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Prevention of Simian Virus 40 Tumors by Hamster Fetal Tissue: Influence of Parity Status of Donor Females on Immunogenicity of Fetal Tissue and on Immune Cell Cytotoxicity

(primiparous and multiparous hamsters/lymph node cells/peritoneal exudate cells)

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ABSTRACT **Fetal tissue from primiparous hamsters prevented simian virus 40 (SV40) tumorigenesis in male hamsters, whereas fetal tissue from multiparous hamsters did not. The parity status of normal (uninoculated) hamsters also influenced the cytotoxicity of their lymphoid cells against tumor cells. Lymph node cells from nonpregnant primiparous and multiparous animals were cytotoxic in microcytotoxicity tests against SV40, polyoma, and adenovirus 7 tumor cells, but were not active against control BHK cells. Lymph node cells from virgin female donors were inactive. Peritoneal exudate cells from these donors reacted in similar fashion against SV40 tumor cells *in vitro* and in adoptive transfer tests *in vivo*. However, the cytotoxicity of peritoneal exudate cells from multiparous hamsters was greatly reduced during pregnancy, a time when noncytotoxic humoral antibody reactive with surface antigen of SV40 tumor cells is present. This humoral antibody is not detected during first pregnancy, and peritoneal exudate cells obtained from pregnant primiparous hamsters demonstrated a high degree of cytotoxicity.**

Many reports have described antigens that are shared by normal fetal cells and tumor cells (reviewed in ref. 1), and some described protection against tumor formation in hosts inoculated with fetal tissues (2–9). In addition, normal pregnant multiparous hamsters develop antibody against surface antigens of simian virus 40 (SV40) hamster tumor cells (5, 8, 10), and serum obtained from guinea pigs immunized with unfertilized mouse eggs is cytotoxic for SV40-transformed mouse cells (11).

Inoculation of adult male hamsters with irradiated hamster, mouse, or human fetal tissue produces immunity to challenge by SV40 tumor cells and can induce cytostatic antibody formation (4–6, 8). Adenovirus 31 and SV40 tumorigenesis could be interrupted in males by inoculation of hamster fetal tissue administered to hamsters infected at birth before the appearance of tumors (5, 8). In addition, SV40 tumorigenesis could be interrupted in about 50% of hamsters by immunization with irradiated human embryo kidney (6).

Prehn (2) noted that prior immunization with fragments of isogeneic mouse embryos inhibited growth of syngeneic tumor transplants. Hanna *et al.* (7) reported that immunization with mouse fetal tissues suppressed growth of cells infected with Rauscher leukemia virus and plasma cell tumors. Brawn (12) reported that multiparous mice possessed cytotoxic lymph node cells that destroyed several methylcholanthrene tumors *in vitro* and that soluble fetal extracts desensitized cytotoxic effector cells.

In this report, we extend the initial observations (5) that hamster fetal tissue could delay the appearance of, and reduce the final incidence of, SV40-induced tumors. Further, we show the differential effect obtained with embryonic tissue derived from primiparous and multiparous females. Also, we studied cell-mediated immunity to SV40, adenovirus, and polyoma tumors, using lymphoid elements from normal multiparous and virgin female hamsters.

MATERIALS AND METHODS

Hamsters. LVG/LAK, Syrian golden hamsters, less than 24-hr old, were inoculated subcutaneously into the interscapular space with $10^{7.5}$ plaque-forming units of SV40, and at weaning were segregated by sex and randomized. Immunization was by intraperitoneal inoculation of test vaccines before the appearance of tumors. *Normal embryos* were harvested when 9–10 or 14 days of age. A finely minced preparation was made, washed with minimum essential medium (MEM), and finally aspirated through a 17-gauge needle. The final suspension was adjusted to contain 1 embryo equivalent per 1 or 2 ml, held in an ice bath, and irradiated in a cesium source at 5000 R delivered in 28 min. One embryo equivalent was inoculated per hamster at each of three weekly intervals beginning 5–6 weeks after administration of SV40 at birth.

Microcytotoxicity Procedure. The SV40 hamster tumor cell line, F5-1 (13), the adenovirus 7 tumor line, Pinckney (14), and the polyoma-transformed hamster line, TT109 (Flow Lab.), used as target cells were cultured in medium 199 containing 10% calf serum (heat inactivated). Microcytotoxicity tests were performed according to Brawn (12, 15), with several additional modifications (16). Target cells were grown overnight in suspension culture in Waymouth's medium (GIBCO) with 10% fetal-calf serum, enriched by addition of 1 ml of 100 mM sodium pyruvate (Microbiological Ass.), 1 ml of nonessential aminoacid solution, and 14.6 mg of L-glutamine per 100 ml of medium, and buffered to pH 7.2–7.4 with $NaHCO_3$. The target cells were washed twice before they were suspended in Waymouth's medium without serum for experiments with lymphoid cells.

Animals that served as effector cell donors were stimulated intraperitoneally with 1 ml of 1% oyster glycogen initially and again 24 hr later, 1 hr before harvesting. At harvest of

Abbreviation: SV40, simian virus 40.

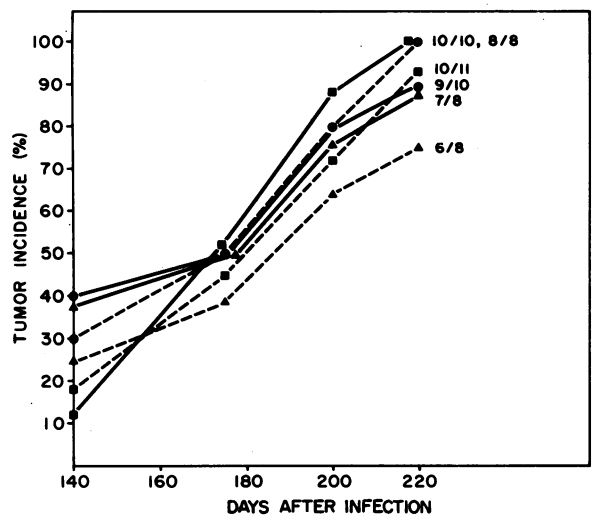

FIG. 1. The lack of immunization against SV40 tumorigenesis with hamster-fetal tissues derived from multiparous females. ●, controls; ■, 9-day fetus; ▲, 14-day fetus; - - -, male; ——, female.

peritoneal exudate cells, animals were injected with 15 ml of warm medium plus heparin (0.5 units/ml), and the fluid was aseptically collected by paracentesis with a 16-gauge needle. After the peritoneal exudate cells were washed twice with medium, they were incubated for 2–3 hr at 37° in a plastic petri plate for removal of adherent macrophages. The cells in the supernatant fluid were collected and suspended in serum-free medium to a concentration of 10^7 cells per ml.

For lymph node cell suspensions, cervical, inguinal, and mesenteric lymph nodes were removed aseptically and pressed gently through stainless steel wire mesh, washed three times with 10 ml of enriched Waymouth's medium, and suspended to a concentration of 10^7 cells per ml in serum-free medium.

Tumor cells in 0.5 ml of medium (3750 or 7500 cells; >95% viable by dye-exclusion test) were placed in a screw-cap plastic tube with 0.5 ml of peritoneal exudate or lymph node cells (5×10^6 cells), incubated for 4–5 hr in a 37° shaker-incubator, and suspended by repeated pipetting. 0.1 ml was placed in each of eight wells of a microtest tissue-culture plate (Falcon Plastics). Peritoneal exudate and lymph node cells were also "plated" in the absence of tumor cells. The plates were incubated for 2 hr, after which 1 ml of Waymouth's medium with 20% heat-inactivated fetal-calf serum was added to each well. The plates were then incubated for 2 days at 37°, fixed with methanol, stained with crystal violet, and washed. The number of attached cells was determined by visual count under 100× magnification.

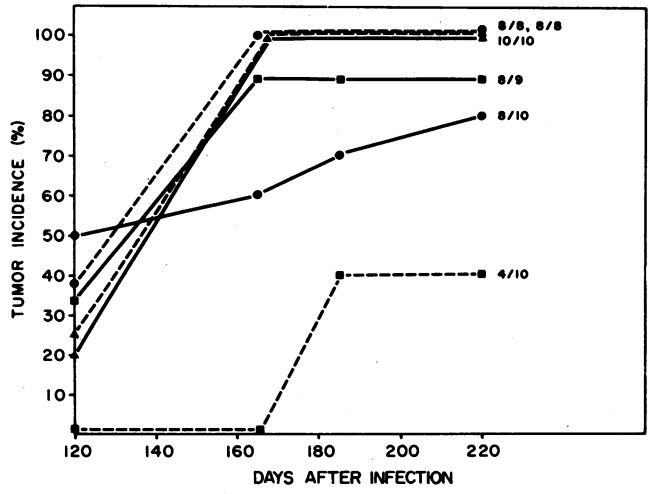

FIG. 2. The effect of immunization with hamster-fetal tissues derived from primiparous females on SV40 tumorigenesis. Symbols: same as in Fig. 1.

RESULTS

In Vivo Immunization Studies. Figs. 1 and 2 compare the results of two studies in which either multiparous or primiparous females were the source of fetal tissue. There was no significant delay (Fig. 1) in SV40 tumorigenesis in either male or female recipients of 9- or 14-day fetal tissue deriving from multiparous females, and the final tumor incidence was essentially unaffected. However, when primiparous females served as the source of fetal tissue (Fig. 2), males inoculated with 10-day fetal tissue responded with both a delay in the appearance of the first tumor and a significantly reduced incidence of tumors; females showed no such response. The differences noted were reproducible.

In both studies, normal hamsters subcutaneously inoculated with fetal tissues did not develop embryomas, indicating that adequate irradiation procedures for the fetal vaccines were used.

In Vitro Cytotoxicity Tests. Brawn (12, 15) reported that multiparous mice possessed cytotoxic lymph node cells that destroyed several methylcholanthrene tumors *in vitro*. Soluble components from fetal-mouse tissues in midgestation desensitized *specifically* immune lymph node cells, preventing cytotoxicity against tumor cells.

In our studies, normal multiparous (4 times pregnant) hamsters and age-matched virgin females were examined for cytotoxic cells. The results (Table 1) indicate reproducible and significant cytotoxicity against SV40 hamster tumor target

TABLE 1. *Cytotoxicity of peritoneal exudate cells from multiparous and age-matched virgin hamsters against SV40 hamster tumor target cells*

Target cells	Parity state	No. cells per well ± *SE**	% Cytotoxicity	*P*
SV40 tumor cells	Virgin	26 ± 3.6	—	—
	Multiparous	10 ± 2.2	62	<0.01
	Virgin	18 ± 1.8	—	—
	Multiparous	7 ± 1.8	62	<0.001
	Virgin	190 ± 24.3	—	—
	Multiparous	106 ± 16.8	44	<0.02
	Virgin	209 ± 20.0	—	—
	Multiparous	101 ± 14.2	52	<0.001
	SV40 Immune	104 ± 26.4	50	<0.01
	SV40 Immune	123 ± 25.6	41	<0.050
BHK-21 cells "normal cells"	Virgin	86 ± 7.6	—	—
	Multiparous	113 ± 6.9	0	

—, Control value.

* Standard error: results are average counts of 20 wells.

cells but not against "normal" cells with peritoneal exudate cells from normal primiparous or multiparous females. Peritoneal exudate cells from virgin female hamsters were active only after immunization with SV40 on three weekly occasions as adults. Peritoneal exudate cells from normal virgin females were not cytotoxic; the results of cell counts with them served as the 100% viability control for all the calculations.

The results shown in Table 2 with lymph node cells from primiparous and multiparous hamsters compare favorably with those with peritoneal exudate cells. The activity was directed against SV40, adenovirus 7, and polyoma tumor cells. Lymph node cells from normal virgin females were not cytotoxic. It has been noted that lymph node cells were sometimes less reactive than peritoneal exudate cells. Lymph node cells from virgin females immunized with SV40 were specifically cytotoxic (37–50%) against SV40 tumor cells, but were inactive against polyoma tumor cells.

In Vivo Cytotoxicity (Adoptive Transfer) Tests. Peritoneal exudate cells were collected from several female donors in different states of parity, washed without removal of adherent macrophages, standardized, mixed with SV40 tumor cells, and incubated in Waymouth's medium for 30 min at 37°. The cells were gently resuspended; a sample was counted and injected subcutaneously in the subscapular space of 4- to 6-week-old normal male hamsters. Each hamster received a mixture of 10^4 target tumor cells and 10^6 peritoneal exudate cells. Animals were palpated weekly for tumors. The results (Fig. 3) indicate that 10-day *pregnant* primiparous as well as nonpregnant multiparous donors possessed peritoneal exudate cells cytotoxic for SV40 tumor cells, whereas virgin donors did not. However, the cytotoxicity of peritoneal exudate cells of the multiparous females was absent during pregnancy. One is reminded that multiparous hamsters, *while pregnant*, develop cell-surface antibody (5, 8, 10), and this antibody might be responsible for the observed blocking of the cytotoxicity of peritoneal exudate cells. *Primiparous*, 10-day pregnant hamsters do *not* have detectable titers of this antibody, and, as noted in Fig. 3, their peritoneal exudate cells were as cytotoxic as those obtained from animals immune to SV40 tumors.

TABLE 2. *Cytotoxicity of lymph node cells from primiparous, multiparous, and virgin hamsters against SV40, polyoma, and adenovirus hamster tumor cells*

Target cells	Parity state	No. cells per well ± *SE**	% Cytotoxicity	*P*†
	Primiparous lymph node cells			
SV40	Virgin	166 ± 10.4	—	—
	Primiparous	108 ± 11.4	35	<0.001
Adenovirus 7	Virgin	24.4 ± 2.6	—	—
	Primiparous	10.0 ± 1.9	58	<0.001
Polyoma	Virgin	47 ± 3.2	—	—
	Primiparous	20 ± 2.8	42	<0.001
BHK-21	Virgin	204 ± 19.9	—	—
	Primiparous	251 ± 21.9	0	
	Multiparous lymph node cells‡			
SV40	Virgin	160 ± 12.0	—	—
	Multiparous	87 ± 6.3	45	<0.001
Adenovirus 7	Virgin	24.5 ± 3.7	—	—
	Multiparous	9.6 ± 1.7	61	<0.01
Polyoma	Virgin	38 ± 8.0	—	—
	Multiparous	14 ± 2.7	63	<0.02
BHK-21	Virgin	140 ± 27.0	—	—
	Multiparous	161 ± 7.0	0	

—, Control value.
* Standard error: 20 wells counted and averaged per value.
† Probability determined by Student's *t*-test.
‡ 3–4 times pregnant.

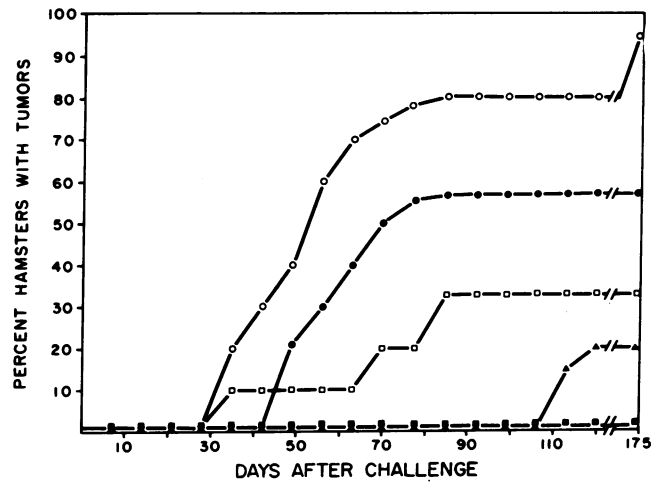

FIG. 3. SV40 tumor development in male hamsters challenged subcutaneously with 10^4 SV40 tumor cells mixed with 10^6 peritoneal exudate cells from hamsters in various states of parity. ○, Virgins; ●, 10-day pregnant multiparous; □, nonpregnant multiparous; ▲, 10-day pregnant primiparous; ■, SV40.

DISCUSSION

Our results on the prevention of SV40 tumorigenesis extend previous studies establishing that males are more responsive than females to inoculation with irradiated 9- to 10-day fetal tissue and that irradiated 14-day fetal tissue is ineffective in both sexes. Furthermore, they indicate the advantage of embryonic cells from primiparous females.

Multiparous animals possessed lymph node and peritoneal exudate cells cytotoxic for hamster tumor cells *in vitro*, while virgin animals did not. This finding confirms the principle of autosensitization during pregnancy reported by Brawn (12, 15), extending it to three tumors transformed by oncogenic DNA viruses. Also, primiparous as well as multiparous hamsters possessed cytotoxic peritoneal exudate cells capable of protecting normal male hamsters against SV40 tumor cell challenge in adoptive transfer tests, supporting *in vitro* results. Direct challenge of the *donors* of peritoneal exudate cells resulted, however, in equivalent tumor development in all donors, and one might postulate that blocking factors are present coordinately with cytotoxic cells, impairing the toxicity of sensitized cells in the donors. The data in Fig. 3 would support such a hypothesis, since the cytotoxic peritoneal exudate cells from multiparous hamsters had greatly reduced activity *during* pregnancy when it is known that noncytotoxic antibody, reactive with the cell surface of SV40 tumors, is present (5, 8, 10). Enhancement of SV40 tumor takes coordinate with increased numbers of pregnancies has been noted recently in other experiments.

In addition, normal females sensitized to fetal tissue developed such cytostatic antibody (as did males), but showed no capacity to reject tumors, either autochthonous or transplanted (5, 8). These immunologic findings possibly have evolutionary significance in females, representing a two-way protective mechanism in the maternal–fetal relationship. The cytostatic antibody developed normally during pregnancy may have a dual function: (*a*) to protect the fetus from cell-mediated rejection, and (*b*) to prevent "metastases" at ectopic sites from accidentally released fetal cells. Perhaps females have evolved this humoral mechanism more efficiently in order to suppress its ability to respond *functionally* with cellular immunity to fetal homograft, even though active peritoneal exudate and lymph node cells can be demonstrated to be present normally (Table 2 and Fig. 3). Thus, males but not females would be free to respond protectively when inoculated with appropriate fetal tissue.

Why then do females respond to inoculation of irradiated SV40 tumor cells with tumor resistance if the protective (surface) tumor transplantation antigen of SV40 tumor cells is the same antigen present in 10-day fetal tissue? Perhaps the embryonic cells contain many antigenic sites involved in stimulating the humoral antibody response, which blocks cell-mediated immunity, while the tumor cells may contain masked or fewer of these sites and stimulate the cell-mediated response more selectively. Also, fetal cells may respond to hormonal stimuli in adult female tissue and undergo membrane maturation and masking of the antigen inducing tumor resistance. In recent studies we have demonstrated that females develop weaker transplantation resistance than males when immunized with SV40 tumor cells (9).

The humoral response of the *donor* female may play an important role in determining whether the tissue of her fetus can effectively immunize recipient hamsters. During early embryogenesis in *primiparous* females, the autosensitive antibody response may be quite low, presumably due to inexperience of the females with the antigen; thus, 10-day fetal tissue would be immunogenic. During late term, the 14-day fetal tissue would be ineffective if the primary antibody response resulted in blocking. Although the antibody has not been detected *in circulation* in 14-day *primiparous* females, a sufficient amount might be absorbed on fetal tissue *in utero*. In *multiparous* females where humoral antibody is detected, an anamnestic response could ensure production of sufficient antibody to cover embryonic sites even at day 9, thereby explaining the lack of protective immunization with such tissue. Some support for this concept was obtained in preliminary experiments. Females splenectomized to reduce the humoral response react protectively to fetal vaccine (9). In addition, quantitative cytofluorograph measurements of the amount of antibody on fetal cells indicate a 4-fold increase in antibody on fetal cells in multiple pregnancy as compared to that on primiparous fetuses (9).

Alternatively, the important immunizing antigen might not be expressed during late embryogenesis, or if expressed, could be masked by other than immunologic means. Mild trypsinization of ineffective fetal tissue may remove either cellular components or antigen–antibody complexes that block the sites important for development of cell-mediated immunity. Then even females might be expected to react with tumor resistance after inoculation of fetal tissue. This seems to be the case in our initial study previously reported (17).

The observed phenomenon of specific resistance stimulated by tumors and the broad, crossreacting response of active fetal tissues may have an explanation in that different repressed genetic sites may become activated by different oncogenic agents. This could result in tumors that vary from each other in antigenic reexpression, but that crossreact with fetal tissue since, in the latter, the various sites would be expressed normally, Neither the data reported here nor in previous publications contest the idea that oncogenic stimuli may, in themselves, induce new antigens of a "nonfetal" type (18), but they do stress that antigenic sites important for tumor (and fetus?) survival or rejection are expressed during normal embryogenesis and during tumorigenesis.

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