IMMUNOGENIC VARIANTS OBTAINED BY MUTAGENESIS OF MOUSE MASTOCYTOMA P815

I. Rejection by Syngeneic Mice

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It is now well known that the presence of transplantation antigens capable of eliciting a rejection response in syngeneic hosts on tumor cells is dependent on the cause of the tumoral transformation. Most mouse tumors induced by viruses or by carcinogens like methylcholanthrene carry strong transplantation antigens (1). On the other hand, mouse tumors obtained without explicit experimental interference usually show little or no immunogenicity (2, 3).

We reported that, upon treatment of a nonimmunogenic malignant mouse teratocarcinoma cell line with a mutagen, we obtained immunogenic variant cells that were incapable of forming tumors in syngeneic mice (tum^-).[1] These variants undergo a process of immune rejection and confer a long-lasting immunity (4). Cross-immunization patterns observed in vivo indicate that they carry singular transplantation antigens (5). Moreover, mice that have rejected these tum^- variants are partially resistant to a challenge with the original teratocarcinoma cells that were capable of forming progressive tumors in syngeneic mice (tum^+), even though no protection can be induced either with irradiated tum^+ cells or with living tum^+ cells injected subcutaneously and later removed by surgery (6). The results obtained with teratocarcinoma have been extended to a Lewis lung carcinoma cell line: tum^- variants with similar properties have been obtained after mutagenesis (7).

To analyze the mechanism of the immune rejection of tum^- variants, we considered that it would be useful to obtain such variants in an ascitic tumor cell line. This should allow for a better monitoring of the tumor cells and the effectors of the rejection process, as both should be recoverable from the peritoneal cavity. We report here that we have obtained tum^- variants from mastocytoma P815-X2, a weakly antigenic ascitic tumor cell line derived from a tumor induced with methylcholanthrene in mouse strain DBA/2 (8). Cross-immunization experiments performed in vivo indicate that some of these variants carry singular transplantation antigens. In the accompanying report, we show that singular antigenic specificities can also be demonstrated in vitro on these tum^- variants by T lymphocyte-mediated cytolysis.

Materials and Methods

Animals. DBA/2 mice were derived from breeders obtained from J. L. Guénet (Institut Pasteur, Paris). The mice used in the experiments were between 8 and 12 wk old.

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[1] *Abbreviations used in this paper:* tum^+, variant cells that were capable of forming progressive tumors in syngeneic mice; tum^-, variant cells that were incapable of forming progressive tumors in syngeneic mice.

Cells and Culture Conditions. Our P815 subline was P815-X2. From this permanent line, we isolated a malignant clone called P1 by a limiting dilution procedure. The P815 cells were cultured in Petri dishes (Falcon 1001; Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) in Dulbecco's modified Eagle's medium (1600; Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal calf serum, in an 8% CO_2 atmosphere.

Mutagenesis. The cells were incubated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich Europe, Beerse, Belgium) at a concentration of 3 μg/ml in Earle's medium at 37°C in an 8% CO_2 atmosphere for 10 min. This medium was replaced by the culture medium described above and the cells were grown for 10 d. The cells were then cloned by a limiting-dilution method in culture medium supplemented with 30% fetal calf serum. The fraction of the cells that survived the mutagenesis was estimated to be 0.2% by extrapolating to day 0 the number of cells obtained at day 10 in the exponentially growing culture.

Injections of Cells and Tumor Analysis. Cells were injected intraperitoneally in medium that contained no fetal calf serum. The mice were examined every 3 d. They were considered as definitely positive and killed when the volume of tumor ascites was ≃5 ml. Mice that showed no sign of tumor growth after 3 mo were considered negative.

Adoptive Transfer. Spleens were teased in Hanks' balanced salts solution supplemented with 1% fetal calf serum. Cell suspensions were filtered through a nylon-mesh gauze (80 μm; Nylon Swiss; Staniar, Manchester, England). The cells were centrifuged and resuspended in Eagle's medium with 1% serum and injected in a vol of 0.3 ml intraperitoneally into mice that had been given 700 rad of gamma irradiation a few hours before. The reconstituted mice were challenged with tumor cells 4 d later.

Collection and Colony Assay of Intraperitoneal P815 Cells. Mice were injected intraperitoneally with 3 ml of Eagle's medium. A 0.3-ml sample of intraperitoneal fluid was immediately collected with a syringe and put in a tube that contained 6 U of heparin. The cells were diluted in Eagle's medium that contained 1% fetal calf serum. Aliquots of 0.2 ml were mixed in a Petri dish (Falcon 1007) with 2 ml Eagle's medium that contained 10% fetal calf serum and 0.4% Bactoagar (Difco Laboratories, Detroit, Mich.). After a 3-d incubation at 37°C, the colonies that contained more than six cells were counted. The number of living intraperitoneal P815 cells was estimated taking into account a cloning efficiency of ~0.3.

Results

We isolated a clone (P1) from the P815 mastocytoma subline P815-X2. This clonal line was maintained permanently in vitro. Its doubling time was ~10.5 h. When P1 cells in culture were injected intraperitoneally into the syngeneic DBA/2 mice, they regularly produced ascitic tumors at doses as low as 60 cells (Table I).

High Frequency of tum⁻ Cells in a Mutagenized P815 Population. P1 cells were treated with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in conditions that allowed for the survival of 0.2% of the cells. 10 d later, the mutagenized population was cloned. 76 clones were injected intraperitoneally into DBA/2 mice at a dose of 600 cells/mouse. 11 clones (14%) showed a decreased tumorigenicity: they formed either no tumor or occasional tumors that progressed much more slowly than the control tumors. The inability of these tum⁻ clones to form tumors in syngeneic mice was confirmed in numerous experiments. They failed to produce tumors even when the injection dose was 1,000 times higher than a dose that regularly allowed the P1 tum⁺ cells to form tumors. The results obtained with seven tum⁻ clones are shown in Table I.

To check that the appearance of tum⁻ clones was a result of the mutagen, we injected 17 untreated control clones of P1. All these clones formed progressive tumors in all the mice.

The inability to form tumors in syngeneic mice is a stable characteristic, unaffected by 3 mo of continuous culture. In vitro, the tum⁻ clones have the same generation

TABLE I

Tumors Produced by tum$^+$ and tum$^-$ P815 Cells in DBA/2 Mice

Cell clone	Dose	No. mice with tumors/No. mice injected	
		Unirradiated mice*	Irradiated mice*
P1 (tum$^+$)	6×10^4	23/24 (33)‡	28/28 (15)‡
	6×10^2	109/116 (36)	47/51 (17)
	60	19/20 (30)	28/30 (19)
P21	6×10^4	0/21	20/20 (16)
	6×10^2	2/55 (114)	53/61 (22)
P32	6×10^4	1/20 (80)	20/20 (15)
	6×10^2	4/51 (37)	40/43 (19)
P35	6×10^4	0/20	20/20 (15)
	6×10^2	0/52	69/70 (23)
P60	6×10^4	2/20 (41)	23/23 (15)
	6×10^2	1/32 (98)	21/21 (21)
P76	6×10^4	10/41 (54)	24/24 (17)
	6×10^2	15/42 (46)	20/20 (21)
P89	6×10^4	2/19 (58)	24/24 (16)
	6×10^2	2/31 (62)	21/21 (21)
P91	6×10^4	1/22 (80)	23/23 (16)
	6×10^2	0/32	20/21 (21)

* Normal adult mice (unirradiated) and mice given 700 rad of gamma radiation 3–6 h earlier (irradiated) were injected intraperitoneally with living P815 cells. These data were pooled from all the injections performed in 1977, 1978, and 1979.

‡ The average number of days after injection at which the mice display acute peritoneal swelling caused by the ascitic tumor is shown in parentheses.

time as P1 and their morphology is identical. The karyotype of the tum$^-$ clones does not differ significantly from that of P1, which has a major population of cells with 42 telocentric and one metacentric chromosome.

Rejection of the tum$^-$ Variants. The tum$^-$ variants invariably produced tumors in mice that had received 700 rad of gamma radiation (Table I). This suggested that their inability to form tumors in normal mice was a result of an immunological rejection process.

To follow the fate of P815 cells injected intraperitoneally, we repeatedly collected samples from the peritoneal cavity of the same mouse and estimated the number of viable tumor cells with a colony assay (Materials and Methods). The results obtained in an experiment involving tum$^+$ clone P1 and tum$^-$ clones P21 and P35 are shown in Fig. 1. We have chosen three representative mice that were injected with 6,000 cells for each clone: two normal and one irradiated.

In the irradiated mice, both the tum$^+$ and the tum$^-$ cells multiplied exponentially to reach a number of $\sim 10^8$ cells by day 10 after injection. Later, the number of cells continued to increase, and the animals usually died between day 15 and 20.

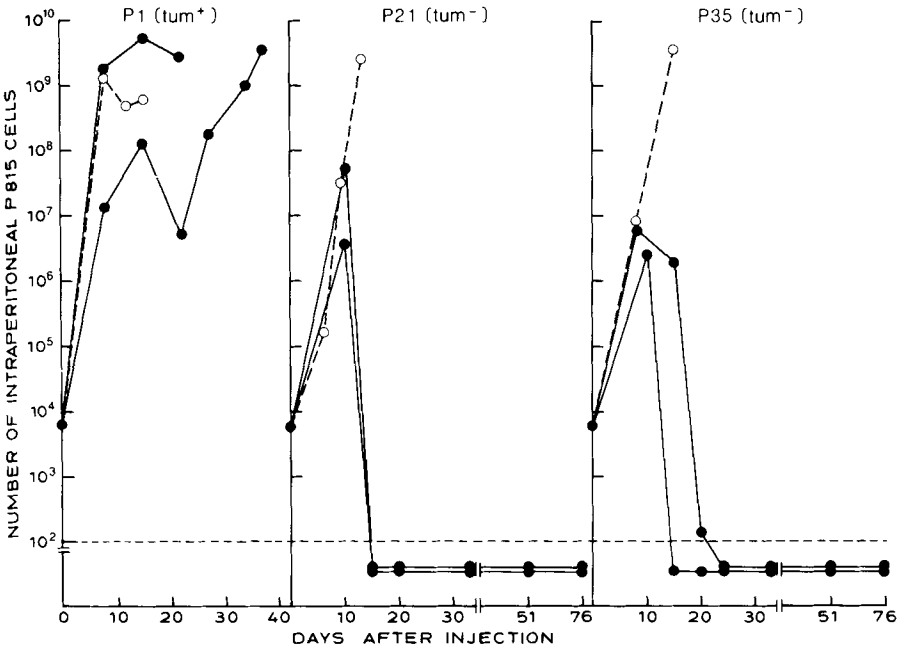

FIG. 1. DBA/2 mice were injected intraperitoneally with 6×10^3 living P815 tum$^+$ (P1) or tum$^-$ (P21, P35) cells. At various times thereafter, samples of the intraperitoneal fluid were collected (Materials and Methods) and the number of living P815 cells was estimated in an agar colony assay. Each curve represents, on a logarithmic scale, the number of living P815 cells found in the same mouse that was tapped on the days indicated on the abscissa. The lowest number of cells that can be estimated in the colony assay is 100. Points <100 indicate that no colony was observed. Some curves are interrupted before the last day mentioned on the abscissa because of death caused by the tumor. ●, normal mice. ○, mice given 700 rad of gamma radiation 3–6 h before injection.

When unirradiated mice were injected with tum$^+$ cells, some showed a multiplication of tumor cells similar to that found in irradiated mice. These mice died early. For others, a decrease in the number of tumor cells was observed starting between day 10 and 15. This decrease was very variable from one mouse to another. In some mice, the number of tumor cells dropped to $<10^5$. However, the number of tumor cells invariably rose again and the mice died. It appears, therefore, that the tum$^+$ cells underwent a partial rejection in some DBA/2 mice.

In the unirradiated mice injected with tum$^-$ variants P21 and P35, the fate of the tum$^-$ cells was quite constant: between day 10 and 15, a rapid and complete elimination of the tumor cells in all the mice occurred. No tumor cell reappeared later and all the mice survived. During the rejection phase, there was an ~10-fold increase in the number of lymphocytes and macrophages present in the peritoneal cavity. Similar results were obtained with other tum$^-$ variants.

Immune Protection against tum$^-$ Variants. Mice that rejected a first injection of living or irradiated tum$^-$ cells acquired a radioresistant immune protection against a challenge with the immunizing clone and other tum$^-$ clones (Table II). It was also possible to transfer adoptively the protection to irradiated animals with spleen cells from immune donors (Table III).

These experiments were used to analyze the pattern of immune protection. There was a significant cross-protection between all the variants. In addition, the mice

TABLE II

Tumors Produced in DBA/2 Mice Immunized with tum⁻ Cells

Experiment	Immunizing clone*	Percentage of mice with tumors‡; challenging clone				
		P21	P32	P35	P89	P91
I	P21 living	0 (0/10)§	50 (6/12)			
	P32 living	14 (1/7)	18 (2/11)			
	None	100 (11/11)	100 (10/10)			
II	P21 living	0 (0/10)				20 (2/10)
	P91 living	25 (2/8)				0 (0/11)
	None	100 (8/8)				100 (10/10)
III	P21 irradiated	8 (1/13)		86 (12/14)		
	P35 irradiated	50 (6/12)		30 (3/10)		
	None	71 (10/14)		64 (9/14)		
IV	P21 irradiated	7 (1/15)	60 (9/15)	38 (6/16)		25 (3/12)
	P32 irradiated	40 (6/15)	58 (7/13)	73 (11/15)		27 (4/15)
	P35 irradiated	67 (10/15)	87 (13/15)	21 (3/14)		38 (3/8)
	P91 irradiated	64 (9/14)	71 (10/14)	50 (7/14)		7 (1/14)
	None	100 (9/9)	100 (8/8)	86 (6/7)		100 (8/8)
V	P21 irradiated	0 (0/15)			85 (12/14)	40 (6/15)
	P89 irradiated	54 (7/13)			30 (4/13)	23 (3/13)
	P91 irradiated	40 (6/15)			93 (14/15)	6 (1/15)
	None	93 (14/15)			93 (14/15)	100 (16/16)

* Mice were injected intraperitoneally with 2×10^5 living tum⁻ cells (experiments I and II) or with 6×10^6 cells killed with 10,000 rad of gamma radiation from a Cs source (experiments III, IV, and V). Control mice received the same amount of injection medium.

‡ 3 wk after immunization, the mice were irradiated with 700 rad and challenged with 2,000 cells injected intraperitoneally.

§ The No. mice with tumors/No. mice injected is shown in parentheses.

immunized with variants P21, P35, and P91 rejected preferentially the immunizing variant. The converse was true: the best protection against each of these three variants was observed in the mice that had been immunized with the challenging variant. These results indicate that in addition to one or more common antigens shared by all tum⁻ variants, a singular transplantation antigen is present on each of variants P21, P35, and P91. No evidence for such a singular antigen was obtained with variants P32 and P89.

Cross-Protection against the Original P1 tum⁺ Clone. Even though we occasionally observed a partial rejection of the tum⁺ P1 cells in the peritoneal cavity, we did not find any significant immune protection after immunization with irradiated tum⁺ cells (Table IV). It was therefore of interest to examine whether mice immunized with tum⁻ variants were protected against P1. As shown in Table IV, a significant protection was conferred by living tum⁻ variants, and even by some irradiated variants. This protection was not abolished by irradiating the immune mice. This implies that at least some of the common antigens present on the tum⁻ clones are already present on the original tum⁺ cell.

TABLE III

Tumors Produced in DBA/2 Mice Protected by Adoptive Transfer

Experiment	Clones used for immunization of donor mice*	Percentage of mice with tumors‡; challenging clones				
		P21	P32	P35	P89	P91
I	P21	0 (0/12)§	58 (7/12)	83 (10/12)		
	P32	33 (4/12)	44 (4/9)	64 (7/11)		
	P35	56 (5/9)	50 (6/12)	18 (2/11)		
	None	100 (12/12)	100 (12/12)	100 (12/12)		
II	P21	0 (0/12)			50 (6/12)	8 (1/12)
	P89	0 (0/12)			0 (0/12)	0 (0/11)
	P91	73 (8/11)			100 (11/11)	0 (0/12)
	None	100 (12/12)			100 (12/12)	100 (12/12)
III	P21	58 (7/12)	100 (12/12)	83 (10/12)		75 (9/12)
	P32	92 (11/12)	83 (10/12)	100 (12/12)		100 (11/11)
	P35	82 (9/11)	92 (11/12)	50 (6/12)		64 (7/11)
	P91	64 (7/11)	100 (12/12)	75 (9/12)		44 (4/9)
	None	75 (9/12)	83 (10/12)	92 (11/12)		100 (12/12)

* DBA/2 mice were immunized intraperitoneally with 2×10^5 living tum$^-$ cells or with injection medium. 3 wk later, their spleen cells were collected and used to reconstitute irradiated DBA/2 recipients. The number of spleen cells transferred to the recipients was 1.3×10^7 in experiment I and II; and 10^6 in experiment III.

‡ DBA/2 mice were irradiated with 700 rad and reconstituted intraperitoneally a few hours later with immune or normal spleen cells. 4 d later, they were challenged intraperitoneally with 2×10^3 living tum$^-$ cells.

§ The No. of mice with tumors/No. of mice injected is shown in parentheses.

Discussion

By treating mastocytoma P815-X2 with a mutagen, we were able to obtain stable cell variants that failed to form tumors in the syngeneic mice even when the injection dose was 1,000-fold higher than the minimal tumorigenic dose of P815. These tum$^-$ variants multiplied rapidly for ~10 d in the peritoneal cavity and reached a number of $\sim 10^7$ cells. Shortly after that, the tum$^-$ cells disappeared completely within a few days. No tumor recurrence occurred for at least 3 mo. The elimination of the tum$^-$ variants was immunological in nature as indicated by its radiosensitivity and by the presence of specific memory cells in the spleen. These results are similar to those observed previously with teratocarcinoma and Lewis lung carcinoma cells treated with the same mutagen (4, 7). Therefore, it seems likely that tum$^-$ variants can be obtained by mutagenesis of any mouse tumor cell line.

Cross-immunization experiments performed either directly or by adoptive transfer clearly showed a preferential rejection of the immunizing variants for three of the five tum$^-$ clones that were tested. This implies that each of these three variants has acquired a singular transplantation antigen. Similar results have been obtained in the teratocarcinoma and Lewis lung carcinoma systems (5, 7). In addition, the cross-reactions indicated the presence of an antigen that is common to the five tum$^-$ variants and to the tum$^+$ cells. No specific transplantation antigen was found on variants P89 and P32. No such variant had been observed in the two previous systems. This may be because P815 is more immunogenic than the two other tumors as shown

TABLE IV
Protection against tum$^+$ Cells

Experiment	Immunizing cell*		Percentage of mice with tumors‡
	Type	Dose	
I	P21	6×10^2	58 (11/19)§
		2×10^5	50 (9/18)
	P32	6×10^2	60 (12/20)
		2×10^5	22 (4/18)
	None		100 (20/20)
II	P1 irradiated	6×10^6	93 (15/16)
	P21 living	2×10^5	50 (8/16)
	P21 irradiated	6×10^6	81 (13/16)
	P35 living	2×10^5	43 (7/16)
	P35 irradiated	6×10^6	87 (14/16)
	P89 living	2×10^5	0 (0/16)
	P89 irradiated	6×10^6	50 (8/16)
	None		84 (27/32)

* DBA/2 mice were injected intraperitoneally with living cells or with cells killed with 10,000 rad of gamma radiation. Control mice were injected with the same amount of culture medium.

‡ 23 d after immunization, the mice were challenged intraperitoneally with 600 living tum$^+$ P1 cells.

§ No. mice with tumors/No. mice injected is shown in parentheses.

by the partial rejection of tum$^+$ cells. A small increase in the immunogenicity of the initial antigen may be sufficient to obtain tum$^-$ variants. We present in the accompanying report (9) an analysis of the T lymphocyte-mediated cytolysis against the P815 tum$^-$ variants described here. This analysis shows the existence of a significant correlation between the detection of singular specificities in vitro and their presence in vivo.

No significant protection against a challenge with living tum$^+$ cells could be obtained by immunization with irradiated tum$^+$ cells. However, a very significant protection was obtained by immunizing with living tum$^-$ cells. This was also observed with teratocarcinoma and Lewis lung carcinoma tum$^-$ variants. With some P815 tum$^-$ clones, a significant protection was also obtained with irradiated tum$^-$ cells. Similar observations have been reported with xenogenized tumor cells obtained by viral infection (10) or by treatment with chemical compounds (11).

A remarkable aspect of the tum$^-$ variants is their extremely high frequency after mutagenic treatment. It is at least four orders of magnitude higher than that of metabolic mutations like azaguanine resistance. To our knowledge, the only other instance where similar frequencies have been described involves myeloma cell lines that produce antibodies with an altered heavy chain (12, 13). If one assumes the cellular genome to have a uniform sensitivity to the mutagen, the genetic target that determines the tum$^-$ character on P815 can be estimated to be >2.5% of that governing the ability to multiply in vitro. The involvement of hypermutable regions may represent a more plausible alternative. On the basis of this high frequency, we believe that there is a definite possibility that the antigenicity of tumors caused by

chemical carcinogens is caused by a mutation that is completely independent from that causing the tumoral transformation.

Summary

We have reported that it is possible to obtain variants that are incapable of forming progressive tumors in syngeneic mice (tum^-) by mutagenesis and cloning of a teratocarcinoma and a Lewis lung carcinoma cell line. These observations were extended to the ascitic P815 mastocytoma of mouse strain DBA/2. After a treatment with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, we obtained a high frequency (14%) of tum^- clones. A colony assay indicated that after a period of rapid multiplication extending to ~ 10 d after injection, the P815 tum^- cells were rejected by a process that was usually completed by day 15. No rejection was observed in sublethally irradiated animals. The immunological nature of the rejection of the P815 variants was further inferred because, upon rejection, the mice acquired a radioresistant specific protection that could be transferred adoptively with spleen cells.

Cross-immunization patterns demonstrated the presence of singular antigenic specificities on three of the five variants that were examined. In addition, a common antigen was found on all the tum^- variants and the original cells that were capable of forming progressive tumors in syngeneic mice (tum^+). Mice injected with tum^- cells were significantly protected against a tum^+ challenge, even though no significant protection was generated by irradiated tum^+ cells. A study of the T lymphocyte-mediated cytolysis against the P815 variants described here is presented in the accompanying report (9).

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