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Immunology

Tumor cell variants obtained by mutagenesis of a Lewis lung carcinoma cell line: Immune rejection by syngeneic mice

(tumor immunology)

ALINE VAN PEL*, MYRIAM GEORLETTE*, AND THIERRY BOON*

Cellular Genetics Unit, International Institute of Cellular and Molecular Pathology, Avenue Hippocrate, 74, B-1200 Bruxelles, Belgium

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ABSTRACT **It has been reported that, by mutagenesis of a malignant mouse teratocarcinoma cell line, it is possible to obtain cell variants that are incapable of forming progressive tumors in syngeneic mice. These variants, which were called "tum$^-$," are eliminated from the host by an immune rejection process. We report here that similar variant cell clones can be obtained at high frequency from a Lewis lung carcinoma cell line treated with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Syngeneic C57BL/6 mice reject these tum$^-$ clones and acquire a strong radioresistant immune protection against the immunizing clone. When the challenging tum$^-$ clone differs from the immunizing clone, a weaker radioresistant immune protection can be demonstrated with some, but not all, combinations. All the tum$^-$ clones induce a significant protection against the original Lewis lung malignant cells. These results imply that each Lewis lung tum$^-$ variant carries on its surface a singular antigen in addition to one or more weak antigens already present on the original tumor cell line. This antigenic pattern is similar to that found on teratocarcinoma tum$^-$ variants. Our results suggest that the procedure of using a mutagen in order to generate tum$^-$ variants carrying new transplantation antigens may be generally applicable to cancer cells.**

We reported previously that, by treatment of a malignant teratocarcinoma cell line with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, it is possible to obtain a number of variant clones that are incapable of forming progressive tumors in the syngeneic 129/Sv mice (1, 2). These variant clones were called "tum$^-$" (nontumorigenic) as opposed to the tum$^+$ (tumorigenic) initial malignant teratocarcinoma cells.

The tum$^-$ character of the teratocarcinoma variants is linked to a process of immune rejection. Indeed, these variants produce tumors as readily as does the tum$^+$ control in sublethally irradiated mice. They also induce the production of specific immune memory cells: when mice are injected with a tum$^-$ variant and 3 weeks later then are sublethally irradiated and challenged with the same tum$^-$ cells, few or no tumors appear. Every one of four independent tum$^-$ teratocarcinoma clones that were tested confers a strong immune protection against itself and a weaker protection against the other clones. Moreover, mice immunized with tum$^-$ cells are also partially protected against the original teratocarcinoma tum$^+$ cells. These results indicate that each tum$^-$ variant carries a singular transplantation antigen as well as a weak common antigen already present on the tum$^+$ cell.

The protection against the tum$^+$ cells was unexpected because no protection is observed in syngeneic mice injected with tum$^+$ teratocarcinoma cells killed by irradiation. We believe that the availability of tumor cell variants capable of promoting the rejection of the original tumor cells, which are too weakly antigenic to be rejected on their own, could open new possibilities in cancer immunotherapy. This has prompted us to investigate whether tum$^-$ variants can be obtained from tumor cell lines other than mouse teratocarcinoma.

Another reason for testing the generality of the tum$^-$ phenomenon was provided by the analysis of the primary cellular modifications involved in the tum$^-$ phenotype. Stable teratocarcinoma variants endowed with a potent new transplantation antigen are produced by the mutagenic treatment at a very high frequency: of the 0.1% of the initial population that survives after mutagenesis, about 20% of the cells are tum$^-$ variants. This remarkable frequency could result from a mutational process affecting a part of the genome that is by many orders of magnitude larger or more sensitive to the mutagen than are those involved in the usual metabolic mutations. On the other hand, this frequency could be due to a differentiation process—that is, a stable modification without a chromosomal mutation. If the tum$^-$ phenomenon were observed only in teratocarcinoma this would strengthen the differentiation hypothesis because teratocarcinomas differ from other tumors by their differentiation potential, which is equivalent to that of early embryonic cells (3–5).

In view of these considerations, we attempted to obtain tum$^-$ variants from Lewis lung carcinoma, a metastasizing lung adenocarcinoma that arose spontaneously in a C57BL/6 mouse (6). This tumor has neither the special differentiation properties nor the absence of H-2 antigen that characterizes mouse teratocarcinoma. Our starting material was a malignant clone that we derived from a permanent Lewis lung cell line, hereafter called 3LL. The results described below show that when Lewis lung carcinoma cells are treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine they yield, at high frequency, tum$^-$ variants that are similar to the teratocarcinoma variants.

MATERIALS AND METHODS

Mice. Our C57BL/6 inbred mice were derived from breeder animals obtained from J. L. Guénet (Institut Pasteur, Paris). The mice used in our experiments were between 12 and 16 weeks old.

Cell Line. We obtained from J. C. Leclerc a cell line derived from Lewis lung carcinoma (3LL). From this permanent cell line, we isolated *in vitro* by a limiting dilution procedure (1) a clone called LI. This clone was found to be free of mycoplasma.

Culture Conditions. The 3LL cells were cultured in Falcon "tissue culture" dishes in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, in an atmosphere of 91% air/9% CO_2. For transfers, the culture medium was re-

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

* Present address: Cellular Genetics Unit, Ludwig Institute for Cancer Research, Avenue Hippocrate, 74, U.C.L. 7459, B-1200 Bruxelles, Belgium.

placed for 3 min by Ca- and Mg-free phosphate-buffered saline supplemented with 2 mM EDTA. The cells were then detached by pipetting.

Mutagenesis. The mutagenesis and cloning procedures have been described in detail (1). The cells were incubated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine at a concentration of 3 μg/ml in Earle's medium at 37°C in 91% air/9% CO_2 for 60 min. This medium was then replaced by culture medium. After 10 days of culture, the surviving cells were cloned by distributing a dilute suspension of cells into a large number of wells containing medium supplemented with 30% fetal calf serum. Each clone was later injected into C57BL/6 mice at a dose of 3×10^5 living cells, and aliquots were frozen for further analysis.

Injection of Cells and Tumor Analysis. The cultured cells were detached by the usual EDTA treatment. They were centrifuged, resuspended in medium containing 1% fetal calf serum, and injected subcutaneously in a volume of 0.2 ml. Mice were examined every 3 days. When tumors had a diameter of about 0.5 cm, the mice were considered to be definitely positive and were sacrificed. Mice showing no signs of tumor after 2 months were considered to be negative.

Adoptive Transfer. Spleens were teased in Hanks' medium supplemented with 5% fetal calf serum. Cell suspensions were filtered through a nylon mesh gauze. The cells were centrifuged and resuspended in Eagle's medium without serum and were injected in a volume of 0.3 ml intraperitoneally into mice given 660 rads (6.6 grays) of γ radiation a few hours previously. The reconstituted mice were challenged with tumor cells 3 days later.

RESULTS

tum⁻ Clones Are Obtained by Mutagenesis of 3LL. The clonal cell line LI, derived from a permanent Lewis lung carcinoma cell line, was used throughout this study. This clone can be permanently maintained in culture. When 3×10^4 LI cells were injected subcutaneously into C57BL/6 mice, they produced progressive tumors that appeared after 10–15 days (Table 1).

A culture of LI cells was incubated *in vitro* with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine so as to obtain 0.2–1% of surviving cells able to multiply *in vitro*. The surviving population was cloned 10 days later and each clone was injected into three C57BL/6 mice. Fifty-five independent clones isolated from the mutagenized population were tested. Forty-nine of them showed decreased tumorigenicity: one or more mice per group either failed to develop a visible tumor or acquired a tumor that later regressed. These clones were called "tum⁻" (not tumorigenic) as opposed to the tum⁺ (tumorigenic) initial LI cells. Ten control clones, derived from a LI population that had not been mutagenized, were also injected into three mice. Each of these control clones produced progressive tumors in the three mice.

Table 1. Tumors obtained after injection of tum⁻ variants into C57BL/6 mice

Clone	No. mice with tumors/no. mice injected	
	Unirradiated	Irradiated
LI(tum⁺), a	64/64 (19 days)	21/21 (17 days)
LI(tum⁺), b	77/79 (23 days)	12/12 (22 days)
L12	7/58 (51 days)	9/9 (18 days)
L20	1/50 (50 days)	9/9 (20 days)
L24	6/23 (38 days)	12/12 (23 days)
L48	0/44	10/10 (26 days)
L50	1/37 (43 days)	10/10 (22 days)
L62	1/53 (49 days)	8/9 (26 days)

C57BL/6 mice ("unirradiated mice") were injected subcutaneously with 3×10^5 (a) or 3×10^4 (b) of tum⁺ control or 3×10^5 cells of tum⁻ clones. Mice given 640 rads of γ irradiation from a cesium source 1–3 hr earlier ("irradiated mice") received similar injections. The number of days shown in parentheses represents the average time after injection at which tumors of about 0.5-cm diameter were observed. Data are pooled from four experiments.

A number of tum⁻ clones regularly produced very few tumors, even at a dose 10-fold higher than the dose that allowed the tum⁺ cells to generate a tumor in almost every mouse. The results of the injection of six such clones are shown in Table 1. The few tumors that were produced by these tum⁻ clones progressed much more slowly than the control tumors. We were also able to confirm the decrease in tumorigenicity of a number of other variants. However, for most of them the tumorigenicity was less markedly decreased: the failure to produce tumors occurred only when low doses of cells were injected.

The tum⁻ variants had the same appearance *in vitro* as the tum⁺ cells and the same generation time, about 13 hr. The tum⁺ and nearly all the tum⁻ clones tested had an aneuploid karyotype with a mode at ≈105 chromosomes.

Some 3LL tum⁻ clones were not stable: after a few months of continuous culture, their cells produced significantly more tumors than did the initial cells. This was analyzed with clone L12. Upon recloning after 4 months of culture, the L12 population was found to contain a mixture of tum⁺ and tum⁻ cells. This phenomenon had not been observed with the teratocarcinoma cell variants. It may be a consequence of the aneuploidy of the 3LL cells.

tum⁻ Variants Produce Tumors in Sublethally Irradiated Mice. tum⁻ variants were injected into mice that had received a sublethal but strongly immunosuppressive dose (640 rads) of γ radiation a few hours before the injection. All the tum⁻ variants produced tumors and these tumors grew nearly as fast as the tum⁺ controls (Table 1). This result suggests that the 3LL tum⁻ variants fail to produce tumors because they induce an immune rejection response in the host.

tum⁻ Variants Confer a Strong Immune Protection Against Themselves. C57BL/6 mice were injected with living cells of tum⁻ clone L20. No mouse formed a tumor. Three weeks later these mice and a group of control mice were sublethally irradiated. Both groups were challenged with the same L20 tum⁻ cells. Only one tumor appeared in the 15 animals that had been immunized with L20 whereas tumors appeared in 14 of the 15 control animals. Similar results were obtained with tum⁻ variants L50, L62, L12, and L48 (Tables 2 and 3). Lewis lung tum⁻ clones are therefore able to confer a strong immune protection against themselves. A similar protection was observed

Table 2. Cross reactions among tum⁻ clones

Immunizing clone	% mice with tumors (no. with tumors/no. injected)		
	L20	L50*	L62*
L20	**7 (1/15)**	*62 (8/13)*	*64 (7/11)*
L50	93 (14/15)	**7 (1/15)**	*64 (9/14)*
L62	73 (11/15)	93 (14/15)	**0 (0/15)**
—	93 (14/15)	92 (23/25)	100 (13/13)

Results of a single experiment in which a homogeneous group of C57BL/6 mice were injected subcutaneously on the left side of the abdomen with 3×10^5 living cells of tum⁻ clones L20, L50, and L62 ("immunizing clone"). The controls were injected with an equal amount of injection medium. Twenty-six days later, the mice received 640 rads of γ radiation. A few hours later, they were injected on the right side of the abdomen with 3×10^4 (L20), 5×10^4 (L50), or 8×10^4 (L62) living tum⁻ cells. The results that differ from the controls at the 95% confidence level (χ^2 test) are in *italics*.

* Challenging clone.

Table 3. Crossreactions among tum$^-$ clones

Immunizing clone	% mice with tumors (no. with tumors/no. injected)				
	L12*	L20*	L50*	L62*	L48*
L12	**35 (6/17)**	100 (16/16)	80 (12/15)	86 (18/21)	89 (8/9)
L20	*53 (10/19)*	**5 (1/19)**	*67 (12/18)*	*54 (12/22)*	*71 (20/28)*
L50	81 (13/16)	94 (17/18)	**5 (1/20)**	*50 (7/14)*	*64 (14/22)*
L62	95 (19/20)	94 (17/18)	95 (18/19)	**0 (0/24)**	89 (16/18)
L48	79 (15/19)	95 (19/20)	82 (14/17)	*56 (10/18)*	**5 (1/20)**
—	90 (18/20)	100 (20/20)	90 (18/20)	83 (20/24)	91 (21/23)

C57BL/6 mice were injected subcutaneously on the left side of the abdomen with 3×10^5 living cells of tum$^-$ clone L12, L20, L50, L48, or L62 ("immunizing clone"). Controls were injected with the same amount of injection medium. After 22 days, the mice were irradiated with approximately 600 rads of γ radiation from a cesium source. Three hours later, the mice were injected on the right side of the abdomen with 10^5 (L12, L62) or 5×10^4 (L20, L50, L48) living tum$^-$ cells. Results that differ from controls at the 90% confidence level (χ^2 test) are in *italics*.
* Challenging clone.

in mice immunized with 4×10^6 tum$^-$ cells irradiated at 4000 rads (data not shown).

The production of immune memory cells by tum$^-$ variants was also demonstrated by adoptive transfer. Mice were immunized with living cells of tum$^-$ variant L50. Three weeks later, their spleen cells were collected and injected intraperitoneally into syngeneic animals that had been sublethally irradiated a few hours earlier. A group of irradiated control mice were reconstituted with the same amount of spleen cells taken from normal mice. A third group of irradiated mice were not reconstituted. Three days later, all the mice were challenged with cells of tum$^-$ clone L50. Almost all the irradiated mice that either were not reconstituted or were reconstituted with normal spleen cells formed progressive tumors, whereas almost all the mice reconstituted with immune anti-L50 tum$^-$ spleen cells failed to do so (Table 4).

Some tum$^-$ Variants Confer a Partial Protection Against Other Variants. Mice were injected with living cells of a tum$^-$ clone. Three weeks later, they were sublethally irradiated and injected with living cells of other tum$^-$ clones. Two independent experiments are reported in Tables 2 and 3.

The two experiments are in good agreement. Some combinations of immunizing and challenging clones failed to provide any protection. Other combinations showed a significant protection, even though this "cross protection" invariably was weaker than the protection obtained when immunization and challenge were performed with the same tum$^-$ variant. Some clones, such as L20 and L50, appeared to be good protectors. Others, such as L62, were easily protected against. No positive or negative correlation between these two properties could be established. The best protectors were those that also conferred the best protection against the tum$^+$ cells (see below). This difference in immunizing ability had not been observed with the teratocarcinoma tum$^-$ variants.

These results indicate that strong singular antigens are present on each variant and that additional weaker common antigens are shared by some variants.

tum$^-$ Variants Confer Immune Protection Against the Original tum$^+$ Cells: In order to find out whether the tum$^-$ variants can protect against the tum$^+$ cells, we immunized mice with living cells of tum$^-$ clone L12, L20, L48, L50, or L62. Twenty-four days later, the mice were challenged with tum$^+$ cells. The immunized mice formed significantly fewer tumors than did the controls (Table 5). This protection was confirmed in numerous other experiments. It indicates that the tum$^-$ variants share a common antigen with the original LI cells.

The degree of protection observed decreased rapidly when the number of challenging cells increased. The protection was long lasting: 100 days after immunization it still was approximately equal to that observed after 21 days. This was also seen in the teratocarcinoma system. However, unlike what was observed with teratocarcinoma, 600 rads of γ irradiation of the immunized animals abolished nearly completely the protection against a tum$^+$ challenge (data not shown).

Lewis lung carcinoma cells have been reported to be unable to confer any immune protection to syngeneic hosts (7). In agreement with this, we have been unable to demonstrate any immune protection in mice that were injected with tumor cells in the footpads and whose tumors were later removed surgically (data not shown). However, we obtained a significant degree of immune protection in mice injected with irradiated tum$^+$

Table 4. Protection against the tum$^-$ L50 variant by adoptive transfer of immune spleen cells

Spleen cells transferred in the reconstituted mice		% of mice with tumors (no. with tumors/ no. injected)
Type	Dose	
Immune	3×10^7	6 (1/18)
anti-L50	1×10^7	20 (3/15)
Normal	3×10^7	92 (11/12)
	1×10^7	89 (16/18)
—	—	100 (12/12)

C57BL/6 mice were injected subcutaneously with 3×10^5 living cells of tum$^-$ L50 variant. Twenty-five days later, their spleen cells were collected and injected intraperitoneally into a group of mice given 660 rads of γ radiation a few hours before the injection. A second group of irradiated mice were injected with the same number of normal spleen cells collected from control mice. A third group of irradiated mice were not reconstituted with spleen cells. Three days later, all mice were challenged with 6×10^4 L50 cells.

Table 5. Cross protection of tum$^-$ clones against tum$^+$ cells

Immunizing clone	% of mice with tumors (no. with tumors/ no. injected)
L12	65 (24/37)
L20	40 (12/30)
L48	57 (17/30)
L50	38 (14/37)
L62	68 (23/34)
—	97 (38/39)

Results of a single experiment in which a homogeneous group of C57BL/6 mice were injected subcutaneously with 3×10^5 living cells of different tum$^-$ variants (immunizing clones). Control mice were injected with the same amount of injection medium. Twenty-four days later, all the mice were injected subcutaneously on the other side of the abdomen with 3×10^4 tum$^+$ LI cells (challenging clone).

Table 6. Protection against tum$^+$ cells

Immunizing cells	% of mice with tumors (no. with tumors/ no. injected)
tum$^+$ LI, irradiated, 4×10^6	55 (11/20)
tum$^-$ L50, living cells, 3×10^5	9 (1/11)
	90 (18/20)

C57BL/6 mice were injected subcutaneously with irradiated tum$^+$ cells or living tum$^-$ cells. Three weeks later, they were challenged subcutaneously with 3×10^4 tum$^+$ LI cells.

cells (Table 6). Nevertheless, mice that were immunized with living L50 tum$^-$ cells in the same experiment displayed a higher level of protection.

Rejection of tum$^+$ Cells in Immunized Mice Is Dependent on a Variable Factor Related to the Challenge. We failed to obtain conditions of immunization with tum$^-$ variants that would protect all the mice against the tum$^+$ cells. In order to find out whether this was due to a failure to immunize some of the mice effectively, groups of immunized mice were challenged subcutaneously with tum$^+$ cells in two well-separated sites. If the mice are uniformly immunized, the tumors should be distributed randomly over all the injection sites. If, on the contrary, the group of mice contains two subgroups with significantly different levels of immunization, this should bias the distribution of tumors, resulting in an excess of mice with two tumors and mice with none.

Mice immunized with L24 tum$^-$ cells were challenged with tum$^+$ cells. Almost all the control mice acquired tumors at both injection sites (Table 7). In the two groups of immunized mice the proportions of mice carrying two, one, and no tumor did not differ significantly from those expected if the tumors were distributed at random among the injection sites of all the mice. This implies that the rejection of the tum$^+$ cells is affected by a variable factor related to the challenge. This could be the precise location of the injection site or some property of the few tum$^+$ cells that initiate the tumor, such as their karyotype.

DISCUSSION

The mutagenic treatment that produces tum$^-$ variants from a mouse teratocarcinoma cell line clearly leads to a similar result when applied to a Lewis lung carcinoma cell line: clones with decreased tumorigenicity are obtained at a frequency exceeding 50%. These clones clearly undergo an immune rejection response in syngeneic C57BL/6 mice. Immunization with living tum$^-$ variants confers a strong protection against the immunizing variant. A weaker but significant protection against some other tum$^-$ variants was observed with three of the five clones that were tested. Finally, mice immunized with tum$^-$ variants show a partial but significant protection against the tum$^+$ cells. This last protection is less noteworthy than the corresponding one observed with teratocarcinoma because a weak protection is also observed in mice immunized with irradiated Lewis lung tum$^+$ cells. However, our results suggest that immunization with living tum$^-$ cells is more effective. The pattern of immune protection obtained with Lewis lung tum$^-$ variants indicates that each tum$^-$ variant carries a strong singular transplantation antigen, the "tum$^-$ antigen," together with one or more weaker common antigens shared by many tum$^-$ variants and by the tum$^+$ cells. Similar conclusions were drawn from previously reported experiments involving teratocarcinoma tum$^-$ variants (2).

Table 7. Protection of tum$^-$ variant against challenge by tum$^+$ cells injected in two separate sites

Immunizing clone	No. tumors per mouse	% mice (no. mice/ no. mice injected)	Expected random distribution, %
L24, 1×10^5	2	16 (3/19)	10
	1	32 (6/19)	43
	0	53 (10/19)	47
L24, 5×10^4	2	36 (8/22)	38
	1	50 (11/22)	47
	0	14 (3/22)	15
—	2	93 (14/15)	
	1	7 (1/15)	

Two groups of mice were injected subcutaneously in the middle of the abdomen with 10^5 or 5×10^4 living cells of tum$^-$ L24 clone. Controls were injected with the same amount of injection medium. Twenty-one days later, all the mice were injected subcutaneously on the left and on the right side of the abdomen with 3×10^4 living tum$^+$ LI cells. Mice were scored for the presence of two, one, or no tumor per animal. The frequencies indicated under "Expected random distribution" are those given by the binomial distribution.

Unlike tumors of spontaneous origin, tumors induced with carcinogens such as methylcholanthrene are usually immunogenic, carrying so-called tumor-specific transplantation antigens (TSTA) (8–10). The high frequency of occurrence of tum$^-$ variants may provide an explanation for this observation. Indeed, because most carcinogens are also mutagens, they may produce tum$^-$ variants at high frequency. We suggest that a large fraction of the cells transformed by carcinogens acquire tum$^-$ antigens by a coincident but independent mutation. This could explain, for instance, why low doses of methylcholanthrene produce tumors that are less antigenic than those produced by high doses, which should favor the occurrence of double mutations (9). This interpretation implies that most tumor-specific transplantation antigens found on tumors induced by carcinogens are not related to the cellular changes involved in the tumoral transformation.

The observation that tum$^-$ variants can be obtained in Lewis lung carcinoma shows that neither the absence of *H-2* determinants nor the differentiation potential of the teratocarcinoma cells is required. This decreases the likelihood that the tum$^-$ phenotype results from a differentiation process as opposed to a mutation. Because tum$^-$ variants arise in two completely unrelated tumor cell lines, it is quite possible that such variants may be derived from many tumors. Preliminary results show that tum$^-$ variants can be obtained with a third mouse tumor, the P815 mastocytoma.

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