Sex-related Differences in Tumor Progression Associated with Altered Lymphocyte Circulation

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ABSTRACT

Male and female ACI rats were inoculated with the syngeneic H-4-II-E hepatoma, and the natural history of the tumor, histopathology, and lymphocyte migration were studied. The tumor formed a s.c. mass in all 16 males and in 22 of the 26 females given injections. In the males, tumors progressed, and all animals died with the mean survival time of 54 days. Complete tumor regression was observed in all but two females. In the females, there was prominent lymphocytic infiltration of the tumor, while males had no cellular reaction at the tumor site. The regional lymph nodes in males usually contained metastases and were nonreactive. The female lymph nodes did not contain metastases but contained many lymphocytes within the peripheral sinus and sinusoids. Six male-female pairs were castrated before tumor inoculation. Castration had no effect on the natural history or the histology of the tumor.

Comparing seven normal control male-female littermate pairs, there were no differences in lymphocyte accumulation in the lymph nodes 22 hr following injection of 51Cr-labeled syngeneic lymphocytes. In seven tumor-bearing male-female littermate pairs, there was a significant decrease in lymphocyte migration to the lymph nodes (p < 0.001) in tumor-bearing males as compared to that in both their female littermates and control males. Depressed lymphocyte circulation in the males was associated with rapid progression of tumors resulting in the death of the animals. Unimpaired lymphocyte mobilization in the tumor-bearing females was associated with complete regression in most animals.

INTRODUCTION

The exact role played by the immune system in the surveillance and regression of tumors has been the subject of lively debate (10, 12). The evidence for immune surveillance derives mostly from experimental studies using antigenic tumors (1). The relevance of the in vitro data to these questions seems to be uncertain (12).

It is generally accepted, however, that immune cells must make direct physical contact with the target cells. Therefore, mobilization of circulating immune cells to the tumor site and to the regional lymph node is required. However, there are surprisingly only a few studies of lymphocyte circulation in tumor-bearing animals (9) and, to our knowledge, none examining the changes in lymphocyte circulation in a syngeneic tumor model.

We have recently observed striking differences in the progression of the H-4-II-E hepatoma in male and female ACI rats. In the present paper, we describe the natural history of this transplanted tumor in male and female recipients and suggest that the observed differences in tumor progression relate to the different abilities of male and female tumor-bearing animals to mobilize circulating lymphocytes to the peripheral lymph nodes.

MATERIALS AND METHODS

Materials

Experimental Animals. Inbred ACI rats (AXC9935 or Irish) originated from the laboratory of Curtis and Dunning at the Columbia University Institute for Cancer Research in 1926. The animals are susceptible to estrogen-induced tumors and exhibit a high incidence of urinary tract and genital abnormalities (2, 11). The ACI rats used in the studies reported here were obtained from Laboratory Supply Co., Indianapolis, Ind.

The animals were housed in metal wire cages in an air-conditioned room and were fed a commercial diet and water freely. The males and females were always separated.

Experimental Tumor. The experimental tumor used was an H-4-II-E hepatoma. The H-4-II-E cell line was derived originally from the Reuber H35 hepatoma (14) which was induced in male ACI rats following the feeding of N-2-fluorenyldiacetamide. The primary cell culture was subcultured 5 times and passed to female ACI rats, giving rise to a transplantable tumor and a second cell culture (H-4-II-E). This second culture was then passed through female ACI rats and was subcultured and colony cloned to give rise to the H-4-II-E cell line. These cells have been passaged alternatively between culture and animal to maintain optimal clonogenic characteristics, thus preventing adaptation to either in vivo or in vitro environments. The tumor is a poorly differentiated hepatoma, which has retained its function of bile pigment secretion. The in vivo and in vitro properties of the H-4-II-E tumor have been described in detail by Evans et al. (5, 6).

The tumor cell suspension used in this study was prepared from a tumor-bearing male ACI rat supplied to us by Dr. C. J. Kovacs. The s.c. tumor mass measuring 3 x 3 x 2 cm was removed and finely minced. The cells were grown in vitro in monolayers at 37°C using a minimal essential medium containing 20% fetal bovine serum. They were passaged 4 times and then harvested, aliquoted, and preserved in liquid nitrogen. For each subsequent experiment, one or more vials of cells were thawed and grown in vitro for 10 to 14 days, passaged once, and then harvested for in vivo use. This was done in order to prevent in vivo changes in lymphocyte circulation in a syngeneic tumor model.

Received August 25, 1980; accepted March 5, 1981.

1 Supported by American Cancer Society Grant PDT118 and NIH National Institute of General Medical Sciences Grant 18674.
2 To whom requests for reprints should be addressed.

First received August 25, 1980; accepted March 5, 1981.

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vitro selection of variants, which might differ from the original tumor or from one experiment to the next.

Methods

Experimental Groups. The animals were divided into 4 experimental groups: group A, to study the natural history and the histopathological features of the tumor in vivo; Group B, to study the effects of castration on tumor growth; Group C, to study lymphocyte migration in normal male-female littermates; and Group D, to study lymphocyte traffic in tumor-bearing male-female littermates.

Tumor Inoculation. In all studies, the H-4-II-E cells were harvested from culture, and 1 x 10^6 cells were suspended in 0.1 ml Eagle’s medium. This inoculum was injected s.c. into the left hind footpad for all studies, with the exception of 5 male and 5 female Group A animals that received s.c. injections of this inoculum on the back.

Group A: Natural History and Assessment of Tumor Growth and Spread. Group A consisted of 16 male and 26 female ACI rats inoculated s.c. with tumors at approximately 9 to 11 weeks of age. The growth and spread of the tumor were observed during the lifetime of 6 male and 17 female animals. After the tumor had first appeared, the superficial tumor mass was measured with calipers, and photographs were taken at regular intervals to assess the tumor size. At death, the animals were autopsied. Gross and microscopic studies were done on the tumor injection site; the popliteal, inguinal, iliac, axillary, and cervical lymph nodes; and all thoracic and abdominal internal organs.

Ten males and 9 females were sacrificed between 18 and 40 days after inoculation, and complete autopsies as above were done to assess growth, spread, and host responses to the tumor in this period.

Group B: Castration Effects. Twelve animals, 6 male-female pairs aged 9 to 11 weeks, were castrated. In the females, a median vertical lower laparotomy incision was made, and the ovaries and the fallopian tubes were dissected, ligated, and removed.

In the male, a longitudinal incision was made over the perineum. The testicles were dissected, the vascular pedicle and the vas deferens were ligated, and then the testicles were resected. All animals had uneventful recoveries from the surgery and then were inoculated with tumor 2 days after the operation. The natural history and histopathology of the tumor were observed.

Groups C and D: Lymphocyte Migration Experiments. Group C consisted of 14 control animals (7 male-female littermate pairs). Group D also consisted of 14 animals (7 male-female littermate pairs). Lymphocyte migration experiments were carried out 27 ± 2 (S.E.) days following inoculation of the H-4-II-E hepatoma. Both males and females in Group D had visible tumors at injection sites.

Lymphocyte Collection and Labeling. Popliteal, inguinal, axillary, brachial, iliac, mesenteric, and cervical lymph nodes were carefully dissected from donor animals maintained under light ether anesthesia. The removed nodes were rinsed in 0.9% NaCl solution, blotted, and placed in a glass Petri dish immersed in an ice bath and containing Roswell Park Memorial Institute Medium 1640 [Grand Island Biological Co., Grand Island, N. Y.; special preparation with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and NaHCO_3, 0.85 g/liter] fetal bovine serum, L-glutamine, and penicillin-streptomycin. Subsequently, the lymphocytes were teased out of the nodes using a scalpel and forceps. The contents of the Petri dish were filtered through a fine mesh screen into a second glass Petri dish. The suspension then was distributed evenly into test tubes and centrifuged for 10 min at 2000 rpm. After centrifugation, the cells were resuspended in 1 ml of medium and combined into one test tube. Medium was then added, and a total cell count was determined using a hemocytometer.

The suspension was again centrifuged and resuspended to a final concentration of approximately 2 x 10^6 cells/ml. At this time, 100 µCi ^51Cr per ml were added, and the suspension was incubated for 45 min at 37°. Following this incubation, the cells were washed 3 times with medium. At the time of the third wash, a second cell count was obtained, and the cell viability was assessed using trypan blue. Following the third wash, the cells were resuspended in 0.9% NaCl solution. A 0.05-ml aliquot was set aside to be used as a standard.

Lymphocyte Migration. The recipient rat was anesthetized with ether, and 1 to 1.5 x 10^6 labeled lymphocytes were injected into the tail vein. The rat was allowed to recover and was placed in a cage.

Twenty-two hr following the injection of labeled lymphocytes, the rats were killed with an overdose of ether and were weighed and dissected. The organs removed included the cervical, popliteal, inguinal, and iliac lymph nodes; spleen; lungs; and liver. The nodes were carefully trimmed of excess fat, rinsed in 0.9% NaCl solution, placed on aluminum foil, and kept on ice. The organs were then weighed on an analytical balance, following a standardized procedure which involved removal of excess moisture by blotting. Finally, the nodes were placed in formyl alcohol, and the amount of recovered radioactivity was counted in a gamma well counter at the appropriate window setting. The organs were subsequently studied histologically.

In all experiments using littermate female and male pairs, all animals received the tumor cells and the lymphocytes from the same inoculum. Care was taken to use the same syringe to rule out the possibility that further differences could be attributed to differences in the inocula.

RESULTS

Group A

Natural History. The natural history of tumor growth was studied in 6 males and 17 females. The tumor cell suspension was inoculated in the left footpad in 5 males and 11 females, and in the back in one male and 6 females. There was evidence of a tumor mass at the inoculation site within 14 to 25 days (18 ± 2.5) in all males and within 14 to 22 days (20 ± 4.5) in 13 of the 17 females. One tumor in the female group appeared at Day 63. The difference between these means is not significant (Student's t test, p > 0.05). Three females inoculated in the back and one inoculated in the footpad did not show any evidence of tumors at any time.

Of the 13 female animals that developed tumor masses, 10 regressed completely within 70 days after inoculation, one regressed completely within 120 days (Chart 1), and 2 had partial regression. Of the 11 animals with complete regression, no deaths occurred during the first 120 days (Chart 1). One of
Tumor Growth and Lymphocyte Migration in H-4-II-E Hepatoma

Chart 1. Ordinate, cumulative mortality from cancer and footpad tumor size; abscissa, time in days after inoculation. Males died with progressively enlarging tumors. Females survived, as tumors enlarged and then regressed. Bars, S.E. Two females that showed no regression were omitted from the chart.

these animals subsequently died from sepsis at 9 months after injection with no evidence of tumor. The remainder are still alive at 14 months. The 2 female animals with only partial regression died 9 months following inoculation. Postmortem examination showed disseminated tumor.

In the males, tumors grew progressively at the inoculation site forming bulky masses, and all of these animals died within 70 days of inoculation (Chart 1). The mean survival time was 54 days. All males had metastases.

The sizes of measurable tumors in males and females are illustrated in Chart 1. The tumors in males were substantially larger than those in females. Those in males continued to enlarge over time, while the masses in females increased and then regressed to an unmeasurable point. Fig. 1, B and C, illustrates the gross appearance of a regressing tumor in a female.

Assessment of Tumor Growth and Spread. After the natural course of tumor growth in males and females was established, histological evaluation during the period of differential growth was carried out. In males from Days 18 to 40, the measurable tumor mass increased in size. In all 10 males sacrificed during this period, the tumor at the inoculation site was growing as a distinct gross mass with the histological appearance of a well-differentiated hepatoma (Fig. 2, A and C). These tumors all had the same histological appearance as did the tumor we received from Dr. Kovacs. The cells were arranged in nests surrounded by thin-walled vessels resembling a lobular pattern at low magnification. Within some of these nests, the cells were arranged in chords simulating hepatic architecture. The cells had eosinophilic granular cytoplasm. Nuclear atypism was characterized by a dense chromatin layer at the nuclear membrane, clumped chromatin, and prominent nucleoli. Mitoses were frequently seen. There was no inflammation at the interface of the tumor and s.c. tissue. Within the tumor, occasional areas of necrosis were present with a few surrounding polymorphonuclear leukocytes. Most of these areas were seen where the skin over an area of tumor had broken down. Lymphocytes were not seen in the tumor. The tumor illustrated in Fig. 2, A and C, is at 31 days after inoculation.

Of the 6 males sacrificed up to 30 days after inoculation, 4 had metastatic tumors to the left popliteal lymph nodes. Of the 4 males sacrificed between 30 and 40 days, all had metastases to the left popliteal nodes; 2 also had left iliac and inguinal node metastases, and one also had metastases to the lung.

In all 9 females sacrificed, the morphological appearance of the tumor at the inoculation site was completely different from that of male tumors. The gross mass was much smaller; on sectioning, thickened s.c. tissue was seen with areas of underlying necrosis. Microscopically, the s.c. tissues had a marked inflammatory response. Areas of necrotic tumor cells were surrounded by a mantle of granulation tissue with many lymphocytes and macrophages (Fig. 2B). In areas where viable tumor cells remained, lymphocytes were prominent and in some areas surrounded individual tumor cells (Fig. 2D). At 18 days, the granulation tissue and mixture of lymphocytes and tumor cells were more prominent than was necrosis. Day 31 was described above and is illustrated in Fig. 2, B and D. At Day 35 and later, the inflammatory response was present with increased necrosis, fibrosis, and fewer recognizable tumor
cells which looked viable. However, these tumor cells were always surrounded by lymphocytes.

In both male and female animals, the popliteal lymph nodes on the left side were larger than those on the right. In comparing the histology of popliteal nodes of males and females, prominent differences were noted. In the male nodes, metastatic tumors were seen, and the peripheral sinuses and sinusoids were relatively free of lymphoid cells. The cortices contained loosely packed follicles, and the medullae contained mixtures of lymphocytes and macrophages (Fig. 3A). Female popliteal lymph nodes had no metastatic tumors, but many lymphocytes were seen within the peripheral sinus and sinusoids. Follicles had prominent reactive-appearing lymphocytes. Large pyroninophilic blast cells were seen in the thymus-dependent areas. The medullary areas were packed with lymphocytes, macrophages, plasma cells, and large multinucleate giant cells (Fig. 3B). The spleens were not significantly different in weight between males and females, but the spleens of the females did have distinctive microscopic features. Giant histiocytes and multinucleate giant cells were seen in the red pulp of the female spleens, and the white pulp in some areas appeared depleted of lymphocytes compared to that in male spleens (Fig. 4). All of the described changes in both lymph nodes and spleens were seen in all female animals sacrificed; the severity increased from Days 18 to 40.

Group B: Castration Studies

Group B consisted of 6 male-female pairs 9 to 11 weeks old, castrated 2 days before tumor inoculation. Tumor growth and regression for each pair are illustrated in Chart 2. All males in this group developed tumors and died 32 to 49 days after inoculation. All females had initial tumor growth followed by complete regression. These females are still alive 14 months after inoculation. The males had growth at the inoculation site with the same histology as seen in normal animals. All had popliteal metastases. Four of 6 had iliac and inguinal metastases, and 2 had lung metastases.

Groups C and D: Lymphocyte Migration Experiments

These studies were carried out both in normal control (Group C) and tumor-bearing (Group D) male-female littermate pairs. In Group C, 7 pairs of male-female littermates 9 to 11 weeks old were given injections at the same time of 1 to 1.5 x 10^9 51Cr-labeled donor lymphocytes from the same batch of donor cells. The 51Cr activity present in the various lymph node groups is expressed as the percentage of the injected dose per g of tissue. The results for the cervical, popliteal, inguinal, iliac, and the total of all lymph nodes are summarized in Table 1. There was no significant difference in lymphocyte migration between males and females in this group (p < 0.8).

Group D included 7 male-female littermates. The lymphocyte migration experiments were carried out following tumor inoculation and in the presence of palpable tumors. The percentage of radioactivity present in the lymphoid organs 22 hr following injection of donor cells is also shown in Table 1. The migration of donor lymphocytes to recipient lymph nodes in the tumor-bearing males was significantly lower (p < 0.001) as compared to that in their female littermates.

The lymphocyte migration was also significantly reduced in tumor-bearing males as compared to that in control males (p
In this study, we observed significant differences in the behavior of the H-4-Il-E hepatoma between male and female ACI rats. In males, the tumors proved to be invariably fatal; in females, the histological appearance of the tumor at the inoculation site was completely different from that of the males. The s.c. tissues adjacent to the tumor showed marked cellular response. The tumor was surrounded by a thick mantle of granulation tissue with many lymphocytes and macrophages. In the males, on the other hand, there was no cellular response at the interface of the tumor and s.c. tissues, with only occasional polymorphonuclear leukocytes present. The 2 females with partial regression died 9 months after inoculation. The tumor at the inoculation site had lymphocytes within the tumor, but the metastases did not.

Comparing the lymphocyte migration between tumor-bearing male-female littermate pairs, our experiments indicate significant impairment of lymphocyte migration in the tumor-bearing males.

The importance of circulation of lymphocytes in the initiation and propagation of immune responses to conventional antigens is well documented (4, 8). Only a few studies, however, have examined the changes occurring in lymphocyte migration in tumor-bearing hosts (9). Lymphocyte migration experiments in Moloney sarcoma virus-injected mice have shown that an increased concentration of labeled cells occurs in the lymph nodes draining the site of the virus inoculation, which coincides with or precedes the onset of tumor regression (9). Although the nature of the antigenic target in Moloney sarcoma virus is not fully determined, it is most probably associated with the virus or with a virus product (12).

The nature of a target which would be antigenic in females but not in males is unclear. Since the tumor originated in a male, we considered expression of the H-Y antigen in the tumor to be a possibility (16). However, preliminary experiments carried out on tumor cells from tumor-bearing male and female animals indicate no evidence of H-Y expression. We have completed a preliminary study of transplanting male skin grafts to female recipients; 12 months following the grafting, no macroscopic or microscopic signs of rejection were noted. The details of the H-Y antigen and skin graft studies in this tumor model will be the subject of a future report. While we suspect that the benign biological behavior and unimpaired lymphocyte migration in the females is an immunological phenomenon, further studies are required to clearly establish this point.

The higher incidence and poorer prognosis of some cancers in males are also documented in other experimental systems (13) and in humans (7, 15). The cellular and molecular bases of sex-associated differences are, however, still obscure.

Our investigation indicates that depressed lymphocyte migration in the tumor-bearing males is associated with rapid regression and death, while female animals with unimpaired lymphocyte circulation will show complete regression of the tumor. Our model, therefore, may be useful for further study of immune-mediated tumor rejection and for assessing immunological impairments on tumor progression.

ACKNOWLEDGMENTS

The authors are indebted to Dr. C. J. Kovacs for supplying a tumor-bearing rat, to Deborah H. Williams for technical assistance, and to Laura Garbett and Nancy Harrison for their assistance in preparation of the manuscript.

REFERENCES

Fig. 2. Histological appearances of male and female footpad tumors 31 days following inoculation. A, tumor growing s.c. in a male. The s.c. tissues show no inflammation, the tumor-s.c. tissue interface is distinct, and the lobular appearance of the tumor can be seen. H & E, × 100. B, s.c. footpad tumor in a female. A mantle of granulation tissue with lymphocytes and macrophages surrounds necrotic tumor cells. H & E, × 100. C, cellular morphology of the tumor shown in A at higher magnification. The vasculature and chord-like arrangement of the cells in males can be seen. Mitoses are present. The cells have granular cytoplasm and nuclei with malignant features. No lymphocytes are present in the tumor. H & E, × 250. D, tumor-s.c. tissue interface in B at higher magnification. Lymphocytes surround viable-appearing tumor cells in the females. Other cells are vacuolated and necrotic. H & E, × 400.
Fig. 3. Left popliteal lymph nodes in males and females 31 days after inoculation. A, left popliteal lymph node in males. Subcapsular sinus and sinusoids are relatively free of cells. The cortex and medulla are nonreactive. B, left popliteal lymph node in females. The subcapsular sinus and sinusoids are filled with lymphocytes. The follicles are reactive, and giant cells are present. H & E, x 100.

Fig. 4. Spleens in males and females 31 days after inoculation. A, males. Distinctive red and white pulps are seen. B, females. Prominent red pulp with many macrophages and multinucleate giant cells. White pulp is decreased and appears depleted of lymphocytes. H & E, x 100.
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