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Growth and Morphology of Mouse Lymphoma Cells in Diffusion Chambers.* (29492)

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Algire *et al*(1) demonstrated that mouse tumor allografts could survive in non-immune and immune hosts when placed in diffusion chambers that presumably allowed passage of fluid and not of cells through their membranes. These authors postulated that the host cells carrying the capacity to reject the allograft could not pass the membrane, gain access to the donor cells and destroy them. Shelton and Rice(2) have shown that transmitted lines of mouse ascites lymphoma cells could remain viable, *i.e.,* retain their ability to produce tumor, when left in diffusion chambers for as long as 10 months in allogeneic hosts. Lieberman(3) has described studies in which normal thymus from AKR and C57 black mice was cultivated intraperitoneally in diffusion chambers in isogeneic and allogeneic hosts. Viable cells representing the reticular-epithelial elements of the thymus were found to persist for as long as 8-10 months. Chambers containing leukemic thymus fragments showed cells which retained their distinctive appearance and ability to produce a disseminated lymphoma for as long as nine months. In all of these studies it was assumed that growth of leukemic and normal allogeneic tissues occurred because the chambers were impermeable to host cells which might cause allograft rejection. Conversely then, it has been assumed that the cells in the chamber could not escape, and lack of tumor growth in isogeneic hosts which were used in the last two of the above mentioned studies would be evidence for this.

More recent studies of Osoba and Miller (4) and Levey *et al*(5) have shown that normal thymus fragments placed in "cell tight" diffusion chambers of 0.30 and 0.45 μ mean pore size respectively restore immunologic function and lymphoid tissue to mice thymectomized when newborn. They have observed persistence only of the epithelial reticular elements of the thymus tissue within

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the chambers. These studies led the authors to conclude that since no cells can escape through these chambers, the thymic function of promoting normal lymphoid development and consequently normal immune response is mediated through a humoral factor.

In the observations reported here diffusion chambers containing both allogeneic and isogeneic normal thymus and lymphoma tissue have shown similar patterns of cell growth within the chambers. They have, however, also been associated with the development of peritoneal and generalized lymphoma in some of the chamber bearing animals, casting doubt on the assumptions that such chambers are impermeable to the passage of cells.

Materials and methods. The mice used in these experiments for both donors and recipients were animals of 2-5 months of age. The strains were inbred AKR/JAX and $C3H_f$/Bi animals taken from the breeding colonies maintained in our laboratory. The AKR/JAX mice have an 80% incidence of a spontaneous thymic lymphocytic lymphoma occurring after 6 months of age. The $C3H_f$/Bi mice have a 1% incidence of lymphoma occurring only in mice over 14 months of age. Since both strains were utilized as donors and recipients the term "isogeneic" is used when the chamber's contents were of the same strain and "allogeneic" used when the donor and recipients were of the 2 different strains.

The chambers were constructed by a modification of Algire's method(1) using a lucite ring of 20 mm outside diameter and an inside diameter of 8 mm and a depth of 1 mm. The rings were stored in aqueous Benzalkonium chloride 1:750 until used. To construct the chamber, sterile Millipore membranes, 18 mm in diameter, with 0.45 μ pore size were glued to both sides of the ring using Acryloid (B-7) adhesive. The membranes had a grid marked on one side and were always mounted with the grid facing the outside to facilitate microscopic examination of the inner surfaces of the membranes after they were removed. After each gluing the chamber was placed under a 700 g lead weight for 5 minutes, to assure a tight seal and complete drying of the glue. A 1 mm fragment of tissue in one drop of Tyrode's solution was placed inside the chamber before the top membrane was glued in place. The chambers were implanted in the abdominal cavity in mice anesthetized with nembutal. Aseptic technique was used throughout the procedure and extreme care was exercised to prevent the outer surface of the chambers from coming in contact with the tumor cells. They were removed from anesthetized animals at varying intervals and were inspected carefully for gross evidence of leakage. Rarely both gross and microscopic evidence of erythrocytes was found within the chambers or the membranes were noted to have separated from the lucite rings. The animals with these defective chambers were eliminated from the study. When a chamber was taken from the animal all tissue was removed from its outer surface. It was then opened by cutting the membrane from the ring and was usually found to contain a homogeneous opalescent material. Sometimes a remnant of the tissue fragment was seen in the chambers removed within a few weeks. This center material was in some instances removed aseptically and implanted intraperitoneally into young adult mice to test for the presence of viable tumor cells. The membranes and sometimes the center material were fixed in Bouin's fluid, stained with hematoxylin and eosin and examined microscopically. The presence of the grid marks allowed complete assurance as to which side of the membrane was being examined. The animals were observed until natural death or sacrificed at one year of age. In all cases autopsies were performed, and hematoxylin and eosin stained sections of the liver, spleen, kidney, lymph nodes and thymus were examined in each case.

Observations. Chambers containing normal thymus fragments. Nine C3H mice at 2-6 months of age were implanted with diffusion chambers containing fragments from normal thymus. Five animals were given chambers containing fragments of normal thymus tissue from 5- and 14-week-old AKR mice. Four mice received chambers containing fragments of 8-week-old C3H thymus. The chambers

TABLE I. Cell Types Within the Chamber at Time of Removal in 51 Mice Bearing Diffusion Chambers.

Types of tissue transplanted	No. of mice	Lymphocytes	Lymphoma cells	Histiocytes and fibroblasts only	No. growth
Allogeneic normal	5	1	0	4	0
Isogeneic normal	4	0	0	4	0
Allogeneic lymphoma	32	0	18	12	2
Isogeneic lymphoma	10	0	6	4	0

were removed from 1-20 weeks after implantation. All of them were found to show abundant histiocytes and fibroblasts on the stained Millipore membrane. In only one instance there was a cluster of normal lymphocytes also present on a membrane removed at 3 weeks. This chamber contained allogeneic thymus.

Chambers containing allogeneic lymphoma tissue fragments. Thirty-two mice received diffusion chambers containing fragments of allogeneic thymus and lymph nodes from animals with spontaneous or transmitted lymphoma. Eighteen of them, in place for 1-32 weeks, showed viable lymphoma cells with a background of histiocytes and fibroblasts. Twelve chambers removed at 1-24 weeks showed only histiocytes and fibroblasts on their membranes and no growth was observed in 2 chambers removed at 12 and 18 weeks. The contents of 3 of the chambers removed at 4, 7 and 12 weeks showing viable lymphoma cells on the membranes were implanted intraperitoneally in young AKR mice. Generalized lymphoma developed in these animals within 3 weeks. The contents of 2 chambers shown to contain only stromal cells did not result in lymphoma when implanted in AKR mice after their removal from the chamber at 16 and 20 weeks after implantation.

Chambers containing isogeneic lymphoma tissue fragments. Ten mice were implanted with chambers containing fragments of thymus and lymph nodes from isogeneic transmitted lymphomas. Chambers removed at 3, 4, 7, 14 and 16 weeks were found to contain viable lymphoma cells with a background of histiocytes and fibroblasts. The contents of 2 of these chambers removed at 4 and 7 weeks produced progressive lymphoma 3-6 weeks after their intraperitoneal implantation in C3H mice. The remaining 4 chambers contained only stromal cells when removed at 5, 6, and 44 weeks respectively. The contents of 2 of these chambers did not result in lymphoma when implanted intraperitoneally in C3H mice. All of the preceding observations are summarized in Table I.

Development of lymphoma in mice with diffusion chambers. None of the 9 mice with normal thymus in diffusion chambers developed lymphoma. Nor was any lymphoma seen in 3 C3H mice with empty diffusion chambers left in place for as long as 40 weeks. Lymphoma did occur in 9 of the 42 animals implanted with diffusion chambers containing leukemic tissue fragments. Table II presents the incidence of lymphoma related to the length of time the chambers were left in place in these 9 animals. The pattern of lymphoma development was not at all consistent but these animals can roughly be divided into 2 groups.

The first group of lymphomas occurred in 6 mice within 3-8 weeks after the diffusion chamber was implanted. The animals were 2.5-4 months of age at the time the tumors were noted. Three chambers contained allogeneic and 3 isogeneic tumor fragments. All

TABLE II. Incidence of Lymphoma in Mice with Diffusion Chambers Containing Fragments of Thymic Lymphomas.

Duration of chamber (wk)	No. of lymphomas /No. of animals
1	1/4 *
3–18	6/25†
12–20	2/8
22–44	0/5

* This animal developed generalized lymphoma of host type at 8 mo of age 17 wk after chamber was removed.

† These animals all developed lymphoma involving primarily peritoneal surfaces while chamber was in place.

of the animals showed primarily a striking involvement of the peritoneum with tumor, the tumor surrounded the chamber and ascites was frequently present. The tumor masses on the peritoneal surfaces of the organs invaded them locally. The diffuse intrinsic infiltration of liver, spleen, and kidney so characteristic of spontaneous lymphoma was not seen. The thymus and lymph nodes contained tumor cells in some instances but were not greatly enlarged. The histological pattern was that of lymphocytic lymphoma.

The second group consisted of 3 lymphomas occurring in C3H mice 16-18 weeks after the chambers were implanted. Somewhat different characteristics were found in each so they will be discussed separately.

The first mouse developed a generalized thymic lymphoma at 8 months of age. This lymphoma was morphologically identical to that seen to occur spontaneously in AKR mice. A diffusion chamber containing a fragment of lymphomatous AKR thymus from a mouse with spontaneous disease was implanted in this animal 18 weeks earlier at 4 months of age and left in place for one week. Upon removal the chamber showed viable lymphoma cells and a background of histiocytes and fibroblasts. Mitotic figures were frequently seen in all cell types.

The second animal developed a lymphoma at 9 months of age. This tumor extensively involved the peritoneal surfaces and retroperitoneal and mediastinal nodes. There was abundant ascitic fluid containing tumor cells. The thymus was only slightly enlarged but was replaced by lymphoma cells. The spleen appeared normal but showed microscopic evidence of tumor cells. This mouse had had a diffusion chamber containing a fragment of thymus from a transmitted AKR lymphoma implanted at 5 months of age and the chamber was removed at the time the animal was sacrificed with symptoms of lymphoma, 16 weeks later. There was only amorphous necrotic material in the center of the chamber and on the Millipore membrane.

The third mouse died at 6 months of age, a diffusion chamber containing a lymphomatous C3H thymus fragment from an animal with cell transmitted disease had been implanted and left in place 16 weeks previously. At autopsy the animal showed a soft white enlargement of the thymus filling the entire thoracic cavity, the lymph nodes were not enlarged and the other organs were completely normal. There was no tumor in the area of the diffusion chamber. Microscopically this was a typical lymphocytic lymphoma of the thymus. The Millipore membrane in this animal showed sheets of viable lymphoma cells with rare mitoses.

The diffusion chambers in all of these 9 animals with lymphoma appeared intact; when examined after the removal, there was no visible break in the seal. There were no red blood cells, segmented neutrophils, or mature lymphocytes in the chambers to indicate entrance of host cells.

Discussion. These studies have shown that normal adult mouse thymus fragments in diffusion chambers of 0.45 μ pore size lose their lymphoid component after one week. The persistent cellular growth is that of fibroblasts and histiocytes which cover the membrane in a layer of 1-2 cells deep and persist for as long as 20 weeks. The loss of lymphocytes from the normal thymus fragments could have occurred because these cells could not survive in the chambers or underwent metamorphosis to the cell types observed. Studies of suspensions of thymus cell *in vitro* frequently show a loss of lymphocytes and a persistent growth of fibroblasts, presumably due to one of these two mechanisms. However, the third and very likely possibility is that the loss of lymphocytes resulted from their escape through the 0.45 μ pores of the membrane. Capalbo *et al*(6) have demonstrated migration of mouse peritoneal cells through Millipore membranes of 0.45 μ pore size into empty diffusion chambers. As will be discussed presently the results with lymphoma cells lend further support to the possibility of cell escape.

Lymphomatous thymus and lymph node fragments showed a growth pattern somewhat different from normal in the chambers. Lymphoma cells, the malignant counterpart of the lymphocyte, were present in over half of the chambers. They maintained their nor-

mal morphology, the ability to divide and to produce tumors in susceptible hosts after isolation in diffusion chambers for several months. Histiocytes and fibroblasts were also present in the chambers with lymphoma tissue fragments. Some of the lymphoma fragments behaved like the normal tissue, *i.e.* with loss of the lymphoid elements and maintenance of growth of stromal cells. The growth patterns of isogeneic and allogeneic cells were the same. Nine mice with diffusion chambers containing lymphoma tissue developed the disease. Empty diffusion chambers have been associated with the development of subcutaneous fibrosarcomas and plasma cell tumors but not of lymphoma(7, 8). It would seem then that the tumors must have resulted from the transmission of a subcellular oncogenic agent released from the lymphoma cells or from the escape of virulent tumor cells from the chambers.

The action of a subcellular oncogenic agent (virus) can be considered in the 3 lymphomas developing 16-19 weeks after chamber implant. In one animal the chamber was removed 18 weeks prior to development of tumor and the tumor grew progressively in the host and not the donor strain. In the second animal the chamber contained no viable lymphoma cells. In the third chamber viable cells were seen at the time of lymphoma development in the host, but the only evidence of tumor was thymic lymphosarcoma. That these few lymphomas were spontaneous neoplasms is unlikely for in the C3H strain, the 1% of the animals developing lymphoma do so only after 14 months of age. The tumor bearing animals here were 4-9 months of age. However, if these lymphomas did develop as a result of tumorigenic virus liberated earlier by the growing lymphoma cells in the chamber, their development is atypical for the virus induced disease occurring in these strains. Cell free filtrates of lymphoma tissues of AKR mice have been shown to result in this disease only after their inoculation into newborn mice, resistance having been demonstrated in the adult animals(9). The latent period of 16-18 weeks is compatible with that found in the virus induced mouse lymphoma arising from the AKR lymphoma tissue filtrates and the host genetic status of one of the tumors is like that seen in virus induced neoplasms. Therefore, the liberation of a virus by these lymphoma cells grown in diffusion chambers resulting in the development of lymphoma in this group of animals remains a possibility which has not been ruled in or out by these studies.

It seems likely that the 6 lymphomas which developed within 3-8 weeks of chamber implantation resulted from the escape of viable cells. The short latent period and the presence of local growth on the peritoneal surfaces could occur if cells had left the chamber shortly after implantation. These membranes were cell tight in that they showed no evidence of host cell entry and no break in their surfaces or of the seal. Therefore, the migration of the cells must have occurred through the 0.45 μ pores of the membrane itself.

Confirmation of the possibility of cell escape was found in a report by Algire(10) which demonstrated that chambers maintaining mouse lymphoma cells from the transmitted L1210 line constructed with Millipore membranes with 0.30 μ and 0.45 μ mean pore size resulted in lymphoma within 2-5 weeks in half of the DBA/2 mice so treated. He concluded that cells of this highly virulent tumor had passed through the pores of the Millipore membranes. In the studies reported here the low incidence of lymphomas presumably resulting from cell escape and persistence of lymphoma cells in the chambers would indicate that the larger malignant cells had greater difficulty traversing the membrane pores than did the lymphocytes from the normal thymus fragments.

Summary. Experiments have been reported which show that diffusion chambers constructed of Millipore membranes of 0.45 μ average pore size can support the growth of lymphoma cells from thymus fragments and stromal cells from both normal and lymphomatous fragments of thymus for as long as 32 weeks. Certain animals carrying chambers with tumor fragments developed lymphoma from 4-18 weeks after the chambers were implanted. It is concluded that the

majority of these neoplasms probably were the result of the escape of the cells from the diffusion chambers. This conclusion is based upon the localization of tumor growth to the region near the chamber, and the short temporal relationship of tumor development to chamber implant. It is the author's opinion that migration of both normal and malignant lymphocytic cells through Millipore membranes of the mean pore size employed here remains a possibility which must be considered in all experiments of this type. Liberation of a subcellular tumorigenic agent by the viable lymphoma cells within the chambers could possibly explain the development of some of the malignancies observed.

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Iron Metabolism in Acute Starvation. (29493)

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One consequence of starvation, the cause of a number of metabolic abberations(1,2), is a diminution of erythropoiesis(3). This is manifested by parallel decreases in marrow erythroblasts, reticulocytes in the peripheral blood and presumably hemoglobin synthesis since there is decreased red blood cell radioiron incorporation(3,4). These changes are partially reversible by erythropoietic stimulation and starvation has been utilized to diminish erythropoiesis in animals prepared for bioassay of erythropoietic activity(4,5). Coincidental alterations in iron metabolism which occur with starvation would be reflected in the results of these assays and leave interpretations uncertain(6,7).

This report details preliminary observations on iron absorption, plasma iron, plasma iron clearances, iron turnover, blood volume and iron partition in normal and acutely starved rats. Acute starvation did not alter blood volume nor gastrointestinal uptake of radioiron. Starvation produced a prolongation in plasma iron clearance and a diminution in plasma iron turnover. Starved rats had decreased radioiron in spleen and bone marrow. These alterations were partially reversed by erythropoietic stimulation.

Materials and methods. Female Sprague-Dawley rats were utilized in all experiments. Iron absorption studies were undertaken in 200-300 g rats, whereas 180-200 g animals were used in the other experiments. Fed animals were provided a regular Purina Rat Chow* diet. Starved rats previously fed a diet of Purina Rat Chow* were deprived of all food for 96 hours prior to study. The fed animals used in the iron absorption experiment were deprived of food for 2 hours prior to study in order to clear the upper gastrointestinal tract. Water was allowed all animals *ad libitum*.

Blood volumes were measured in fed and in starved rats by a method previously described(8). Iron absorption, plasma iron(9)

* Iron content 275 p.p.m.