The factors affecting the development of tumor metastases have been the subject of many recent experimental investigations (1, 3, 6, 11, 12, 16, 22-25). In the study of a large number of spontaneous and induced primary hepatomas in mice we have not observed metastases in other organs, although a few metastases of hepatomas to the lungs have been described (8, 17). Furthermore, eight different transplantable hepatomas developed in this laboratory have not been observed to spread to other organs when implanted subcutaneously in the axillary region. In a study of the comparative capacity of several organs to support the growth of these transplantable tumors we found that two of the hepatomas grew much more readily in the spleen than elsewhere and that one but not the other was accompanied by the development of secondary nodules in the liver (18, 15). A comparison of these two hepatomas suggests that the difference in their capacity to metastasize is related to differences in cell adhesiveness. The spleen seems to be structurally well adapted to permit the release of nonadhesive cells into the blood stream.

MATERIALS AND METHODS

Two strains of mice were employed, a subline of the C3H strain (C3H/St/Wi), maintained in this laboratory for 38 generations, and the BUB strain, developed in this laboratory and now in its 44th generation. The mice are kept in air-conditioned rooms at 80°F. and fed Purina Laboratory Chow.

The two transplantable hepatomas were developed in this laboratory. One, hepatoma SS3, arose spontaneously in a C3H mouse (14) and has been maintained by subcutaneous serial passages for 60 transplant generations. The other, hepatoma BC3, was induced in a BUB mouse by repeated oral administrations of carbon tetrachloride and has been maintained by intrasplenic serial passages for nineteen transplant generations (15). To compare these hepatomas with another type of transplantable tumor we used a subline of Sarcoma 37 which we obtained in 1954 from the Cancer Clinic at the Boston Children’s Hospital and which we have maintained in both C3H and BUB mice for 79 transplant generations.

The tumors were routinely transplanted by injecting 0.02-0.05 ml. of minced tissue, moistened with sterile 0.85 per cent saline, either intrasplenically or subcutaneously in the axillary region by means of a syringe and 18-gauge needle. Implants of tumors into the spleen were made by way of an incision through the dorsal body wall in mice under light ether anesthesia, and, subsequently, the spleens and other organs were examined at various intervals. Tissues were fixed in Bouin’s fluid and stained with Delafield’s hematoxylin and eosin. To obtain serial longitudinal sections of the portal vein with a minimum of manipulation and distortion, that segment of the vein extending roughly from the region of convergence of its tributaries to its entrance into the liver was allowed to adhere to a piece of blotting paper before it was severed and fixed.

RESULTS

Hepatoma SS3.—Serial subcutaneous transplantations of this tumor are routinely made at approximately 4-week intervals when the tumor is 10-15 mm. in length. No macroscopically visible metastases from the axillary region have been observed in 56 transplant generations, during which from six to 50 mice were used in each passage. A subline of the hepatoma was maintained by intrasplenic transplantation for eighteen serial passages. In the spleen the tumor reached transplantable size 3 weeks after implantation, and many hosts succumbed at this time. In addition to the large intrasplenic growth, secondary nodules were found in the livers of most but not all of the hosts (Fig. 1). The nodules were situated most frequently at the edges of the liver lobes. They varied from 1 to 6 mm. in diameter. Serial sections of the portal

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veins of eighteen of these hosts killed 21–42 days after tumor inoculation revealed the presence of free, isolated, intact hepatoma cells (Fig. 7) and occasionally small clumps of these cells (Fig. 8) floating free in the lumen among blood cells, apparently carried by the current of blood flow. There was no indication of an advancing growth of a solid cord of tumor cells up the vein or of implantation or sticking of the cells to the wall of the portal vein. Microscopic examination of the livers of these mice, however, revealed small tumor nodules attached to the walls of intrahepatic branches of the portal vein (Fig. 9), where they frequently form intravascular emboli (Fig. 10). The large nodules compress the surrounding normal liver cells (Fig. 11), and occasionally some tumor cells grow beyond the walls of the vein into the surrounding parenchyma, as illustrated in Figure 12 with another hepatoma.

To determine whether tumor cells are forced directly into the liver at the time of implantation in the spleen, groups of six to eight recipient mice each were splenectomized and others sham-splenectomized immediately after tumor implantation and 1, 3, 6, 9, 12, and 24 hours after implantation, whereas other injected spleens were manipulated as little as possible. Three weeks later metastatic nodules were macroscopically visible in the livers of all the mice (Fig. 2).

The livers of other recipient mice, four in each group, were fixed immediately and 1, 3, 6, and 9 hours after intrasplenic tumor implantation, and sections from several parts of each lobe were examined. Throughout this period fragments of the hepatoma, including clumps of cells, isolated and apparently intact cells, and obviously disrupted cells and free nuclei, occurred in both large (Fig. 6) and small (Fig. 5) branches of the portal vein within the liver.

Sarcoma 37.—For comparison with the hepatomas, Sarcoma 37 was implanted intrasplenically, and splenectomies and sham-splenectomies were performed immediately and 9 and 22 hours after tumor implantation, whereas other spleens were manipulated as little as possible. The livers of all 21 hosts, when examined 12–22 days later, contained numerous macroscopically visible nodules (Fig. 3) as well as smaller emboli which filled many intrahepatic branches of the portal vein.

Hepatoma BC3.—Serial intrasplenic transplantations of this tumor are routinely made at 2-month intervals (15) into a minimum of twelve and up to 30 mice. The hosts usually can survive 3 months and occasionally longer, and the intrasplenic hepatoma may reach a diameter of 30 mm. In seventeen transplant generations we have found neither secondary nodules in the liver (Fig. 4) nor intact cells in serial sections of 22 portal veins. None was found, furthermore, in a total of seventeen mice after manipulation of the spleen and splenectomies performed immediately and 1, 4, 7, and 10 hours after tumor implantation. Serial sections of the portal veins and samples of each lobe of the liver of the latter groups of hosts revealed only free hepatoma nuclei and cell debris in the lumen of the portal vein and its intrahepatic branches.

In an attempt to enhance the spread of hepatoma BC3 from the spleen to the liver, in one experiment the donor tumor was prepared for implantation as follows: it was minced with fine scissors, as usual, and a portion was injected into the spleens of 30 recipient mice; after storage in saline at 5°C for 1 hour, the rest was homogenized in a loosely fitted tissue homogenizer, then pressed through bolting cloth, mesh size #8, 90 meshes per linear inch, and a portion injected into the spleens of a second group of 30 mice; the remainder was pressed through finer bolting cloth, mesh size #25, 200 meshes per linear inch, and injected into a third group of 30 mice. Animals from each group were killed at intervals of 10, 12, and 16 weeks after transplantation. As would be expected from earlier observations, no nodules of the tumor were found in the livers of the first 30 mice which received nonhomogenized minced tumor. Among those that received the tumor pressed through bolting cloth #8, four out of 28 survivors, and bolting cloth #25, five of 29 survivors were found to have a small number of tumor nodules in the liver (Fig. 12). Some were macroscopically visible, usually about 1 mm. in diameter, but two larger ones measured 5 mm. and 9 mm. in diameter. They always occurred at the edges of the various liver lobes. Other smaller nodules were detected by microscopic examination of serial sections through samples of each lobe of the liver.

**DISCUSSION**

The steps in the process of metastasis as set forth by Coman (3), Zeidman (24), and others (10–12, 22) involve the detachment of tumor cells from the primary tumor or transplanted tumor, their invasion of surrounding tissues and penetration of blood vessels, their transport in the blood stream and lodgment in the blood vessels of distant organs and, finally, their invasion of the surrounding parenchyma. The separation of isolated cells or small groups of cells from the main mass of the tumor appears to be determined by the relative adhesiveness among the cells (2, 4, 5, 7). Experimental analysis of the other steps is under way.
in many laboratories to determine the relative importance of the inherent activity of the invasive cells, mechanical factors which may affect the movement and lodgment of the cells, and the influence on tumor emboli exerted by the host tissues at the site of lodgment (1, 10-12, 22, 23).

The implantation of Hepatoma SS3 or Sarcoma 37 into the spleen by forcible injection of minced tumor tissue actually constitutes a mode of intravascular injection directly into the liver. This is demonstrated by the fact that tumor nodules invariably developed in the liver when intrasplenic injection of the tumor was followed immediately by surgical removal of the spleen. Furthermore, apparently undamaged cells of Hepatoma SS3 were found in branches of the portal vein within the various lobes of the livers that were fixed immediately after injection of the tumor into the spleen. This is not the only time that tumor cells reach the liver, however, because intact cells and groups of cells were found in passage through the portal vein after growth of the intrasplenic transplant had continued for 3-4 weeks. Therefore, at least part of the metastatic process, that is, the detachment of tumor cells from the main mass and their transport through the blood stream to another organ, takes place during the growth of Hepatoma SS3 in the spleen. The time of onset of this metastatic process (11, 16) has not been determined. The secondary nodules observed in the liver originate in part from the cells which passed through the spleen into the liver at the time of implantation and, if the hosts survive long enough, probably also in part from cells which later became separated from the growing tumor in the spleen. The latter would represent true metastatic nodules.

Hepatoma BC3, on the other hand, did not metastasize spontaneously from the spleen to the liver. No intact hepatoma cells were found in serial sections of portal veins and of samples of all lobes of the liver during the growth of the intrasplenic transplants. Even patent sinuses of the spleen contained no free cells. Furthermore, passage of intact cells through the spleen and portal vein directly to the liver at the time that the tumor was injected into the spleen occurred only when the donor tumor first was fragmented in a homogenizer and passed through fine-meshed bolting cloth. It was only under these conditions that secondary tumor nodules developed in the liver. Thus, it appears that this hepatoma differs from Hepatoma SS3 in the ease with which individual cells or small clumps of cells can be separated from one another so that passage through vascular channels can ensue. Coman and his associates (2, 4, 5, 7) have demonstrated that decreased mutual adhesiveness is characteristic of cells of invasive tumors and that this property is associated with a decreased calcium content of the cells and a structural modification of the cell surface. Hepatoma SS3 arose spontaneously in a C3H/StWi mouse, and spontaneous hepatomas are generally easy to transplant in this strain of mice. Hepatoma BC3 was induced by repeated carbon tetrachloride injury to the liver in the BUB strain, and we experienced considerable difficulty in establishing a transplantable tumor of this type (15). The difference in capacity of these two hepatomas to metastasize, therefore, might be related to the differences in their origin or there may be a strain difference in cell adhesiveness.

Once intact cells from either Hepatoma SS3 or Hepatoma BC3 enter the liver via the portal vein, they stick to the walls of the larger branches of the portal vein and appear to be mechanically blocked in the smaller branches at the edges of the lobes. A similar disposition of blood-borne carcinoma T150 cells has been described in pulmonary arterioles and capillaries (1). In another carcinoma (V2), on the other hand, in vivo observations (22) demonstrated that adherence to capillary walls and not mechanical blocking was involved, but the subsequent behavior of these cells differed from that of carcinoma T150 (1) and of our hepatomas. Intravascular growth of the hepatomas took place, and emboli were formed which obliterated the lumina of the veins. Penetration of the parenchyma was seen only at occasional sites along the periphery of the larger nodules (Fig. 12). Compression of the normal liver cells around the large nodules always occurred (Fig. 11), and this suggests that invasive growth of these hepatomas takes place primarily by expansion of the entire tumor mass, as proposed by Willis (21) and Young (22), rather than by active migration of motile cells, as demonstrated in other types of tumors (10, 22).

The spleen appears to be peculiarly well adapted to permit cells of tumors, e.g., those of Hepatoma SS3, which do not normally metastasize to penetrate the blood stream. Weiss (19) has shown that the red pulp of the spleens of man and of the rat consists entirely of branching and anastomosing vascular sinuses. At any one time some sinuses are patent and others are collapsed to form the splenic cords. Under some conditions (20) all the sinuses may become opened, and the red pulp becomes one large vascular bed. In such an environment tumor cells which are only loosely adherent to one another could easily be swept away in the current of the blood stream as a collapsed sinus opens to permit the passage of blood and the...
escape of stored blood cells. There have been several hypotheses proposed to account for the general observation that metastases of tumors occur relatively infrequently in the spleen. These include the concepts that the spleen is resistant in some way to the growth of metastases (6, 21), that the motility of the organ prevents lodgment of emboli (9, 18), and that relatively fewer emboli ever reach the spleen because of the paucity of lymphatics (18) and the presence of barriers which reduce the number of emboli in the systemic circulation (9). Since the spleen may readily permit passage of implanted tumor cells into the blood stream, presumably because of its unique vascular pattern and activity, it seems possible that isolated tumor cells or small aggregates of tumor cells might not easily be trapped there long enough for establishment of metastatic growth. Our intrasplenic implants take well, because truly massive numbers of cells are introduced so that all the cells are not swept away in the circulating blood. Once lodgment in the spleen is achieved, the stroma and blood supply apparently are competent to support tumor growth, since hepatoma BC3 grows so much more readily there than elsewhere.

SUMMARY

Two transplantable hepatomas, one which arose spontaneously (Hepatoma SS3) and one which was induced by repeated carbon tetrachloride poisoning (Hepatoma BC3), grew more readily in the spleen than when implanted subcutaneously. The spleen seems to be structurally adapted to permit tumor cells which usually are not invasive to penetrate the blood stream. Intrasplenic growth of Hepatoma SS3 was accompanied by the development of secondary nodules in the liver. Some of these nodules undoubtedly developed from cells which passed directly to the liver at the time that the tumor was forcibly injected into the spleen, because they developed even when the spleen was removed surgically immediately after intrasplenic tumor implantation. Some of the intrahepatic nodules may also represent true metastases, because intact tumor cells and emboli were found being transported through the portal vein and adhering to the walls of the intrahepatic branches of the portal vein during the period of growth of the tumor in the spleen. Hepatoma BC3, on the other hand, did not metastasize spontaneously from the spleen to the liver. Furthermore, immediate passage of intact tumor cells into the liver when the tumor was injected into the spleen occurred only if the tumor was first homogenized and passed through fine-meshed bolting cloth. This is interpreted as evidence that the cells of Hepatoma BC3 are more adhesive, thus less readily separable, than those of Hepatoma SS3.

REFERENCES

lymphoma but rare in FL. The viral leukemia of Gross is indistinguishable from spontaneous thymic lymphoma. Whether the thymic lymphomas in Swiss mice are related to a virus, as in Ak mice, remains to be established. The transplantable tumor variants of FL described here are characterized by mono- and multinucleated giant cells, not encountered in transplantable lymphomas or myeloid leukemias of mice.

The leukemia produced by cellular graft of the transplantable tumor variant of FL here described (best done by intravenous injection of tumor cells) is somewhat different from that induced by FV. Leukemia produced by cell graft has a rapid onset and course and is often advanced after 30 days, whereas the characteristic leukemia following virus infection usually occurs after about 3 months and is seldom seen in less than 30 days.

The most characteristic gross anatomic feature of FD is splenomegaly in which the spleen is soft and red with rounded edges, often with frank hemorrhagic areas. In contrast, the splenomegaly induced by cell grafts is indistinguishable from other autonomous leukemias, the organ being gray or gray-red and firm with more sharp (less rounded) edges.

Earlier work called attention to extensive erythrogenic hyperplasia appearing early in FD before any manifestation of anemia. This may be due to a stimulation of erythrogenic cells by virus, suggesting that this was unrelated to the proliferation of reticulum cells. The bone marrow, the usual site of erythrogenesis, is involved infrequently and only at the late stage of the disease, whereas the reticulum cells of the spleen proliferate within a few days after virus infection. In contrast, leukemia produced by cellular grafts is characterized by monomorphous proliferation of large reticulum cells without erythroblastosis or anemia. In many animals the tumors remained localized at the site of graft (Fig. 1), in others they spread by continuity (Fig. 2). It is possible that virus was contained in the cells and, if slowly released, produced immunity which prevented later development of lesions characteristic of the viral disease. In the morphogenesis of viral leukemia, antibody production may play a prominent, hitherto poorly explored, role.

The occurrence of "leukemic" thrombi in the lung is characteristic, but the interpretation of its pathogenesis is conjectural. It may be the consequence of some antibody formation against the grafted cells. This may explain why these lesions are common in transplanted leukemias and relatively rare in spontaneous leukemias. The development of such lesions in spontaneous (including virus-induced) leukemias may be due to autoantibody production or perhaps to mere blocking of the circulation by clumps of tumor cells which have entered the pulmonary arteries.

A word of caution on the designation of the transplantable tumor cells described here as "autonomous": the failure of two tumors, from animals with no gross or microscopic evidence of FD, to yield tumors (or the generalized viral disease) on subpassage suggests the possibility that the presence of virus is a prerequisite for tumor growth. On the other hand, the change in character of the tumor cells, notably in their nuclei, is also suggestive of transformation of virus-driven normal cells to autonomous, virus-independent cells. However, resident virus can conceivably alter the nuclear pattern, and reliance on the latter in determining whether a cell is autonomous should not be dogmatic. Thus, even cells with abnormal nuclear (chromosomal) morphology can be dependent on a "masked" resident virus.

Further work is needed (a) to define the cytogenetic change, suggested by chromosomal abnormalities associated with acquisition of transplantability, (b) on the association of virus with leukemic cells or the conceivable loss of virus in the ariaplastic tumor cells, and (c) on the presumed existence of a dual type of leukemic cell population:

All sections are from tissues fixed with Zenker-formol and stained with hematoxylin and eosin.
Fig. 7.—Isolated tumor cell (arrow) in portal vein of mouse killed 28 days after intrasplenic implantation of Hepatoma SS3. X450.

Fig. 8.—Aggregate of tumor cells, one in mitosis, floating free in portal vein of mouse killed 22 days after intrasplenic implantation of Hepatoma SS3. X450.

Fig. 9.—Nodule of Hepatoma SS3 cells developing in intrahepatic branch of portal vein 23 days after intrasplenic implantation of the tumor. X225.

Fig. 10.—Tumor embolus in intrahepatic branch of portal vein 30 days after intrasplenic implantation of Hepatoma SS3. X225.

Fig. 11.—Liver of mouse killed 22 days after intrasplenic implantation of Hepatoma SS3. Large secondary nodule on the left (edge indicated by arrows) produces compression lines in the normal hepatic parenchyma on the right. X225.

Fig. 12.—Large intrahepatic nodule of Hepatoma BC3, bottom of photograph, 13 weeks after intrasplenic injection of tumor cells passed through bolting cloth. Double arrow indicates margin of tumor contained within branch of portal vein. Single arrow indicates tumor cells infiltrating the normal hepatic parenchymal cells toward top of photograph. X250.
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All sections are from tissues fixed with Zenker-formol and stained with hematoxylin and eosin.

FIG. 1.--Large tumor, localized to graft site (right thigh), without metastasis to regional lymph nodes and without splenomegaly and hepatomegaly.

FIG. 2.--Small tumor at graft site (right thigh), with extensive abdominal metastases but without splenomegaly and hepatomegaly.

FIG. 3.--Small tumor at graft site (right thigh), with metastasis to regional lymph nodes, splenomegaly, and hepatomegaly. Hemorrhagic "leukemic" infarcts in lungs.

FIG. 4.--Generalized leukemia and splenomegaly and hepatomegaly following intravenous injection of tumor cells.


Metastasis of Transplantable Hepatomas from the Spleen to the Liver in Mice

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