However, it is apparent from study of cleared specimens and histological sections that such involvement is based on lymphatic extension and does not represent metastasis. It should be emphasized in interpreting this report that the term "metastasis" is used exclusively to signify discontinuous secondary growth and is differentiated from "lymphatic extension" which in actuality represents no more than a spatial continuum of the primary tumor.

Histologically, the spontaneous tumor was a lymphoblastic lymphoma, and, in general, the transplanted tumor is of the same cell type, but variations to lymphocytic and stem cell types do occur (Fig. 1). The variants do not persist as such and invariably revert to lymphoblastic type on continued transfer. There is no associated leukemia.

The methods employed in studying the low metastatic rate of the tumor were entirely those of transplantation, and experimental design was based on a concept of metastasis as requiring entrance of tumor cells into the blood or lymph stream with transport as viable units to a distant site, followed by stromatization and extravascular growth.

RESULTS

A preliminary series of experiments was undertaken to find the relative incidence of takes following direct transplantation of the tumor to various
organs and sites ordinarily involved in metastasis, and, in addition, representative animals were held under observation to determine any variation in metastatic rate associated with the different transplantation sites. Good growth with no alteration in the frequency of takes was obtained on transfer to muscle, testicle, liver, kidney, brain, and eye; and in no instance was the incidence of dissemination greater than that noted from subcutaneous transplants. The intrathoracic and intraperitoneal inoculation of tumor emulsion resulted in diffuse growth throughout the thorax and abdomen with rapid death of the host, but extension beyond these cavities was not observed.

It is apparent, therefore, that the low incidence of metastasis was not related to the use of the subcutaneous space as a transplantation site and, further, that the cells of the tumor would grow after manual implantation in various bodily organs. Accordingly, the possible influence of blood passage on the viability of tumor cells was tested by the intravenous injection of fresh saline suspensions. This was followed by multiple foci of growth scattered throughout the body, demonstrating that the cells were capable of intravenous survival and that growth and stromatization followed blood deposit as well as direct manual transfer.

The possession of such capabilities appeared to fulfill the conditions of metastasizability, and it remained to determine whether, in actuality, tumor cells were released by the subcutaneous transplant into the blood stream. Accordingly, a series of animals bearing subcutaneous transplants were bled by cardiac puncture at daily intervals after transplantation, and the blood obtained was transferred in 0.25-cc. amounts to the subcutaneous space of normal hamsters. The recipients were then followed for the occurrence of the lymphoma at the site of inoculation.

The incidence is listed in Table 1. Tumor cells were not present in the blood, at least in adequate numbers to produce tumors, until the 7th day after transfer, but, on that day, the blood contained sufficient cells to generate tumors in eleven of sixteen test animals. The growths derived from blood-borne cells were usually apparent between the 10th and 15th days, and their subsequent behavior was in no way different from that of growths originating from transplanted tumor fragments.

Such results show that, subsequent to the 6th day, the transplants released tumor cells into the blood in sufficient concentration to produce growth on subcutaneous transfer of small samples to normal animals. In interpreting the significance of this finding, the possibility arose that the focal localization of the blood in the subcutaneous space of the test animals, incident to manual transfer, might be a meaningful factor in the occurrence of the growth, and that the failure of the growth of the same cells in the tumor-bearing animal might be related to a continuous circulation without arrest or deposit in a suitable nidus. The experiment was, therefore, extended, and blood derived from tumor-bearing animals was transferred back autologously to the subcutaneous space of the respective individual as well as homologously to the same site in normal animals. The fact that none of the 46 tumor-bearing animals used developed a growth at the site of autologous blood transfer whereas typical lymphomas occurred in the vast majority of normal controls (Table 2) demonstrated that the differing behavior of circulating tumor cells in tumor-bearing animals and of injected cells in normal animals was not a function of local concentration (Fig. 2). On the contrary, the suggestion inherent in the results was that the presence of the tumor itself exerted an inhibitory influence on the growth of its cells transplanted either manually or by circulating blood elsewhere in the animal's body.

This suggestion was investigated in a variety of ways. The possibility that the presence of the growing tumor inhibited the growth of its disseminated cells was tested by excision of the tumor. Fourteen individual experiments were carried out involving 177 tumor-bearing animals. The tumor was removed from 110 of these on the 10th day of growth, and the remainder were held
as controls. Many of the experimental animals died during the 2d postoperative week, and all the living members of both groups were killed on the 25th day after transfer. Complete autopsies were performed, and the presence or absence of metastasis was noted.

The results are shown in Table 3. Metastasis occurred in 62.7 per cent of the animals of the experimental group in contrast to only 8.9 per cent of the control group. In the control group, metastases were small and usually limited to a single organ, whereas they were widespread and of sufficient size to cause death in the majority of operated animals.

The growth of disseminated cells was apparent clinically during the 1st postoperative week in many instances. The lymph nodes of the neck, contralateral axilla, groin, and interscapular region were involved early and frequently enlarged to 2-cm. masses before death. Involvement of the cervical nodes was sometimes associated with an edema of the face and neck so intense as to impart a leonine appearance to the animal. At autopsy, pleural and peritoneal effusions of chylous fluid containing tumor cells was common, and, occasionally, both membranes were studded with small nodules of tumor. The thymus, spleen, mediastinal and abdominal lymph nodes were usually massively enlarged, and discrete foci of tumor growth were present in the liver and kidney. The bone marrow was frequently completely replaced by tumor tissue (Figs. 3-5).

Control experiments were carried out to determine whether or not manipulation of the tumor incident to removal or stress associated with the operation were themselves factors in increasing the metastatic rate. In one series involving 26 animals, biopsy with removal of approximately one-fourth of the tumor was performed on the 10th day of growth. The animals were killed on the 30th day, and metastases were found in three—a metastatic rate of 10.7 per cent as compared with 9.5 per cent in controls operated upon (Table 3). In a second control group, an area of skin measuring approximately 3X2 cm. was removed from the contralateral side of 52 animals on the 10th day, and the animals were killed on the 30th day. The incidence of metastasis in this group was 7.7 per cent and 6.6 per cent in controls (Table 3). Accordingly, it was concluded that neither manipulation of the tumor or the operative procedure were significant factors in the increased metastatic rate observed following tumor removal. On the contrary, the increased rate appeared to derive from removal of an inhibitory influence associated with the presence of the growing tumor.

Excision of the transplant on the 10th day resulted in the occurrence of metastases in 62 per cent rather than in all the animals concerned, and a question arose whether the length of residence of the tumor in the animal prior to removal might be a factor in the magnitude of increase of the metastatic rate. Accordingly, tumors were removed from groups of animals between the 4th and 13th days of growth, and observed metastatic rates are recorded in Table 4. Metastases were not found when excision was performed prior to the 6th day, but, between the 6th and 13th days, the incidence increased in linear fashion from 29 per cent to 100 per cent.

The absence of metastasis in animals whose tumors were removed before the 6th day of growth obviously relates to the absence of tumor cells in the blood stream prior to that time. It seems reasonable to suggest that the progressively increasing incidence of metastasis associated with continued residence of the tumor might in turn re-
late to an increasing concentration of tumor cells in the blood stream, but pertinent data have not been obtained. In any case, removal of the tumor on the 13th day of growth was followed by metastasis in all animals.

The technic employed in all excision operations was directed toward complete removal of the growth, but, in nearly one-third of the cases, tumor tissue was inadvertently left behind, and, during the ensuing 10-day period, this grew to produce a mass approximately the original tumor in size. Significantly, the incidence of metastasis was not different in animals with such recurrent tumors, indicating that the small focus of tumor tissue missed at operation did not exert the inhibitory effect that characterized the main tumor mass.

Additional evidence supporting the existence of a relationship between the inhibitory influence and tumor bulk was obtained in another experiment. In this case, tumor tissue was transferred simultaneously to both axillary regions of 75 hamsters. Bilateral takes occurred in all cases, and the animals were divided into two groups. Thirty-four were held without treatment for 30 days and were then killed for examination. During this period, the dimensions attained did not appear to be influenced by the second transplant, and the individual tumors approximated the size of unilateral growths in control animals. At autopsy, no metastases whatsoever were found, whereas, in a control group bearing unilateral tumors, the metastatic rate was 8.5 per cent. The remaining 41 animals bearing bilateral growths were operated upon with the removal of one tumor on the 10th day after transfer, and, at autopsy on the 30th day, the incidence of metastasis was found to be 7.3 per cent (Table 5).

Thus, metastasis did not occur in the presence of a quantity of tumor double that derived from a single transplant, and halving the amount by surgical removal resulted in a metastatic rate approximating that characteristic of a single growth. It would appear, therefore, that the relationship between the amount of growing tumor and the intensity of the influence inhibiting metastasis was a direct one, with larger tumor bulk exerting the greater influence. Correspondingly, tumor tissue may be present in amounts too small to exert an appreciable effect, as evidenced by the fact previously noted that recurrent tumors derived from foci unobserved at operation were associated with the same metastatic rate characteristic of completely removed tumors.

In the latter case, the overlooked foci grew rapidly, and recurrent nodules could be palpated simultaneously to both axillary regions of 75 hamsters. Bilateral takes occurred in all cases, and the animals were divided into two groups. Thirty-four were held without treatment for 30 days and were then killed for examination. During this period, the dimensions attained did not appear to be influenced by the second transplant, and the individual tumors approximated the size of unilateral growths in control animals. At autopsy, no metastases whatsoever were found, whereas, in a control group bearing unilateral tumors, the metastatic rate was 8.5 per cent. The remaining 41 animals bearing bilateral growths were operated upon with the removal of one tumor on the 10th day after transfer, and, at autopsy on the 30th day, the incidence of metastasis was found to be 7.3 per cent (Table 5).

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and 11th days when only four takes occurred in 73 animals. This was followed by a rise to 10 per cent, and this level was maintained throughout the remainder of the experiment.

It is evident from such results that the occurrence of takes on retransfer was inhibited by the presence of a growing tumor and that the degree of inhibition increased with the increase in size of the primary transplant. In this respect, the situation was identical with reference to manual transfer and transfer via the blood stream, and, in both instances, the mechanism of inhibition appeared to be concerned with the "taking" of transferred cells rather than with growth. In actuality, growth of the transplanted fragment did occur with some frequency in refractory animals, but this was short-lived and proceeded in the manner of a tissue culture without vascularization.

In such cases, a palpable increase in size was noted during the first 3 or 4 days but was followed by complete disappearance by the 6th day. Visual examination of the transplants confirmed the occurrence of early growth but, in all instances, failed to show any evidence of reaction on the part of the surrounding tissue, and, microscopically, no growth of new stroma was found. The tumor tissue, however, remained viable for at least 3 days in the refractory host, and retransfer to normal animals resulted in stromatization and progressive increase.

Attempts were made to overcome the apparent inability of such animals to stromatize the transplants by introducing fresh connective tissue derived from normal hamsters. This procedure had been highly effective in other experiments concerned with the transplantation of tissue possessing minimal stroma-inducing ability. For example, the human hypernephroma is an extraordinarily poor stroma-inducer, and when transplanted to the guinea pig's eye fails to excite a connective tissue response and grows in the manner of a tissue culture. However, if embryonic guinea pig tissue is transplanted in association with fragments of the tumor, the embryonic tissue, being an excellent stroma-inducer, obtains a vascular supply and then, by virtue of its high susceptibility to stroma-inducing stimuli, responds to the minimal stimulus of the tumor cells and, in turn, provides them with a stroma from its own substance.

The present experiment was undertaken on the chance that a growing fragment of embryonic hamster tissue might provide a nidus for the lodgment of circulating tumor cells, and, by virtue of its high responsiveness and short exposure to the constitutional influences of the resistant animal, react with the production of stroma. However, it was found that, although the embryonic tissue induced stroma in the resistant animal, it did not become the site of a lymphomatous growth. Similar results followed the transfer of mixed embryonic and lymphoma tissue. It seems probable, therefore, that the influence of the primary tumor extended to the recently incorporated embryonic tissue and inhibited its ability to respond to the presence of lymphoma cells with stroma production.

The fact that the resistant animal reacted to the presence of embryonic tissue with stroma production and, further, that no deficiency in granulation tissue formation occurred in wound healing showed that the loss of responsiveness to stroma-inducing stimuli was restricted and not generalized. In an additional experiment, a variety of tumors, including a melanoma and a fibrosarcoma of hamster origin as well as the Brown-Pearce and V2 tumors of rabbit derivation, were transplanted subcutaneously to hamsters refractory to the lymphoma; and, inasmuch as all of them grew with a normal stromal component, it was concluded that the inhibitory influence was directed specifically against the cells of the lymphoma in question.

The specific character of the inhibitory influence was also evidenced by the results of experiments designed to determine the fate of blood-borne fibrosarcoma cells in refractory animals. Suspensions of fibrosarcoma cells were injected into the enlarged mammary veins emerging from the subcutaneous lymphoma on the 15th day of growth. In all instances, the presence of lymphoma cells in the blood stream was confirmed by transplantation. The animals were killed on the 20th day after intravenous injection and showed wide-

| TABLE 6 | THE INCIDENCE OF TAKES ON THE REINOCULATION OF TUMOR-BEARING ANIMALS |
|---|---|---|
| Reinnoculation (Days of growth) | Results |
|   | No. animals | No. takes | Per cent takes |
| 0–1 | 25 | 25 | 100.0 |
| 2–3 | 30 | 30 | 100.0 |
| 4–5 | 40 | 40 | 100.0 |
| 6–7 | 46 | 46 | 100.0 |
| 10–11 | 73 | 73 | 100.0 |
| 12–13 | 59 | 59 | 100.0 |
| 14–15 | 48 | 48 | 100.0 |
| 16–17 | 14 | 14 | 100.0 |

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spread metastases. Histologically, all the metastases were fibrosarcomatous, and none was derived from the lymphoma. Accordingly, it was clear that the specificity of the inhibitory influence was not a manifestation peculiar to manual transplantation in the subcutaneous space but also pertained to blood-borne transfer in internal organs.

This point was well illustrated in sections derived from the lungs of the animals in question. In a number of fortunate cuts, both stromatized fibrosarcoma metastases and intravascular masses of lymphoma cells were present in the same microscopic field (Figs. 6, 7). The nodules of fibrosarcoma were vascularized, and the cells were actively invading lung parenchyma. In contrast, the masses of lymphoma cells were entirely intravascular, and there was no endothelial reaction to their presence. The cells in the peripheral layers appeared viable, and occasional mitotic figures were found but the central areas were usually necrotic. Sections of lung taken after longer residence of subcutaneous lymphoma sometimes show small intravascular masses of necrotic tissue, and it is suggested that they may represent the end stage of such lymphomatous emboli (Fig. 8). In any case, the point of present interest is that vascular endothelium failed to react, despite intimate contact with a comparatively large growing mass of lymphoma cells.

It is apparent from examination of Table 6 that, although the resistance of tumor-bearing animals to reinoculated tumor cells and to blood-borne tumor cells may both relate to the failure of stromatization, the two situations were not entirely analogous. For example, despite the fact that on the 8th day after transfer more than a quarter of the tumor-bearing animals were susceptible to reinoculated tumor cells, less than 10 per cent proved susceptible to their own circulating tumor cells and died with metastases. The suggestion that the "taking" ability of the cells might be modified by blood passage was investigated in a further experiment recorded in Table 7. Tumor cells were mixed with blood serum derived from refractory tumor-bearing animals, and the suspension was injected into the contralateral axillae of ten animals bearing tumors of 7 days' duration. At the same time, a saline suspension of cells derived from the identical tumor was injected into the contralateral flank. The expected ratio of takes (60 per cent) resulted from the saline suspension, but in no instance did the serum suspension produce a tumor. On the other hand, the two suspensions were equally effective in normal controls, and each produced 100 per cent tumors. Such results indicate that some factor in the blood serum of tumor-bearing animals produced an alteration in exposed tumor cells such as to inhibit their ability to induce stroma in other tumor-bearing animals but not in normal animals.

This factor is under continued study and is of particular interest in that present evidence indicates that it continues to function in some cases even after removal of the primary tumor. In several instances, animals operated upon on the 10th day after transfer with complete removal of the primary tumor have survived to the present date (8 months); and not only does their serum carry the inhibitory factor, but they are refractory to reinoculation.

It would appear, therefore, that two factors are concerned in the low metastatic rate of the lymphoma under consideration. One of these is related to the presence of the primary tumor, whereas the other exists in blood serum and may continue to operate after removal of the primary tumor.

**TABLE 7**

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<thead>
<tr>
<th>Inoculum—Emulsion of Tumor Cells With:</th>
<th>Recipients</th>
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<tr>
<td></td>
<td>Tumor-bearing animals</td>
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<tr>
<td></td>
<td>No.</td>
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<tr>
<td>Serum from tumor-bearing animals</td>
<td>0</td>
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<td>Saline</td>
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DISCUSSION

In recent years, considerable attention has been directed toward a search for tumor cells in the blood of cancer patients, and it is significant that such cells have been found both in the absence of metastasis and in individuals who subsequently failed to develop metastasis (1, 4, 5). It should be emphasized that the basis of identification of the blood-borne cells has been morphological rather than biological, and it is not certain that they are viable elements or, if viable, whether they represent fully evolved cancer or an earlier developmental stage with limited growth potentialities. In the latter event, it must be assumed that vascular invasion and escape into the blood stream precedes the attainment of autonomy, and present evidence is contrary to such a supposition (2). An alternative possibility, exemplified by the
Case reported in this paper is that, under certain circumstances, the tissues of a cancer patient may fail to react to the presence of viable circulating cancer cells with the production of a stroma and vascular supply.

It has been observed by several workers that the surgical removal of transplanted tumors in mice is followed by an increased incidence of metastasis (3, 6), but it is questionable whether or not the findings presented in this report can be interpreted as bearing on the same problem. The tumors used in the mouse experiments consisted of two sarcomas and a melanoma, whereas the present tumor was a lymphoma—a growth with many characteristics different from those of the accepted neoplasms. It should also be noted that the results relate to the specific lymphoma studied. The investigation has been expanded to include lymphomas of other animal species as well as a series of more representative cancers in the hamster. Although experimental data are not yet adequate for final interpretation, it is highly significant that, in all cases, tumor cells can be demonstrated by transplantation of blood to normal animals for long periods of time before the occurrence of metastases and that, in most instances, removal of the primary growth significantly shortens this period.

Whether or not the findings in the case of the hamster lymphoma are unique or of more general application, they represent a situation of biological interest. It is apparent that the presence of the primary tumor inhibited the occurrence of metastasis. Investigation suggested that the inhibitory influence was not directed against growth of the tumor cells but rather against their stromatization and vascularization. The stroma-inducing capacity of the cells was not diminished, as evidence by the response in normal animals, and the failure of stromatization seemed rather to stem from a loss of responsiveness on the part of the connective tissues of the tumor-bearing animal. The loss of sensitivity was specifically concerned with the stimuli exerted by the cells of the tumor in question, and no lack of responsiveness was apparent on the transfer of other tumors or of embryonic tissue.

REFERENCES
Fig. 5.—Metastasis in liver of hamster shown in Figure 3. Mag. ×6.

Fig. 6.—Section of lung from lymphoma-bearing hamster 20 days after the intravenous injection of a suspension of fibrosarcoma cells. Note metastasis of fibrosarcoma in lung parenchyma and intra-arterial masses of lymphoma cells. Mag. ×140.

Fig. 7.—Higher magnification of lymphomatous masses shown in Figure 6. Note necrotic center and complete absence of endothelial reaction. Mag. ×170.

Fig. 8.—Section of lung from a lymphoma-bearing hamster killed after 30 days of growth. Note hyalinized necrotic emboli in large vessel. It is suggested that these may represent the final stage of the intravascular growths shown above. Mag. ×165.
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