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TUMOR IMMUNITY IN HAMSTERS IMMUNIZED WITH FETAL TISSUES[1]

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Hamster and mouse fetal cells were shown to contain antigen cross-reactive with simian virus 40 (SV40)-induced tumor specific transplantation antigen in stimulating a surface reactive, cytostatic (C) antibody against SV40 tumor target cells in diffusion chambers. The antibody was synthesized when 10-day, but not 14-day, irradiated fetal cells were injected into adult, syngeneic hamsters, and these animals were subsequently found to exhibit immunity to SV40 tumor cell challenge. The spectrum of fetal antigens present in hamster fetus was extended to adenovirus 31-stimulated tumors, and the experimental data afford an explanation of the variability previously noted in the status of immunity to tumor challenge in hamsters immunized with syngeneic fetal tissues. The failure of unirradiated fetal cells to induce transplantation immunity was correlated in this work with the development of embryomas or teratomas at the site of immunization. Males responded to fetal immunization better than did female animals although multiparous, pregnant hamsters developed C antibody during pregnancy, and lost it post-partum. The role of fetal antigenic expression in cancer and cancer immunology is considered in relation to these findings.

In several adult human neoplasias, fetal antigens are expressed which stimulate a specific immune response (1–3). We therefore have undertaken a search for fetal antigens in a wide spectrum of human tumors. The fundamental thesis is that tumor specific transplantation antigens (TSTAs) are proteins or other macromolecules normally present only at certain well-defined stages of early embryogenesis. Should this thesis be correct, we must assume that a large number of different antigens exist to account for the variety of antigens seen in chemically induced tumors. Why then do similar or identical antigens appear in tumor cells of different species transformed by the same virus if viral genes do not code for TSTAs? We postulate that the principle that ontogeny repeats phylogeny applies at the molecular level, and that just as the early events of embryogenesis are morphologically almost identical in all vertebrates, the molecular requirements are also very similar. Indeed it is difficult to see how selective pressures which affect adult survival could affect antigens which appear transiently in the early embryo. If early fetal antigens are similar in a variety of vertebrates, and if viruses cause transformation by affecting a specific site in the host genome, then the production of similar TSTAs in different species by the same virus would be expected.

The first and most direct test of these concepts was to attempt to immunize adult hamsters against simian virus 40 (SV40) hamster tumor cell challenge by multiple inoculations with irradiated fetal tissue homogenates of syngeneic, mid-gestation hamster or mouse fetal tissue. Challenge immunity was observed (4). Term fetal cells were ineffective, and irradiation of the inoculum was found necessary to induce cytostatic (or C) antibody and transplantation immunity to SV40 cell challenge. Failure of previously reported attempts to induce immunity to polyoma tumor challenge may be due to the use of unirradiated fetal tissues as the immunogen (5).

The findings that surface (S) antibody against SV40-stimulated surface antigen on hamster tumor cells can be detected in second pregnancy hamster serum (6), that guinea pig anti-C57BL/6

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mouse eggs contain antibodies reactive with several methylcholanthrene-induced mouse tumors (7) and that extracts from 72 different mouse tumors obtained from 18 different mouse strains react with rabbit anti-mouse fetal tissue antiserum (8) all provide additional support for the basic thesis.

Does fetal antigen immunization also provide protection against oncogenesis in animals neonatally infected with oncogenic virus such as SV40 and adenovirus 31? In this report we examine this question and also the experimental conditions for stimulating consistent transplantation immunity. Additionally, we attempt to establish more definitely the relationship of fetal antigens to TSTAs. Finally, we have sought to explain the failure of non-irradiated fetal cells to evoke transplantation immunity.

MATERIALS AND METHODS

Animals. The LVG/LAK strain of Syrian golden hamster was employed and the findings have been confirmed in the LSH/LAK strain (Lakeview Hamster Colony, Newfield, N. J.). Adult, pregnant and neonatal hamsters were handled as previously described (9–11).

Tumor cells. The F5-1 SV40 hamster tumor line was employed in the LVG hamster work and a similar SV40 tumor line was used in the LSH hamster as recorded previously (4). Both lines are free of mycoplasma and were cultured *in vitro* (10).

Viruses. The VA 45–54 strain of SV40 virus prepared in green monkey kidney cells with a titer of 10^9 plaque-forming units/0.2 ml was used to infect neonatal hamsters within 24 hr after birth (11). Adenovirus 31, strain 1315/63, passaged in primary human embryo kidney cells on five occasions was used in one set of experiments. The virus pool contained 10^6 $TCID_{50}$/0.2 ml.

Preparation of fetal tissues. Fetal tissue was prepared and irradiated (5000 R) exactly as previously described (4). Five million intact cells (trypan blue dye-excluding cells) were given at each intra-peritoneal immunization. The percentage of viability of the cell homogenate ranged from 20% to 40% in different experiments. Suitable disaggregation of 14-day gestation fetus can best be obtained following several strokes of a loose-fitting Teflon tissue homogenizer in a 13 x 100 mm tube.

Diffusion chamber assay. The *in vivo* assay for

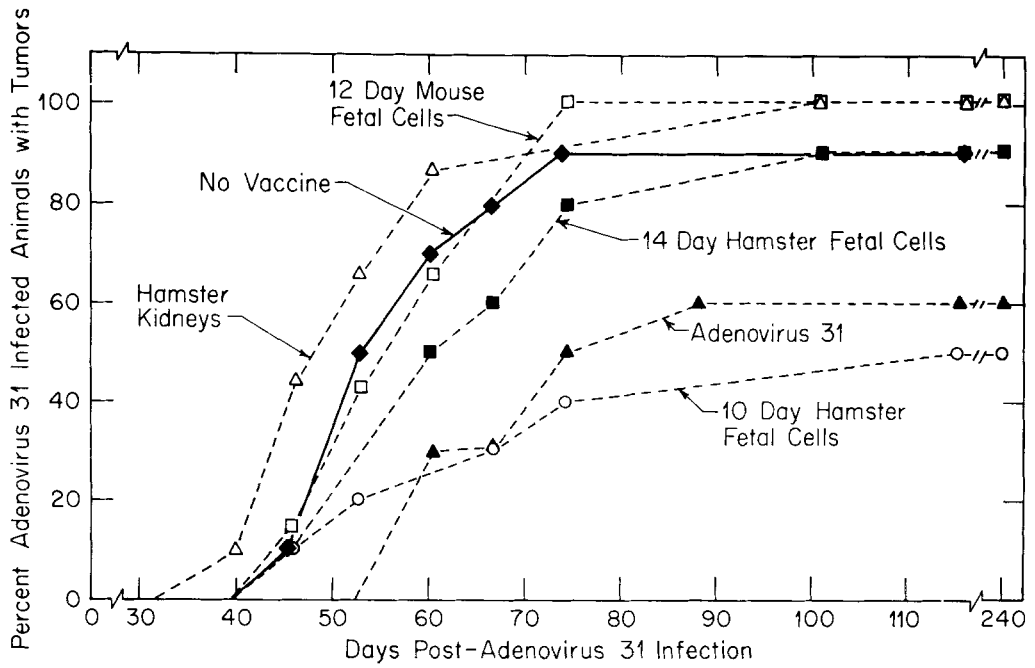

Figure 1. The effect of a single intraperitoneal immunization at 28 days with irradiated hamster or mouse fetal cells or adult hamster kidney on the tumorigenicity of adenovirus 31 in hamsters infected at birth with the virus.

C antibody employing the diffusion chamber technique is described in detail elsewhere (9, 10). Briefly, SV40 tumor cells were sealed into diffusion chambers and implanted into the peritoneal cavity of anesthetized hamsters 10 days after the last immunization. Chambers were left in place 5 days, after which cells were removed with the aid of pronase and the contents of the chambers examined for viable cells by the trypan blue dye-exclusion test. Following removal of the chambers, the hamsters were challenged with 5×10^4 SV40 tumor cells in the right subscapular space, and palpated weekly for tumors.

RESULTS

Adenovirus 31, when injected into neonates subcutaneously, produces tumors in hamsters after 40 to 50 days. Immunization of adenovirus 31-infected animals against tumorigenesis during this short latent period was attempted at 4 weeks of age with a single injection of 10^7 living 10- or 14-day irradiated hamster fetal cells. Control immunogen included 10^7 living adult hamster kidney cells from a syngeneic donor or 0.2 ml of adenovirus 31. The virus preparation was known to induce some transplantation immunity under these conditions. Figure 1 shows that immunization with 10-day fetal cells provided 40% to 50% protection against adenovirus 31 tumor whereas 14-day fetus was ineffective. Immunization with adult kidney potentiated tumor development. Each group of vaccinated hamsters contained approximately equal numbers of males and females.

In a similar experiment an attempt was made to interrupt SV40 tumorigenesis by immunization with hamster fetal tissue. SV40 tumors characteristically have a long latent period (90 to 250 days), which provides ample time for multiple immunizations with either 9- or 12- to 14-day gestation fetus homogenate. Three intraperitoneal injections of 5×10^6 irradiated fetal cells, irradiated F5-1 SV40 tumor cells or irradiated adult kidney cells were employed as immunogens. The results in Figure 2 indicate that 9-day fetal tissue was capable of inducing only modest transplantation immunity to SV40 tumor (28% protection). Again, each group of vaccinated animals was composed of about equal numbers of males and females. Interestingly, when males were compared with females a surprising result was obtained as shown in the inset to Figure 2. Clearly, females were not as responsive as males to immunization with 9-day fetal homogenate.

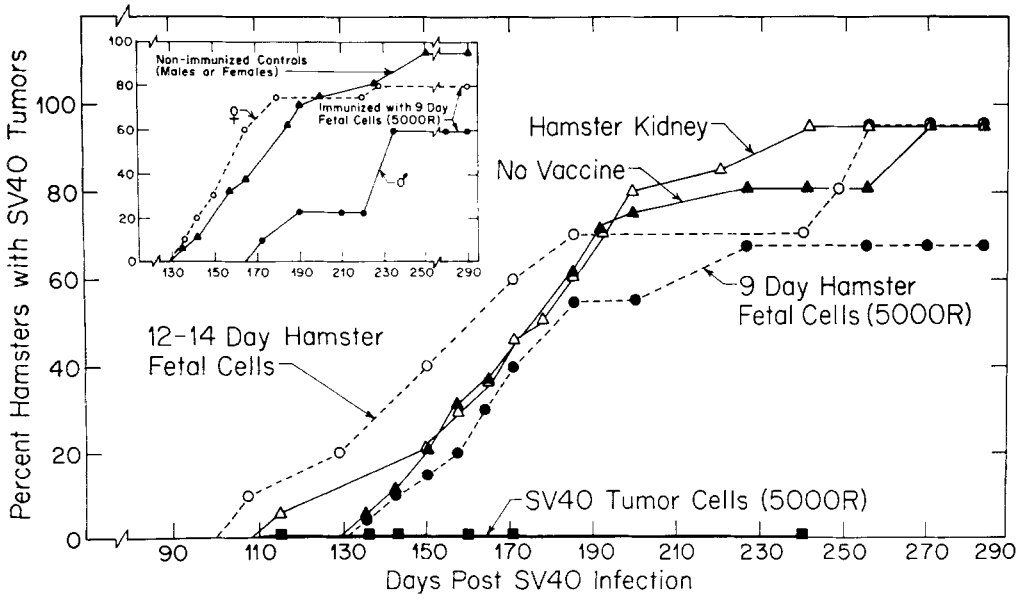

Figure 2. The effect of three intraperitoneal immunizations with irradiated hamster fetal cells, adult hamster kidney, or SV40 tumor cells on the tumorigenicity of SV40 in hamsters infected at birth with SV40 [inset]. Plot of same data in animals immunized with 9-day fetal cells with results segregated for tumor appearance in male and female vaccinees.

In another experiment the effectiveness of immunization with 10-day fetal cells in normal adult male hamsters was evaluated against varying levels of SV40 tumor cell challenge. Each animal received four injections of irradiated fetal cells at 1-week intervals beginning at 3 weeks of age. When challenged, the results given in Figure 3 were obtained. The importance of selecting a suitably low tumor challenge level to detect transplantation immunity induced by fetal immunization is evident. Complete or nearly complete protection was obtained against 1000 or 5000 tumor cells, whereas there was little protection against challenge with 10,000 to 50,000 cells.

Subsequently the response of males and females to immunization with irradiated 10-day fetal tissue was tested, and the findings again confirmed earlier observations in SV40-infected animals. Females were protected against a low cell challenge level (10^4 tumor cells) to some degree (40% protection at 40 days) but males immunized in the same way were completely protected. At high challenge levels (2×10^4 tumor cells) females were not immune to challenge (tumor incidence equal to non-immunized controls—100% whereas only 10% of males developed tumors by 40 days after challenge. These findings show that male hamsters respond quantitatively better to fetal immunization than do females, although females show some degree of transplantation immunity under proper test conditions. Neither males nor females responded to immunization with 13- to 14-day fetal material as previously demonstrated (4). In all these studies irradiated SV40 tumor cells conferred nearly absolute protection to SV40 tumor.

In previous work (4) we assumed that the failure of non-irradiated fetal cells to induce C antibody and transplantation immunity in adult animals might be explained by rapid maturation of the fetal tissues in the adult hormonal environment. This explanation was based in part on the rapid disappearance or masking of the transplantation antigen between the 10th and 12th day of gestation (4). Post-mortem examination of the injection sites of animals that had received subcutaneous preparations of non-irradiated fetal cells showed the presence of small tumors. A titration of tumor production produced by non-irradiated 10-day fetal cells administered to adult male

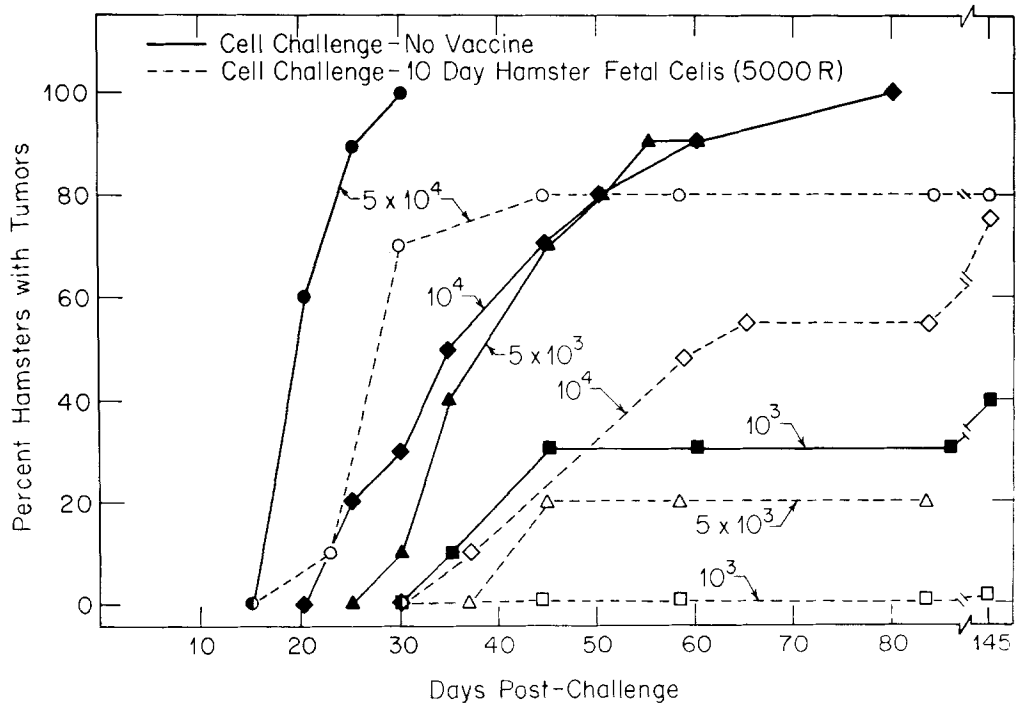

Figure 3. Titration of immunity to SV40 tumor cell challenge in adult male hamsters immunized with three intraperitoneal injections of irradiated hamster embryo cells. Numbers in figure refer to tumor cell challenge dose.

hamsters subcutaneously gave the results presented in Figure 4. Microscopic examination of these inoculation embryomas revealed a wide variety of mature tissue components foreign to the area of their localization and lacking in orderly organization (Fig. 5). These components included, among others, hair follicles, sweat glands, prominent cystic formations lined by skin, nerve tissue including nerve bundles, neurones and glial tissue, cartilage, bone and hematopoietic tissue. The morphologic pattern was like that seen in some true teratomas. These tumors could be produced by injection of 5×10^5 or more viable fetal cells from whole-embryo homogenates. Few palpable tumors (1 mm in size for minimal detection) developed in animals receiving 10^5 or fewer cells. Animals bearing these artificially-induced teratoma-like tumors did not have cytostatic or C antibody against SV40 target tumor cells (Table I) or transplantation immunity, whereas animals that were tumor-free after 140 days post-challenge with 10^5 or fewer fetal cells developed C antibody (rejectors). Surgical removal of the teratoma-like mass permitted the development of C antibody in animals successfully "cured" of the tumor, whereas surgical failures did not have C antibody as determined in the diffusion chamber assay. Among the few hamsters that developed tumors (teratomas) after inoculation of 10^5 or fewer fetal cells, only small tumors were generally observed and these were frequently detected long after challenge. These tumors rarely increased in size beyond 0.5 to 1 cm, whereas tumors induced with higher doses of fetal cells grew progressively and reached 2 to 4 cm in size.

Since Duff and Rapp (6) reported that female hamsters developed S antibodies during pregnancy, female hamsters in their third pregnancy were tested at various stages of gestation for the presence of circulating C antibody. Antibody could be detected in the peritoneum of hamsters between the ninth day of pregnancy and for 5 days post-partum (19th day from conception) as shown in Table II. Control hamsters were virgin females. Since by definition C antibody is a type of S antibody, these data are in agreement with the findings of Duff and Rapp. In our experience female hamsters are not more resistant to a given SV40 tumor challenge than are males, but no data on the tumor resistance of normal multiparous females as compared to virgin females are available.

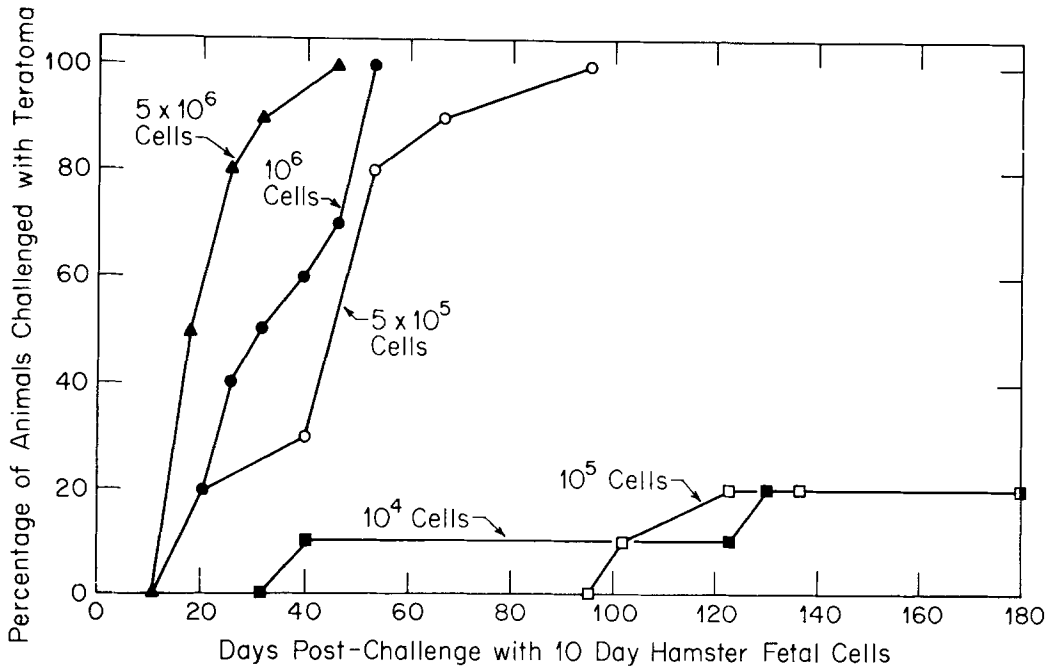

Figure 4. Production of inoculation embryomas or teratomas in adult male hamsters challenged with non-irradiated hamster fetal cells.

DISCUSSION

The results show that viral oncogenesis can be interrupted by immunization with fetal cells, thus providing additional evidence that tumor-specific transplantation antigens induced by viruses are early fetal antigens reexpressed.

Five aspects of the interruption process have been examined: a) the relationship between the level of cytostatic (or C) antibody and the success or failure of interruption of tumorigenesis; b) the effect of length of the tumor-induction latent-period on the success of immunization; c) the effect of variation in the age of the donor fetus on anti-tumor immunogenicity; 4) the basis for differences in the effectiveness of irradiated and unirradiated fetal cells as immunizing agents; and e) the effect of the sex of the recipient on response to immunization with fetal cells.

In confirmation of previous results, cytostatic antibody invariably appeared when tumorigenesis was successfully interrupted, and failed to appear when unirradiated fetal cells (which are ineffective immunogens if left in place) were used. This suggests that the monitoring of C antibody may be useful in man to indicate when a tumor has been completely eradicated or is recurring.

Adenovirus-31 tumorigenesis was interrupted by a single injection of 10- but not 14-day irradiated fetal cell preparations in agreement with our previous findings for SV40 tumor immunity (4). Fetal antigen immunization against adenovirus 31 tumors was superior to that obtained by immunization with live virus.

The latent periods for SV40 and adenovirus 31 tumorigenesis differed markedly, averaging 175 days for the former and 55 days for the latter

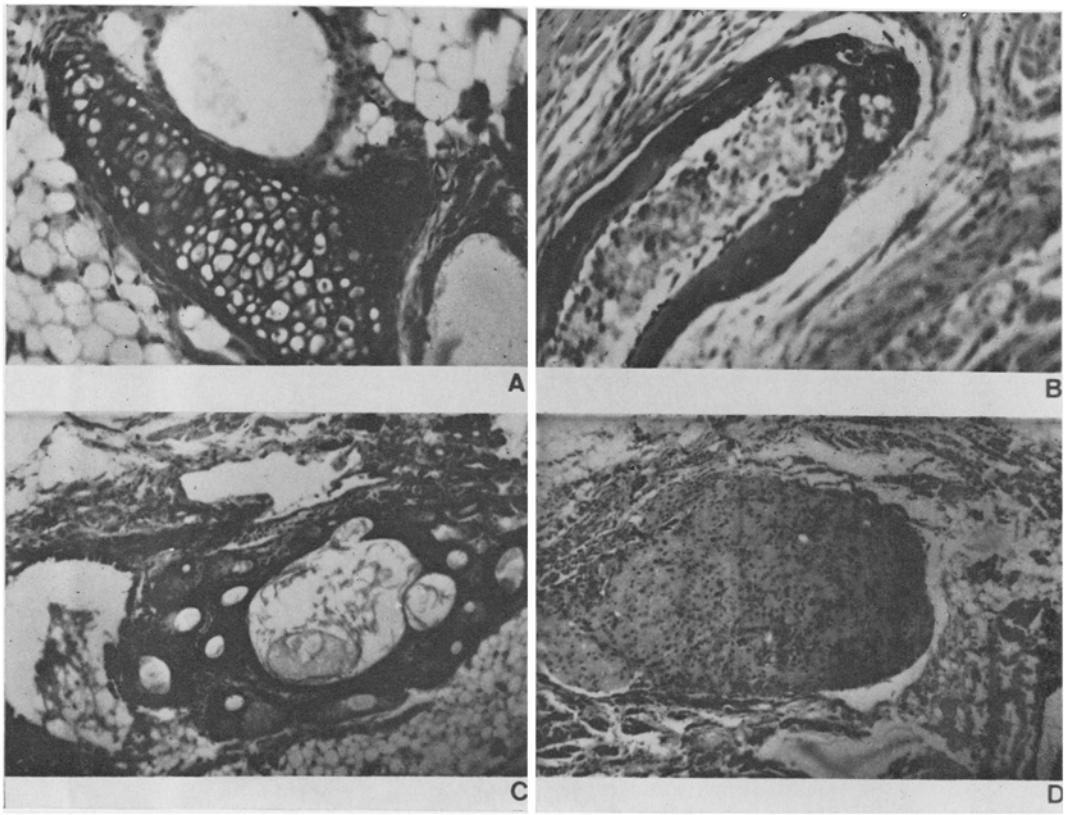

Figure 5. Photomicrographs of the injection site of adult hamster, injected with non-irradiated fetal hamster tissue. *A*, demonstrates a plate of immature, actively growing cartilage. *B*, shows a spicule of bone tissue containing fat and hematopoietic cells within its center. *C*, shows pilo-sebaceous structures which are dilated to form small cyst-like lesions. *D*, shows a collection of nerve tissue, including neurones and glial cells.

TABLE I

Cytostatic antibody in adult hamsters bearing embryomas produced by injection of fetal cells subcutaneously

Animals Status	Percentage Cytostasis Observed against SV40 Tumor Cells[a]	SV40 Tumor Challenge Immune[b]
Hamster bearing		
Large teratoma	0	−[c]
Small teratoma	0	−
Surgically cured hamsters	40	+
Surgical failures	0	−
Rejector hamsters	17	+

[a] Cytostasis determined by comparing the SV40 tumor cell yield from chambers in normal hamsters with yields from chambers in the indicated test animals. Ten or more hamsters constituted each group.

[b] Challenged with 50,000 live SV40 tumor cells subcutaneously.

[c] Similar embryoma-bearing animals challenged with 10,000 live tumor cells were resistant to the lower cell dose; males 100% protection, females 80% protection.

TABLE II

Detection of C antibody in multiparous female hamsters following conception

Days Post Conception[a]	Percentage Cytostasis[b]
Virgins[c]	0
9	0
14	14
18	21
19	23
20	0

[a] Chambers containing 15,000 viable SV40 tumor cells were placed into the peritoneal cavity 5 days before the indicated days below.

[b] Percentage cytostasis was determined by removing the chamber at 5 days implantation at the indicated dates from 10 hamsters and determining the average number of viable cells in the pregnant hamsters or mothers. This figure was compared with cell counts from chambers implanted simultaneously into virgin hamsters and counted on the same sampling date to determine the percentage of cytostasis.

[c] Virgin hamsters were the same age as the multiparous animals and cell counts over 5 days in these animals are identical to those obtained in male hosts (~550,000 viable cells).

The degree of immunity to SV40 tumorigenesis after immunization with irradiated fetal cells was less than with adenovirus 31. This may be due to differences in immunosensitivity to the antigens, to the immunization schedules relative to the length of the latent period, to the age of the hosts when the tumors appeared, and certainly to differences in antigenic (fetal) recognition in females.

Nine- or 10-day irradiated fetal cells were effective immunogens while 14-day cells were not This suggests that a change in tissue antigen composition occurs at this period in development and that the effective antigens are phased out. Detailed studies of the molecular changes associated with this *mid-fetal transition* are in progress. Since many different sets of proteins may develop temporarily during differentiation, those that appear in other adult tumors may occur at other early stages of development.

Fetal cells were originally irradiated to prevent their differentiation in the adult host and also to prevent embryomas or teratomas from developing. In this paper we confirm that the latter occurred when unirradiated preparations were used. Teratoma- or embryoma-bearing animals possessed neither circulating C antibody nor tumor transplantation immunity under the conditions of test, and in this respect were strikingly similar to the virus-induced tumors themselves.

A marked sex difference was seen in the effectiveness of irradiated fetal cell immunization in interrupting SV40 tumorigenesis, females being much less responsive. In this and previous studies the results cannot be attributed to a sex-linked histoincompatibility, such as the H-Y locus on the mouse Y chromosome, which is responsible for the rejection of male skin grafts by females of the same strain. The H-Y locus is unique in that it lacks alleles and is absent in females (12). The reasons why such a locus was not involved here are: a) males, not females, were more responsive to fetal antigens; b) females responded to fetal antigens, but to a lesser degree than males; c) in virus-induced tumors the tumor immunity observed was not complicated by the sex of the tumor (e.g., was of a different sex from that of the bearer) since the tumors were autochthonous; d) females developed C antibody to SV40 tumor to a degree comparable to that of males; and e) immunization of males with adult female tissue did not evoke antibody against SV40 tumor cells or tumor transplantation immunity.

While males appear to respond better to fetal antigens than do females, in nature only females are normally exposed to them, and then only during pregnancy. The implications of the observed female response are profound, and suggest that females may recognize fetal antigens with a rapid antibody response, but exhibit only a weak cell-mediated response. This may be the result of a postulated maturational control mechanism evolved to control responsiveness to the fetal homograft. The mechanism may be analogous to the well-documented, hormonally induced cell surface modifications observed to occur rapidly in several tissues (13). Sex differences in the incidence of human cancer are well known (14) and are partially attributed to hormonal differences. Of special interest are differences in cancer incidence between childbearing and childless women when the incidence in non-reproductive organs is compared. The excess incidence of tumors of the colon reported in nuns (15) suggests that natural exposure to fetal antigens may be protective.

Prehn (16) has suggested that tumors and embryo tissue might share common antigens not found in differentiated cells. He demonstrated a slight suppression of transplanted methylcholanthrene-induced tumor growth in mice preimmunized with live embryo cells when compared with animals immunized with tumor transplants. Immunization tumors and embryomas were surgically removed before challenge, a procedure which would be expected, from the results reported here, to yield both C antibodies and transplantation immunity. Ting's failure to detect TSTA immunity to polyoma tumor challenge in mice immunization with unirradiated living mouse fetal cells is in agreement with our findings of no C antibody in hamsters injected with unirradiated hamster fetal cells (5).

Since, as reported here, immunity to fetal antigens interferes with the formation of autochthonous tumors induced by viruses, it is now important to determine whether similar immunization prevents the appearance of a variety of spontaneous tumors in several strains of experimental animals.

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