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Rejection by syngeneic mice of cell variants obtained by mutagenesis of a malignant teratocarcinoma cell line

(tumor immunology)

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ABSTRACT **Cells from the malignant teratocarcinoma line PCC4.aza1 were treated with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Fifty-five clones were isolated from the surviving cells. Twelve clones are unable to form tumors in the syngeneic 129/Sv mice. However, these "tum$^-$" clones form tumors as readily as the original cells when they are injected into irradiated mice. Moreover, they stimulate the production of immune memory cells, which protect the injected animals and confer resistance by adoptive transfer. The tum$^-$ clones are therefore unable to generate tumors in syngeneic mice because they elicit an immune rejection response.**

A number of transplantable teratocarcinoma tumors have been obtained by Stevens in the inbred mouse strain 129/Sv (1). These progressive tumors contain a chaotic array of ectodermal, endodermal, and mesodermal differentiated tissues. The differentiated areas are separated by regions of undifferentiated, actively multiplying cells. These cells, which are called embryonal carcinoma cells, are malignant and pluripotent: the injection of a single embryonal carcinoma cell can result in the appearance of a tumor containing all the differentiated tissues found in teratomas (2).

It is possible to put cells from ascitic teratocarcinomas in culture and to derive stable permanent cell lines from the embryonal carcinoma cells. Some of these lines are malignant and pluripotent (3–7). The pluripotency suggests that there may be an equivalence between the cells present in early embryos and those of teratocarcinoma lines. Further support for this hypothesis comes from the finding that teratocarcinoma cells elicit in syngeneic mice an antibody response against the "F9" surface antigen, an antigen present on early embryos and absent on the differentiated tissues of adult mice with the exception of the germ line (8). Moreover, like early embryo cells, teratocarcinoma pluripotent cells do not carry H-2 antigens (9, 10).

The work described below has been performed entirely with line PCC4.aza1. This is an azaguanine-resistant clone derived from the permanent line PCC4 (6). When PCC4.aza1 cells are injected into 129/Sv mice, they give rise to large progressive tumors in a few weeks. These tumors contain numerous regions of various differentiated tissues, as found in primary teratocarcinomas.

As a means of investigating the cell determination processes of early embryos, we thought that it would be useful to obtain a number of PCC4.aza1 variants that have lost some specific differentiation potentialities. We treated a culture of PCC4.aza1 with a mutagen. The surviving population was cloned and the properties of a number of clones were analyzed both *in vitro* and *in vivo*. This procedure has yielded a number of variants with a decreased differentiation potential. They have been briefly described elsewhere (11).

Abbreviations: tum$^-$, not tumorigenic in syngeneic hosts; tum$^+$, tumorigenic in syngeneic hosts.

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In the course of this experiment, we noticed that a large number of the mutagen treated clones failed to produce progressive tumors in the syngeneic 129/Sv mice. Further experiments confirmed that these clones had a very much reduced tumorigenicity. We will describe here the isolation and initial characterization of these "tumorless" (tum$^-$) variants. We will show that their inability to generate progressive tumors is not due to an intrinsic growth defect, but to the fact that they trigger an immune rejection response in the syngeneic host.

MATERIALS AND METHODS

Mice. Mice from the inbred 129/Sv line were obtained from J. L. Guénet (Institut Pasteur, Paris). Unless otherwise mentioned, the mice were more than 7 and less than 12 weeks old. *Steel*/+ and +/+ mice were used indifferently.

Teratocarcinoma Cell Lines. Line PCC4.aza1 is a clonal, permanent cell line resistant to a concentration of 15 μg of azaguanine per ml (5). It was derived from line PCC4, which itself was derived from the transplantable teratocarcinoma OTT 6050 obtained by Stevens in 129/Sv mice (1). The isolation and cloning procedures of these lines have been fully described elsewhere (5). PCC4.aza1 and the clones described below were found to be free of mycoplasma.

Culture Conditions. The teratocarcinoma cell lines are cultured in Falcon "tissue culture" dishes with the Dulbecco modification of Eagle's medium supplemented with 15% fetal calf serum in an atmosphere of 12% CO_2. The cells are detached and transferred by pipetting, without EDTA or trypsin treatment.

Injection of Cells and Tumor Analysis. The cells in culture are collected in medium containing only 1% fetal calf serum. The percentage of viable cells, estimated with trypan blue, fluctuates between 75 and 90%. Unless otherwise stated, half the cells are injected subcutaneously in the lower abdominal region, then the needle is pushed into the peritoneal cavity and the other half of the cells is injected. With PCC4.aza1 this procedure produces about 100% tumors in animals injected with more than $5 \cdot 10^5$ living cells. About 90% of the tumors obtained are subcutaneous, 10% are intraperitoneal. Mice are examined every 3 days for tumor growth. Small tumors appear usually after 3 weeks (depending on the injection dose), then they grow progressively and the animals are sacrificed about 10 days later when they show large subcutaneous tumors of about 1 cm diameter or when they show acute abdominal swelling indicative of intraperitoneal tumors. Animals without any sign of tumor formation 2 months after the injection are considered negative.

Mutagenesis. Mutagenesis was performed with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine at a concentration of 3 μg/ml in Earle's medium at 37° in an atmosphere of 15% CO_2. The duration of the treatment varied from 60 to 75 min. The mutagen treated cells were allowed to multiply *in vitro* for 3 days in

order to allow any mutation to segregate. Cloning was performed by distributing a very dilute suspension of cells into a large number of wells of Linbro plates IS-FB-96 in culture medium with 30% fetal calf serum. The dilution was such that fewer than 15% of the clones could have been derived from more than one cell. After each clone had grown, it was injected at a dose of $5 \cdot 10^5$ living cells into 129/Sv mice and aliquots were frozen for further analysis. When tumors were obtained, they were analyzed histologically (5). In one of the mutagenesis experiments, we determined the efficiency of the mutagenesis by measuring the frequency of the mutants able to multiply in a medium containing 1 μg/ml of amanitin. The frequency of these mutants was about 10^{-5}. This represents at least a 40-fold increase over the level found in the control not treated with mutagen.

Adoptive Transfer. Spleens are collected on sterile steel grids, minced with needles, and pressed through the grids with sterile glass tubes. The spleen cells are collected in Eagle's medium without serum. They are washed by centrifugation in the same medium and injected in a volume of about 0.5 ml in a tail vein of a mouse given 600 rads (6 joules/kilogram) of gamma radiation less than 12 hr before. The reconstituted mouse is challenged with tumor cells 4–7 days later.

RESULTS

Clones with reduced tumorigenicity obtained by mutagenesis of PCC4.aza1

Cells from the malignant teratoma line PCC4.aza1 were incubated *in vitro* in a medium containing the mutagen *N*-methyl-*N'*-nitro-*N*- nitrosoguanidine. Between 1 and 0.1% of the initial cells survived after this treatment. A number of single cells were isolated from this population. They were allowed to multiply so as to obtain independent clones. Fifty-five clones were derived from three separate experiments. These clones were injected, each into three 129/Sv mice. For 12 of them, the ability to produce tumors in syngeneic mice was very markedly reduced. They were therefore called "tumorless" (tum$^-$), as opposed to the tumorigenic (tum$^+$) original cells. Twelve out of 55 (22%) is certainly an underestimate of the variants with a reduced tumorigenic potential, because we did not classify as tum$^-$ a number of clones having a slighter but nevertheless significant reduction of tumorigenicity.

Ten control clones were obtained, using the same procedures, from a cell population that had not been treated with the mutagen. When injected, they all produced multidifferentiated progressive tumors.

The results of the injection of some tum$^-$ clones are shown in the second and third columns of Table 1. Obviously, the tum$^-$ clones produce very few tumors under conditions where the tum$^+$ control generates a tumor in every animal. In the rare instances where a tum$^-$ variant does produce a tumor it almost invariably progresses much more slowly than the control tumors. Occasionally, a tum$^-$ variant produces a small tumor that then regresses or remains stationary.

The tum$^-$ phenotype is stable: tum$^-$ clones maintained in culture for over 50 generations remain unable to produce tumors. However, variations in the condition of the mice can lead to erratic results. Recently, a number of groups of more than 30 mice have been injected with $2 \cdot 10^6$ living tum$^-$ cells of clones 20 and 25. In some groups tumors were completely absent. In others a significant number of mice, sometimes more than 20%, acquired a progressive tumor. The health and the age of the mice seem to be important factors. Mice that are less than 5 weeks old are definitely more likely to develop a tumor than mice that are more than 12 weeks old.

Table 1. Tumors obtained after the injection of tum$^-$ variants into 129/Sv mice

Clone	Unirradiated mice		Irradiated mice	
PCC4.aza1 (control)	28/28	(29 days)	24/24	(28 days)
20	2/37	(51 days)	31/32	(24 days)
21	3/15	(74 days)	5/5	(50 days)
25	1/49	(57 days)	38/38	(28 days)
33	3/36	(49 days)	10/11	(25 days)
40	2/16	(35 days)	6/6	(31 days)
42	0/14		8/8	(28 days)
51	2/17	(41 days)	6/6	(26 days)
70	3/14	(50 days)	8/8	(26 days)
133	0/12		6/6	(29 days)

Result of the injection of tum$^-$ cells and control tum$^+$ cells. Normal adult mice (unirradiated) and mice given 600 rads of gamma radiation 3–6 hr earlier (irradiated) were injected with the same number of living teratoma cells. This number varied between $5 \cdot 10^5$ and $2 \cdot 10^6$. The table indicates the number of animals that acquired a progressive tumor over the total number of animals injected. The number of days written between parentheses represents the average time at which the mice either have a solid subcutaneous tumor of about 0.5 cm diameter or —less frequently—display acute peritoneal swelling due to an ascitic tumor. Data are pooled from all the injections performed in 1974 and 1975.

General properties of the tum$^-$ variants 20 and 25

Variants 20 and 25 have a generation time *in vitro* of about 12 hr, equal to that of PCC4.aza1. Like PCC4.aza1, they show a complete absence of density-dependent inhibition. These properties are shared by all the variants listed in Table 1, with the exception of clone 21.

To explain the lack of tumorigenicity of clones 20 and 25, we examined the possibility that they may correspond to some differentiated cell type. The tumorigenicity of teratocarcinoma transplantable tumors is indeed inversely related to their content in differentiated tissue (12), and we have observed that two teratoma-derived differentiated cell lines of myoblasts are unable to form tumors (13). However, many lines of evidence indicate that variants 20 and 25 have not evolved into differentiated cells. In culture they have retained the characteristic morphology of the tum$^+$ control. Their karyotype, as well as that of clones 70, 42, and 133, has a mode at 37 telocentrics and 2 metacentrics, like that of PCC4.aza1, whereas the karyotype of the nontumorigenic myoblasts is aneuploid (13). Moreover, the F9 antigen is present on 20 and 25, whereas it is absent from differentiated cell lines and tissues (8, 13). Finally, the rare tumors obtained from tum$^-$ variants 20, 25, 70, and 133 show multiple differentiations, indicating that these variants have remained pluripotent. This point has been confirmed with the tumors obtained in irradiated mice (see below).

tum$^-$ variants produce tumors in irradiated mice

When mice are given 600 rads of γ radiation from a cesium source before the injection of cells, the tum$^-$ variants produce tumors almost every time, as shown on the right part of Table 1. Moreover, with the exception of clone 21, these tumors grow as fast as the tumors formed by PCC4.aza1. This shows that the tum$^-$ mutants have retained the ability to grow *in vivo*. Because of the sensitivity of the immune system to irradiation, these results suggest that the failure of tum$^-$ cells to form a tumor is due to an immune response of the host.

In an experiment performed with tum$^-$ variant 25, tumors

Table 2. Injection of tum⁻ variants in irradiated 129/Sv mice previously injected with the same variant

Clone	No. with tumors/No. of animals injected	
	Immunized mice	Control mice
20	0/12	11/11
25	0/22	17/17
42	0/4	5/5
133	0/4	6/6

The immunized mice were injected with $2 \cdot 10^6$ living cells of a tum⁻ clone on day 0. On day 21, they were given 600 rads of gamma radiation and were injected 3–6 hr later with $2 \cdot 10^6$ living cells of the same tum⁻ clone. The control mice were not injected on day 0. On day 21 they were irradiated and injected like the immunized mice. The results are pooled from a number of independent experiments.

were always obtained when the mice were irradiated any time between 7 days before and the time of injection. Irradiations performed after the day of injection had a decreasing effectiveness up to the fifth day. Later irradiation did not result in the formation of tumors. Cells from clone 25 can therefore survive 5 days in the animal even though they eventually fail to form a tumor.

Presence of radioresistant memory

To test whether the rejection of the tum⁻ cells is really due to an immune response, we took advantage of the fact that immune memory cells are often relatively radioresistant (12).

Mice were injected with $2 \cdot 10^6$ cells of tum⁻ variant 20. No tumors occurred, as expected. Three weeks later these "immunized" mice together with a group of control mice were given 600 rads of γ radiation. All the mice were then injected with $2 \cdot 10^6$ cells of variant 20. None of the immunized mice acquired a tumor. All the control mice did. Experiments performed with tum⁻ variants 25, 70, 42, and 133 gave the same results, as shown in Table 2.

It appears therefore that the mice injected with a tum⁻ variant acquire a radioresistant memory against this variant.

Protection of irradiated mice by adoptive transfer of immune spleen cells

Mice were injected with $2 \cdot 10^6$ living cells of variant 25. Three weeks later, their spleen cells were collected and injected intravenously into irradiated animals. These "reconstituted" mice as well as irradiated, nonreconstituted control mice were injected 4 days later with $2 \cdot 10^6$ living cells of clone 25. No tumor appeared in the mice reconstituted with $6 \cdot 10^7$ spleen cells. Tumors appeared in all the controls. Similar results were obtained with other tum⁻ variants, as summarized in Table 3. Reconstitution with spleen cells from nonimmunized mice was much less effective. Five irradiated mice reconstituted with $5 \cdot 10^7$ normal lymphocytes were injected with clone 25. All five acquired tumors. A slight protection, noticeable by a delay in tumor appearance was observed with one out of three animals reconstituted with $2 \cdot 8 \times 10^8$ normal spleen cells.

Protection against tum⁻ variants can therefore clearly be transmitted by adoptive transfer of spleen cells from immunized animals. This confirms the presence of memory cells and strongly indicates that the rejection of tum⁻ variants is due to an immune response.

DISCUSSION

Our results show that a population of cancerous teratocarcinoma cells treated with nitrosoguanidine yields nontumoral variants

Table 3. Injection of tum⁻ variants after adoptive transfer of immune lymphocytes

Clone	No. of immune lymphocytes transferred in the reconstituted mice	No. with tumors/ No. of animals injected	
		Reconstituted mice	Control mice
25	$6 \cdot 10^7$	0/9	9/9
	$8 \cdot 10^6$	1/7	
20	$6 \cdot 10^7$	0/11	6/6
	$8 \cdot 10^6$	0/12	
	$2 \cdot 10^6$	3/4	
70	$7 \cdot 10^7$	1/5	3/3
	$9 \cdot 10^6$	1/5	

Adult 129/Sv mice were injected with $2 \cdot 10^6$ living tum⁻ cells. After 3 weeks, their spleen cells were collected and injected intravenously into 129/Sv mice (reconstituted mice) previously irradiated with 600 rads. Control mice were irradiated and not reconstituted with spleen cells. All the mice were challenged 4–7 days later with $2 \cdot 10^6$ cells of the same tum⁻ variant as that used to obtain the immune spleen cells.

with a rather high frequency. These tum⁻ variants are still able to form tumors in irradiated mice, indicating that they have kept the neoplastic properties of the original tum⁺ cells. However, they provoke a strong immune rejection process in the syngeneic 129/Sv mice.

Nitrosoguanidine is a potent mutagen and the tum⁻ phenotype is stable in culture. It is therefore tempting to consider that the tum⁻ character is determined by a mutation on the chromosomal DNA. After mutagenesis, about 0.5% of the initial cells survive and this population contains about 20% tum⁻ variants. Let us assume that all the killing as well as the production of tum⁻ phenotypes is due to random dominant mutations induced by the drug. According to the law of Poisson, this implies that the genetic target governing the tum⁻ phenotype amounts to four percent of the size of the genetic target governing cell multiplication *in vitro*. This surprisingly large relative value becomes even larger if nitrosoguanidine induces some nonspecific killing, not due to its mutagenic action.

Two questions arise immediately from the existence of tum⁻ variants. First, what is the difference between a tum⁻ cell and its tum⁺ equivalent? Second, what is the precise mechanism of rejection of the tum⁻ cells?

The difference between a tum⁻ cell and the corresponding tum⁺ could be either structural or functional. tum⁻ variants may have acquired new surface antigens causing them to be rejected. Alternatively, they may have lost hypothetical functions whereby the tum⁺ cell either avoids stimulating an immune rejection response or becomes resistant to it. Some information regarding this should emerge from a systematic study of the cross-protection patterns existing between different tum⁻ variants.

The mechanism of rejection of tumoral cells has been studied with various tests like those of lymphocyte cytotoxicity, cytostasis, stimulation of lymphocyte multiplication, and inhibition of macrophage migration (13–16). These tests are used to demonstrate *in vitro* an immune cellular response. They have been shown to be positive in syngeneic situations for a number of tumor systems (17). However, the relevance of these tests to the rejection process taking place *in vivo* has proven difficult to establish. The tum⁻ variants may provide a new possibility for a critical evaluation of some of these tests. Indeed, any test that is relevant to the rejection process that we observe *in vivo*

should be positive on the tum$^-$ cells and negative on the tum$^+$ control cells.

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