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EFFECT OF IMMUNIZATION WITH FETAL CELLS ON ADENOVIRUS-12 ONCOGENESIS

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Abstract: The effect of immunization with hamster fetal cells on the tumor induction by adnovirus type 12 was studied by *in vivo* and *in vitro*. The immunization with 10-day old fetal cells showed a recognizable inhibition on the tumor induction by adenovirus type 12. The inhibition was observed only in males but not in females. For the inhibition, immnization with 10^7 or more cells was required. The immunization with same dose of 12-day-old fetal cells were ineffective. The inoculation of the spleen cells from hamsters immunized with unirradiated fetal cells strongly inhibited the adenovirus-12 onocogenesis. Membrane immunofluorescent test, however, failed to demonstrate the fetal antigens in any of adnovirus-12-induced tumor cells, SV40-induced tumor cells and cells from spontaneous hamster lymphoma.

Evidence has been accumulating in the literature that fetal cells have antigens that cross-react with specific antigens in tumor cells (1, 2, 3). Among these fetal antigens, transplantation type antigens are particularly interesting, because, if they really exist, the immunization with fetal tissues might be very useful for the immunotherapy of cancers. Recently COGGIN *et al.* have reported that immunization of adult hamsters with irradiated fetal hamster or mouse cells produced strong immunity to the challenge by SV40-induced tumor cells and simultaneously induced cytostatic antibody formation (4). From these experimental results and the suggestion that only a limited number of proteins could be coded for by the viral genome (5), they postulated that during the process of oncogenesis there is a derepression of fetal genes, resulting in the *de novo* synthesis of antigenic substances detectable as transplantation antigens of tumors. They also postulated that fetal antigens might be the same as tumor-specific transplantation antigens (TSTAs) of SV40-tumors and, probably of many other tumors. Why do similar or identical antigens appear in tumor cells of different species transformed by the same virus of which viral genes do not code for TSTA, and how could we explain the peculiar fact that immunization with mid-gestation hamster fetal cells could confer the resistance to various tumors induced by different oncogenic viruses (4, 6, 7)? As for these questions, COGGIN *et al.* explained that, just as the early events of embryogenesis are morphologically almost identical in all

vertebrates, the molecular requirements are also very similar; thus TSTAs induced by the same virus are identical regardless of animal species. Their report on the inhibition of SV 40 tumorigenesis in hamsters by immunization with human fetal tissue homogenates would support the supposition (8).

The cross-reactivity between the TSTAs of tumors induced by the highly oncogenic group A and the weakly oncogenic group B adenovirus types has been reported (9). Nucleic acid hybridization experiments, however, demonstrated that the complementarity is specific for both DNA of the inducing virus type and other types belonging to the same adnovirus group (10). These findings suggest that the TSTAs found to be common to tumors induced by adenoviruses of groups A and B would not be coded for by the virus but their appearance would rather be due to the derepression of the fetal genes or other cellular genes. Thus, the genetic background of TSTAs of adenovirus tumors is still obscure, and the reports on the fetal antigens or their relationship with TSTAs in adenovirus tumors are very limited (7). As the first step to study the existence and the characteristics of fetal antigens in adenovirus tumors, the author investigated the effect of immunization with hamster fetal cells on adenovirus-12 (Ad-12) oncogenesis.

MATERIALS AND METHODS

Animals: Experiments were carried out with our closed colony of randomly mated Syrian hamsters (*Mesocricetus auratus*) (11). Within 24 hours after birth, 0.1 ml of virus was injected into the dorsal subcutaneous tissue of newborn hamsters. Immunizations with embryonic tissue were performed at 18 days of age by intraperitoneal injection. At three weeks of age the hamsters were weaned and segregated according to sex. All animals were examined three times a week for tumors until five months of age, when the experiments were terminated. In each experiment, nonimmnized animals and animals immunized with adult kidney cells were included as controls.

Virus: Adnovirus type 12 (Huie) was cultured in HeLa cells. The cultures were harvested when cytopatic effect was maximum by three cycles of freezing and thawing. The cell debris was removed by low speed centrifugation and the supernatant was used for experiments. The virus titer was determined in HeLa cells as described by YABE *et al.* (12). The virus used in the present studies contained $10^{2\ 0\text{-}2.5}$ tissue culture infective doses (TCID 50/0.1ml).

Preparation of Fetal Cells: Primiparous mothers were used to obtain embryos, since it has been reported that there was no significant delay in SV40 tumorigenesis in hamsters immunized with fetal tissue from multiparous females (13). Generally a male hamster was placed in a box containing one virgin female in the late evening. Females were observed daily early in the morning for sperms in the vaginal smear. The day of detection of sperms in mated females was taken as day 0 of embryo development, and the approximate age

of the embryo was calculated accordingly. Pregnant hamsters were killed, and the embryos were removed from the uterine sacs and soaked in cold Hank's balanced salt solution (HBSS). The fetuses were aspirated gently through 21-gauge needles into a 10-ml syringe. The preparations of dispersed embryos were further drawn through a 23-gauge needles and transferred into a large centrifuge tube containing cold HBSS to spin down at 500 rpm for five min. x-irradiation was performed in plastic tubes at a distance of 45 cm from the source of the Toshiba KXL-19 x-ray machine equipped with 0.5 mm alminum and 0.5 mm copper filters. The unit was set at 200 kVp, 25 mA, delivering 213 R/min for 23 min 27 sec to perform 5000-R irradiation. Viable cells were counted after irradiation by use of the trypan blue dye exclusion test. The unstained cells usually constituted 25 to 30% of the total. The cell concentration was adjusted by further dilution with HBSS.

Kidney Cells: Retired male breeders were perfused gently with warm phosphate-buffered saline (PBS) through the left ventricle until the kidney became grossly white, then the kidneys were removed, trimmed and washed in HBSS. After thorough mincing under aspetic conditions, the cells were suspended in HBSS and passed gently through a sterile Teflon homogenizer to obtain disaggregated cells. The procedures for viable cell count and x-irradiation were similar to those used for the preparation of embryo cells.

Tumor Cells: Hamsters bearing Ad-12 tumors were killed by cranial dislocation and tumors were removed aseptically. Tumor cell suspension was prepared in the same way as the kidney cell suspension except for perfusion with PBS.

Spleen Cells: Two-month old male hamsters were immunized with irradiated or unirradiated 1×10^7 hamster fetal cells. Animals were killed by dislocation of cervical spine, usually 11 days after immunization. Spleens were dissected free of the surrounding tissue under aspectic conditions, placed in a small amount of medium 199 in a loose-fitting Teflon homogenizer and gently ground by hand. The suspensions were allowed to stand in a refrigerator for one hour. The supernatant was collected and washed with medium 199 and then resuspended in the same fluid. Further purification of lymphocytes was not done, since HEWETSON *et al.* reported that the purified lymphocytes did not show any greater inhibitory activity as did comparable leukocyte samples (14).

Cell Lines: Three lines of tissue culture cells were used in the membrane immunofluorescent test. HT-4, Ad-12 transformed hamster cell line (15), and HT, SV40 induced hamster tumor cell line, were supplied by Dr. C. HAMADA, Kyoto University and Dr. ODA of our Medical School. These cells were grown in Eagle's minimum essential medium (MEM) containing 10% heated fetal calf serum (BDH Chemicals, England). They were previously shown to contain Ad-12 S antigen (16) and SV40 S antigen (17). MLP-1 cells, originated from ascites of a hamster bearing transplantable spontaneous hamster lymphoma of our laboratory (11), were grown as suspension in the medium RPMI 1640 containing 15% heated fetal calf serum (18).

Antisera: Four- to five-week-old Syrian hamsters were immunized by eight

intraperitoneal injections with 2×10^7 viable, x-irradiated and 10-day fetal cells. Another six kinds of antisera were produced in the hamster and the procedures of immunization were listed in Table 4. Sera were collected two weeks after the last injection by cardiac puncture and kept at −80°C. All sera were inactivated at 56°C for 30 minutes befor use.

Antisera and conjugates were routinely absorbed prior to use with an equal volume of packed homogenated lung, liver, kidney and spleen of hamsters or 100 mg of hamster liver powder for one hr at 37°C. Two times of absorptions were sufficient to reduce the non-specific staining.

Immunofluorescent Staining: Live cells were stained in suspension or directly on monolayer by a modification of the method of MÖLLER (19). Cells were washed once with PBS and incubated with 0.1 ml of undiluted antiserum for one hr at 37°C. The cells were then washed three times with PBS and incubated with 0.1 ml of 1 : 10 dilution of rabbit anti-hamster globulin con-

TABLE 1 EFFECT OF IMMUNIZATION WITH X-IRRADIATED

Immunogen		Experimental group			
Cells	Dose	Sex	Tumor incidence	(%)	Average latent period[1]
10 D.G.[2]	1×10^7	♂	6/14	(42.8)	51.3 days
Fetal cells		♀	8/8	(100)	40.3
10 D.G.	1×10^7	♂	16/22	(72.8)	63.9
Fetal cells		♀	N.D.[3]		
10 D.G.	1.2×10^6	♂	20/20	(100)	40.6
Fetal cells		♀	17/19	(89.5)	34.4
10 D.G.	3×10^6	♂	10/14	(71.4)	41.5
Fetal cells		♀	14/16	(87.5)	44.3
12 D.G.	1×10^7	♂	12/12	(100)	51.3
Fetal cells		♀	10/10	(100)	39.0
Adult	1×10^7	♂	15/19	(79.0)	58.0
kidney cells		♀	15/15	(100)	48.3
Adenovirus-	1×10^7	♂	9/15	(60.0)	53.5
12 tumor cells		♀	N.D.		

1) Average days from virus inoculation to appearance of palpable tumors.
2) D.G., days of gestation.

jugated with fluorescein isocynate (Miles laboratories, Kankakee). After three washings with PBS, cells were suspended in a minimal volume of grycerol-PBS mixture (1 : 1), and mounted on a glass slide. Examination was done for circumferential staining of cells by darkground ultraviolet fluorescence microscopy with a colorless barrier filter.

RESULTS

1. *Effect of X-irradiated Fetal Cell Immunization*

The influence of fetal cell immunization on Ad-12 oncogenesis was explored by comparing the tumor incidence and the incubation period for tumor development among immunized and non-immunized hamsters. As shown in Table 1 and Figure 1, duplicated experiments with 1×10^7 x-irradiated 10-day fetal cells showed a slight reduction in tumor incidence (15.5 and 7.2%) and remarkable delay in tumor appearance (9.9 and 24.9 days) in male hamsters. In females, however, no response to immunization was observed. Hereafter, unless otherwise stated, the comparison between the experimental group and the control group is done in males. The possibility of non-specfic response of hosts was excluded by experiments using immunization with adult

FETAL CELLS ON ADENOVIRUS-12 TUMORIGENESIS

Sex	Control group			% Reduction of tumor incidence
	Tumor incidence	(%)	Average latent period	
♂	14/24	(58.3)	41.4 days	15.5
♀	10/13	(76.9)	46.1	−23.3
♂	12/15	(80.0)	39.0	7.2
♀	15/15	(100)	39.8	——
♂	16/16	(100)	40.6	0
♀	10/10	(100)	39.6	10.5
♂	15/20	(75.0)	52.0	3.6
♀	22/22	(100)	49.1	12.5
♂	12/15	(80.0)	39.0	−20.0
♀	15/15	(100)	39.8	0
♂	6/12	(50.5)	51.9	−28.5
♀	11/12	(91.8)	41.0	− 8.2
♂	11/12	(91.8)	48.9	31.8
♀	16/16	(100)	36.9	——

3) N.D., not done.

kidney cells (Table 1). In the experiments with smaller cell doses, there was no detectable effect of immunization.

2. *Effect of Unirradiated Fetal Cells*

Experiments with unirradiated fetal cells done in triplicate showed

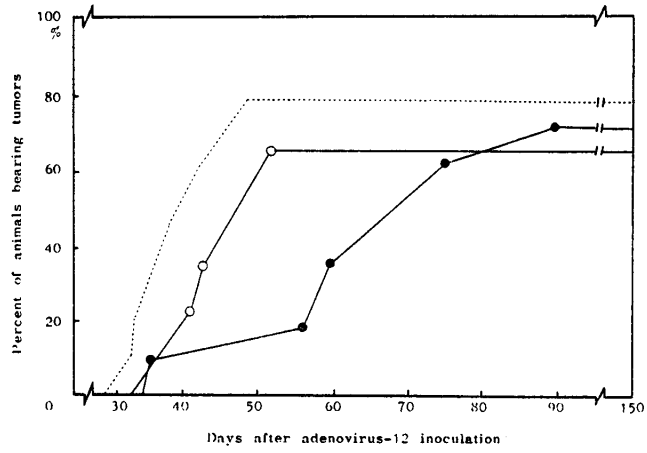

Fig. 1. The effect of immunization with hamster fetal cells on the tumorigenesity of adenovirus-12 in male hamsters. The virus was inoculated at birth and immunization was done once at 17 days of age by single peritoneal injection of the fetal cells. Broken line, control; Solid line with filled circles, animals immunized with irradiated cells; Solid line with open circles, animals immunized with unirradiated cells.

TABLE 2 EFFECT OF IMMUNIZATION WITH UNIRRADIATED

Immunogen		Experimental group			
Cells	Dose	Sex	Tumor incidence	(%)	Average latent period[1]
10D.G.[2]	1×10^7	♂	8/16	(50.0)	66.0 days
Fetal cells		♀	20/20	(100)	36.4
10 D.G.	1×10^7	♂	9/15	(60.0)	55.0
Fetal cells		♀	8/8	(100)	50.1
10 D.G.	1×10^7	♂	12/18	(66.7)	44.7
Fetal cells		♀	8/8	(100)	40.0
10 D.G.	1×10^7	♂	6/10	(60.0)	44.0
Fetal cells	$(\times2)$[3]	♀	6/8	(75.0)	35.6
11 D.G.	1×10^7	♂	8/14	(57.1)	53.5
Fetal cells		♀	8/8	(100)	46.0

1) Average days from virus inoculation to appearance of palpable tumor
2) D.G., days of gestation

variable effects of immunization; 25% and 13.3% inhibition in the tumor incidence in two experiments, and no effect in one experiment (Table 2). But the latent period for tumor development was prolonged in all immunized groups. Another group of hamsters were immunized twice with unirradiated

fetal cells at a two-week interval. In this group, tumor incidence and the latent period were the same as those of the group with a single immunization. Almost all of the hamsters with tumors and all of the hamsters without tumors had a large teratoma-like tumor. Microscopic examinations of these artificially induced teratomas disclosed prominent cyst formation, nerve tissue, hematopoietic tissue, hair follicles and sweat glands. This morphologic appearance was similar to that reported by COGGIN *et al.* (7).

3. *Age of Embryos and Immunizing Effect*

The effect of immunization with unirradiated, 11-days embryos appeared to be slightly inferior to that obtained by immunization with 10-day embryos (Table 2). The immunization with x-irradiated, 12-day embryos showed no suppressive effect on the tumor development (Table 1).

4. *Effect of Sensitized Spleen Cells*

Since immune lymphocytes are primarily responsible for the rejection of solid antigenic tumors and the presence of humoral cytotoxic antibodies seems to be less important for their rejection, spleen cells from hamsters immunized with fetal cells were tested *in vivo* for their inhibitory effect on Ad-12 oncogenesis.

FETAL CELLS ON ADENOVIRUS-12 TUMORIGENESIS

Sex	Control group			% Reduction of tumor incidence
	Tumor incidence	(%)	Average latent period	
♂	15/20	(75.0)	52.4	25.0
♀	22/22	(100)	49.1	0
♂	14/24	(58.3)	41.4	− 1.7
♀	10/13	(76.9)	46.1	−28.1
♂	12/15	(80.0)	39.0	13.3
♀	15/15	(100)	39.8	0
♂	14/24	(58.3)	41.4	− 1.7
♀	10/13	(76.9)	46.1	1.9
♂	15/20	(75.0)	52.4	17.9
♀	22/22	(100)	49.1	0

3) Immunized twice at two weeks' interval

In the first experiment, newborn hamsters were given subcutaneous injection of the virus, and inoculation of the sensitized spleen cells was performed on the 18th day. The results are summarized in Table 3. Injection of spleen cells from hamsters immunized with unirradiated, 10-day fetal cells reduced the tumor incidence in males (63.2%). The tumor incidence

TABLE 3 EFFECT OF SENSITIZED SPLEEN

Exp. No.	Spleen cell donor: Sensitized with[3]	Dose	Experimental group: Sex	Tumor incidence	(%)	Average latent period[4]
I[1]	10 D.G.[5] Fetal cells (un-irradiated)	5×10^7	♂	6/21	(28.6)	48.5 days
			♀	18/18	(100)	46.3
	11 D.G. Fetal cells (un-irradiated)	5×10^7	♂	8/8	(100)	48.3
			♀	10/10	(100)	39.8
	10 D.G. Fetal cells (X-irradiated) X2[6]	5×10^7	♂	15/15	(100)	48.3
			♀	N.D.[7]		
	Normal adult spleen cells	5×10^7	♂	9/15	(60.0)	55.7
			♀	7/7	(100)	43.7
II[2]	10 D.G. Fetal cells (un-irradiated)	5×10^7	♂	0/15	(0)	—
			♀	0/10	(0)	—
	10 D.G. Fetal cells (un-irradiated)	1×10^7	♂	6/15	(40.0)	72.2[8]
			♀	14/18	(77.8)	73.5[8]

1) Spleen cells were inoculated intraperitoneally at 18 days of age into hamsters which received adenovirus 12 at birth.
2) Spleen cells were inoculated intraperitoneally at 11 days of age into hamsters which received adenovirus 12 at 7 days of age.
3) 1×10^7 viable cells were used for sensitization.

remained unchanged in female hamsters inoculated with the same dose of sensitized spleen cells. Spleen cells from hamsters immunized twice with x-irradiated, 10-day fetal cells or with un-irradiated 11-day fetal cells showed no suppressive effects on Ad-12 tumor growth.

In the second experiment, the virus was given intraperitoneally to one week-old hamsters and the spleen cells from the embryo-immunized hamsters were injected intraperitoneally four days after virus inoculation. In this case, no tumor developed not only in the male but also in the female hamsters when 5×10^7 sensitized spleen cells were inoculated into the recipients. Inoculation of 1.0×10^7 sensitized spleen cells also showed 30% reduction in the tumor incidence.

As seen in the control hamsters in Table 3, sex difference was observed in the tumor incidence among the hamsters receiving 0.1ml of Ad-12 within 24 hours after birth, whereas no sex difference was noted among the hamsters receiving the same dose of the virus intraperitoneally one week after birth.

5. *Fetal Antigens on the Cell Surface by Immunofluorescent Test*

Sera from hamsters immunized with hamster fetal cells by seven dif-

CELLS ON ADENOVIRUS-12 TUMORIGENESIS

Sex	Control group: Tumor incidence (%)	Control group: Average latent period	% Reduction of tumor indicence
♂	11/12 (91.8)	48.6 days	63.2
♀	16/16 (100)	36.7	0
♂	11/12 (91.8)	48.6	− 8.2
♀	16/16 (100)	36.7	0
♂	11/17 (64.7)	40.5	−35.3
♀	26/29 (89.7)	40.7	——
♂	11/17 (64.7)	40.5	4.7
♀	26/29 (89.7)	40.7	−10.3
♂	14/20 (70.0)	68.2[8)]	70.0
♀	16/21 (76.2)	73.5[8)]	76.2
♂	14/20 (70.0)	68.2[8)]	30.0
♀	16/21 (76.2)	73.5[8)]	1.6

4) Average days from virus inoculation to appearance of palpable tumors
5) D.G., days of gestation
6) Sensitized twice at 2-week interval
7) N.D., not done
8) Average days from virus inoculation to death by tumors.

ferent procedures were used for the detection of fetal antigens by membrane immunofluorescent technique. As seen in Table 4, specific membrane fluorescence was not observed in any of the tumor cells tested.

TABLE 4 ATTEMPT TO DETECT FETAL ANTIGENS BY MEMBRANE IMMUNOFLUORESCENT TECHNIQUE

Antisera	Immunization procedure	Membrane immunofluorescence: HT-4[1)]	HT[2)]	MLP-1[3)]
1	Single immunization with 2×10^7 x-irradiated 10-days fetal cells	—	—	—
2-3	Seven immunizations with 2×10^7 x-irradiated 10-day fetal cells at one-week intervals	—	—	—
4-6	Eight immunizations with 2×10^7 x-irradiated 10-day fetal cells at one-week intervals	—	—	—
8-10	Sera from pregnant hamsters at 10 days of gestation on their second pregnancy	—	—	—
11-14	Sera from pregnant hamsters at 10 days of gestation on their first pregnancy	—	—	—
16-18	Single immunization with 2×10^7 unirradiated fetal cells into virgin hamsters	—	—	—
20-22	Single immunization with 2×10^7 unirradiated fetal cells and removal of resulting subcutaneous embryoma	—	—	—

1) Adenovirus-12-transformed hamster cell line 2) SV40-induced hamster tumor cell line
3) Spontaneous hamster lymphoma cell line

DISCUSSION

In the present experiments, immunization with fetal cells could confer weak protection against the Ad-12 tumor growth. Injection of the spleen cells from hamsters immunized with fetal cells also gave a strong inhibitory effect on the Ad-12 oncogenesis. These results would mean the expression of the fetal antigens on the surface of Ad-12 tumor cells.

For the inhibition of Ad-12 oncogenesis, the age of embryos used for immunization was an important factor; 10-day fetal cells were effective, but 12-day cells were not. This suggests that the fetal antigens which cross-react with Ad-12 tumor cells disappear after this period in the embryogenesis. The age of embryos, which is effective for inhibition of tumors, is 12 days for SV40 (4), 14 days for polyoma virus (6), and 10 days for Ad-12. This difference of the critical ages of embryo for effective immunogen would suggest that different sets of fetal antigens might be expressed temporarily during embryogenesis.

Large doses of fetal cells, 1×10^7 or more, must be used to obtain enough immunity, and there was no detectable effect of immunization when the cell doses injected were below 3×10^6. Therefore, it is supposed that fetal antigens on the membrane of fetal cells are not so dense as the normal tissue antigens.

A marked sex difference was observed in the effect of fetal cells immunization in interrupting Ad-12 oncogenesis. In female hamsters, reduction of tumor incidence was obtained only when the virus was inoculated at 7 days of age and large doses of spleen cells (5×10^7) from embryo-immunized hamsters were injected four days after the virus inoculation. Other procedures of immunization could not influence the tumor incidence in female hamsters. TING *et al.* claimed that the sex difference in the responsiveness to the fetal cell immunization is one of the important characteristics of the fetal antigens (20). In Ad-12 system, however, the tumor incidence itself is also complicated by the sex of the hosts (21). The fact that the sex difference of the tumor incidence in Ad-12 oncogenesis could be neutralized by thymectomy at one week of age (22) or, as shown in the present study, by delay of the virus inoculation to one week of age would suggest that the sex difference of the tumor incidence is attributable to the sex-dependent difference of immunological maturity. It is likely, therefore, that the sex difference of the effectiveness of fetal cell immunization in Ad-12 oncogenesis is due to the difference in the numbers of the tumor cells developing in the recipients at the time of immunization.

Various techniques *in vitro* have been applied to detect the surface antigens

of the tumor and normal cells. Several authors demonstrated the surface antigens of tumor cells by membrane immunofluorescent test using the antisera to tumor cells (23-25), and in some cases they also found the same antigens in the fetal cells (24, 25). On the contrary, TING *et al.* found some difficulty in producing antisera to fetal antigens (20). Seven kinds of immunization with fetal cells were performed in the present study, and the resulting sera did not react with the the tumor cells tested. However, Ad-12 oncogenesis was inhibited by the adoptive transfer of spleen cells from hamsters immunized with fetal cells. Accordingly, these evidences indicate that the fetal antigens may stimulate the production of cellular antibody rather easily than that of humoral one.

The highly oncogenic group A adenoviruses and weakly oncogenic group B adnoviruses all induce a common TSTA (9). Hamster fetal cells possess a cross-reactive antigen to Ad-31 and Ad-12 hamster tumor cells as reported by COGGIN *et al.* (7) and as in the present study. These facts support the postulation that fetal antigens might be the same as TSTA of tumors induced by adnoviruses. On the other hand, it has been also reported that the TSTAs of tumors induced by other than adnoviruses are virus-specific (26). The antiserum to fetal tissue reacts with various tumor cells, but fetal tissue fails to remove the reactivity of the antisera to tumor antigens (20). Furthermore, anti-embryo sera react with SV40 "cryptic" transformants which contain SV40 genome but do not express its function, while the anti-SV40 tumor sera do not react (27). From these facts, it can be assumed that viral neoplasms may possess at least two types of transplantation antigens; one type may be coded directly by the viruses and the other may be controlled by the function of derepressed fetal genes.

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