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Increased Frequency of Immunogenic Variants Obtained by Repeated Mutagen Treatment of Mouse Mastocytoma P815

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Abstract—*A previous report from this laboratory demonstrated that treatment of mouse mastocytoma P815 with the mutagen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) produces tumor cell variants that are unable to form tumors in syngeneic animals. We examined whether repeated mutagen treatment could increase the frequency of tum⁻ variants above that obtained after a single treatment. This was found to occur with frequencies increasing from a few percent after 1 treatment to more than 90% after 8 treatments. Moreover, uncloned survivor populations obtained after 8 or more MNNG cycles that contained such a high proportion of tum⁻ variants had a markedly decreased tumorigenicity for syngeneic mice. As reported for tum⁻ variants obtained after 1 mutagen treatment, several tum⁻ variants obtained after repeated treatments carried new variant-specific antigens that elicited a specific cytolytic T cell response. Some of these tum⁻ antigens were found to consist of multiple determinants that could be lost independently. We observed that the resistance of the mutagenized populations to MNNG increased gradually with the number of mutagen treatments. In addition, some tum⁻ variants obtained after 8 mutagen treatments showed a reduced sensitivity to mitomycin C.*

INTRODUCTION

IT IS now well established that, by submitting mouse tumor cell lines to *in vitro* mutagen treatment, it is possible to obtain a high frequency of tumor cell variants that are unable to form tumors in syngeneic mice because they elicit a strong immune rejection response. These 'tum⁻' variants have been obtained after MNNG or ethyl methanesulfonate treatment of a large number of mouse tumors that had arisen spontaneously or after induction either with radiation or with chemical carcinogens [1–6]. Tum⁻ variants form progressive tumors in mice that have been immunodepressed by irradiation. The majority of them carry new variant-specific antigens that can be demonstrated either by cross-protection experiments or with specific CTLs [7–10].

In rejecting tum⁻ variants syngeneic mice acquire a significant degree of immune protection against challenge with the original (tum⁺) cells. This is observed not only for tumors such as Lewis lung carcinoma [2] or mastocytoma P815 [3] that are weakly immunogenic but also for tumors such as a teratocarcinoma [7, 11] and two spontaneous mouse leukemias [5] that appear completely incapable of eliciting any syngeneic rejection response [12].

Since the use of tum⁻ variants clearly leads to an extension of the class of mouse tumors that can be demonstrated to express a tumor-associated transplantation antigen, it is tempting to find out whether such variants can be obtained from human tumors for the purpose of reconsidering the presence of human tumor-associated transplantation antigens and possibly of eliciting some

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Abbreviations: tum⁺: variant cells capable of forming progressive tumors in syngeneic mice; tum⁻: variant cells incapable of forming progressive tumors in syngeneic mice; FCS: fetal calf serum; DMEM: Dulbecco's modified Eagle's medium; MNNG: *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; CTL: cytolytic T lymphocyte; i.p.: intraperitoneally; MLTC: mixed lymphocyte tumor cell culture; isc: immunoselected *in vitro*; ist: immunoselected *in vivo*.

therapeutically useful rejection response. However, one major difficulty resides in the detection of antigenic variants among mutagenized human tumor cell clones. Since it will obviously not be possible to inject these clones into the autologous patient to assess their tumorigenicity, it is therefore important to find out whether by repeated mutagen treatments it is possible to increase the frequency of tum$^-$ variants so as to obtain cell populations that consist predominantly of such variants. Our results indicate that after multiple MNNG treatments tumor cell populations containing more than 90% tum$^-$ variants can be obtained.

MATERIALS AND METHODS

Animals

DBA/2 mice were derived from breeders obtained from J. L. Guénet (Institut Pasteur, 28 rue du Dr Roux, Paris, France). The mice used in the experiments were between 12 and 14 weeks old.

Cells and culture conditions

The P815 subline used is P815-X2. From this permanent cell line a malignant clone called P1 has already been isolated by a limiting dilution procedure [3]. The P815 cells were cultured in Petri dishes (Falcon plastic 1001, Division of Becton Dickinson, Oxnard, CA or Greiner cat No. 632102) in DMEM (1600, Grand Island Biological, Grand island, NY) supplemented with 5% fetal calf serum (FCS) in an 8% CO_2 atmosphere. Under these conditions P815 cells grow in suspension.

Mutagenesis and cloning conditions of tumor cells

Exponentially growing P815 cells were harvested and washed twice in 40 ml DMEM without FCS by centrifugation (200 *g*, 10 min) in plastic tubes (Falcon cat 2070, Division of Becton Dickinson, Oxnard, CA).

The cell pellets were resuspended at a concentration of 10^6 per ml in Earle's medium containing a concentration of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich Europe, Beerse, Belgium) ranging from 3 to 6 μg/ml. They were incubated at 37°C in an 8% CO_2 atmosphere for 5–60 min. After exposure to the mutagen the cells were washed twice in Earle's medium and resuspended in DMEM supplemented with 10% FCS. The surviving cells were maintained in culture until their multiplication rate became equal to that of a non-mutagenized control. The fraction of survivor cells was then estimated by comparing the number of cells obtained in the mutagenized populations to that observed in the control. Populations were selected for further mutagenesis from those that contained less than 1% survivors but more than 1000 independent survivor cells. These populations were maintained in culture for 12–22 days before they were cloned or mutagenized again.

Clones were obtained by a limiting dilution procedure at an average of 0.1 cells per well in round-bottom microtiterplates (Titertek, cat No. 76-213-05, Flow Laboratories, U.S.A.) in 200 μl of DMEM supplemented with 10% (vol/vol) FCS.

Injection of cells and tumor analysis

Cells were injected i.p. in 0.3 ml DMEM without FCS. Mice were examined every 3 days. They were killed when the volume of tumor ascites was approximately 5 ml. Mice that showed no sign of tumor growth after 3 months were considered negative.

Mixed lymphocyte tumor cell cultures (MLTC)

Spleen cells (5×10^6) from immune DBA/2 mice injected i.p. 3 months before with 2×10^5 living P815 variant cells were stimulated *in vitro* with 2×10^5 killed cells of the immunizing variant in 2 ml of DMEM supplemented with L-arginine HCl (116 mg/l), L-asparagine (36 mg/l), L-glutamine (216 mg/l), glucose (3.5 g/l), 10 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (HEPES) (Sigma Chemical, St Louis, MO), 5×10^5 M 2-mercaptoethanol and 5% FCS. Stimulator cells were inactivated by irradiation (9600 rad gamma radiation, cesium source). Cultures were incubated for 5 days in 24 wells tissue culture plates (Linbro, cat. No. 76-033-05, Flow laboratories, U.S.A. or NUNC cat No. 1-68357) at 37°C in an atmosphere containing 8% CO_2.

Chromium release assay for cytolytic activity

Chromium release assays [13] were carried out and percentage specific ^{51}Cr-release and lytic units were calculated as previously described [8]. Assays were performed in RPMI-1640 medium (Grand Island Biological, Grand Island, NY) supplemented with 5% FCS and 10 mM HEPES or in DMEM suplemented with glucose (3.5 g/l), 10 mM HEPES and 5% FCS.

CTL clones

The derivation and maintenance of CTL clones specific for P815 or for tum$^-$ variants was carried out as described by Maryanski *et al.* [9]. Briefly, immune spleen cells stimulated in MLTC were incubated in limiting dilution conditions with 10^6 irradiated (2000 rad) DBA/2 spleen cells and 1000 tum$^-$ stimulator cells inactivated by irradiation (9600 rad). The cells were seeded in microtiter

plates of 96 round-bottom wells containing 100 μl of DMEM supplemented as for the MLTC and 100 μl of supernatant from a secondary mixed lymphocyte culture as a source of interleukin 2 [14]. After 7–9 days of incubation at 37°C in air containing 8% CO_2 aliquots (40 μl) of the microcultures were mixed with 2×10^3 ^{51}Cr-labeled target cells (in 160 μl) for 4 hr. Microcultures were considered to be positive when the specific ^{51}Cr-release exceeded the mean spontaneous release obtained in the absence of responder cells by more than 5 standard deviations.

Positive microcultures were transferred to cultures with 5×10^6 irradiated (2000 rad) DBA/2 spleen cells and 10^4 stimulator tum$^-$ cells in 0.5 ml of DMEM supplemented as for the MLTC and 0.5 ml of supernatant from a secondary mixed lymphocyte culture. For subsequent passages (every 3–5 days) 5×10^4 cells were transferred in the same conditions in 24-well tissue culture plates (NUNC cat No. 1-68357). The CTL clones were frozen for storage at −70°C in DMEM supplemented with 10% FCS and 10% dimethylsulfoxide.

Selection of antigen-loss variants with CTL clones

This selection was performed as described by Maryanski and Boon [15]. Cells (10^6) from a P815 tum$^-$ variant were mixed with the appropriate CTL clone (3×10^6 cells) in a round-bottom tube (Falcon 2051) in 2 ml DMEM containing 5% FCS. After a 6-hr incubation at 37°C in air containing 8% CO_2 the cells were washed and resuspended in 10 ml DMEM containing 10% FCS and cloned by limiting dilution in 96-well round-bottom microtiter plates containing 0.2 ml DMEM supplemented with 10% FCS per well.

RESULTS

A tum$^+$ clone (P1) previously isolated from P815 mastocytoma subline P815-X2 was used for repeated mutagenesis. We reported previously that more than 90% of the syngeneic DBA/2 mice injected i.p. with 600 P1 cells develop an ascitic tumor [3].

Increase in the frequency of tum$^-$ clones after repeated mutagen treatments

A P1 cell population was exposed to 9 consecutive treatments with the mutagen MNNG. For each treatment 10^7 cells were exposed to a concentration of mutagen ranging from 3 to 6 μg/ml for a length of time varying from 5 to 60 min so as to obtain a fraction of survivor cells smaller than 1% but containing more than 1000 independent survivor cells. We observed that in the course of the multiple mutagenesis cycles a low degree of resistance to the mutagen gradually developed so that the concentration and the exposure time had to be increased to obtain the same degree of killing of the cells. After mutagen treatment the mutagenized population and a non-mutagenized control population were grown in parallel until the rate of multiplication of the mutagenized population equalled that of the control. The relative amounts of the two populations were then used to determine the fraction of initial cells that had survived mutagen treatment (Fig. 1). At that point parts of the mutagenized population were frozen for conservation, subcloned by limiting dilutions for *in vivo* testing and used as an initial population for a further cycle of mutagenesis.

Seventy-eight clones obtained after 1 MNNG treatment and about 20 clones obtained after 3, 5 or 8 treatments were studied *in vivo*: 2×10^5 living cells of each clone were injected i.p. to 4 normal adult DBA/2 mice and to 1 sublethally irradiated mouse (750 rad). The fraction of clones that failed to form a tumor in the 4 normal mice among those that formed a tumor in the irradiated control increased from 1/78 in the population that was mutagenized once to 16/17 in the population that was mutagenized 8 times. In addition to these tum$^-$ clones, others were obtained that appeared to have a less reduced tumorigenicity since they failed to form progressive tumors in some but not all of the 4 normal mice (Table 1).

A few (3/20) clones obtained after 8 mutagen treatments failed to form progressive tumors in the control mouse that had received 750 rad of gamma irradiation. These clones had a division time *in vitro* that was less than 1 hr longer than that of P1. Two of these clones were injected into groups of mice irradiated at 850 rad. One of them produced progressive ascitic tumors in 3/4 mice whereas the second clone killed 1/4 mice. Moreover, for both clones the survivor mice showed unequivocal signs of tumor growth followed by a clear phase of regression. It is therefore plausible that these clones had a particularly large increase in their immunogenicity so that even irradiated animals could eliminate them by the time their immune system had recovered.

Reduced tumorigenicity of P815 cell populations obtained after several mutagen treatments

We have previously observed that mixtures consisting of equal numbers of tum$^-$ and tum$^+$ cells formed tumors about as readily as equivalent amounts of tum$^+$ cells alone (unpublished results). However, it could be expected that in mixtures containing a large majority of tum$^-$ cells

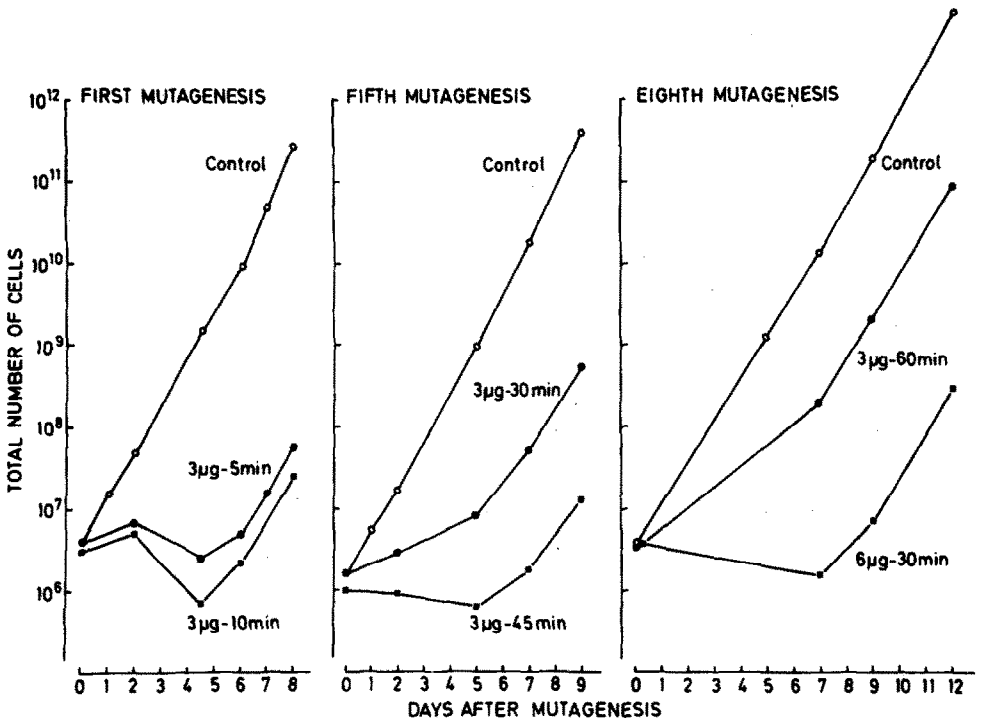

Fig. 1. Growth of P815 cells after the first, fifth and eighth rounds of mutagenesis. A P815 cell population was treated with MNNG at the dose of mutagen and for the time (min) *indicated in the figure (e.g. 3 µg/ml and 5 or 10 min for the first mutagenesis). The mutagen was then removed and the cells were maintained in culture and diluted periodically so as to ensure exponential growth conditions. The number of cells indicated in the figure is corrected for these dilutions. When the growth rate of a mutagenized population had become approximately equal to that of the non-mutagenized control population the frequency of survivor cells was determined and populations with adequate survival rate were selected. These populations were then divided into one population that was mutagenized again and another that was not mutagenized and used as control population. These populations were also subcloned for the analysis of tum⁻ variants. The populations selected after the first, fifth and eighth mutagenesis steps were treated with 3 µg/ml for 10, 45 and 60 min respectively.*

part of the immune rejection response directed against the tumor-associated antigen of P815 would be capable of rejecting the minority of tum⁺ cells. Accordingly, we tested populations obtained after mutageneses 1–9 in normal and irradiated mice (Table 2). We observed a significant increase of the mean survival time with populations mutagenized 3 times or more. From mutagenesis 7 onwards a significant fraction of the mice failed to show progressive tumors and survived for more than 3 months.

Presence of new antigens on tum⁻ variants obtained after multiple mutagen treatments

Since we have previously reported that a majority of P815 tum⁻ variants obtained after 1 mutagenesis cycle carry new variant-specific antigens that elicit a specific cytolytic T cell response, we examined whether such antigens were also present on tum⁻ clones obtained after 5 mutagenesis steps. Spleen cells from DBA/2 mice that had rejected such tum⁻ variants were stimulated *in vitro* with the same variants and

Table 1. Tumorigenicity of P815 clones obtained after repeated MNNG treatments

No. of MNNG treatments	No. of clones injected*	Fraction of clones forming progressive tumors in†: 0/4 mice	1–3/4 mice	4/4 mice
1	78	1.3% (1/78)	22% (17/78)	77% (60/78)
3	20	20% (4/20)	45% (9/20)	35% (7/20)
5	19	42% (8/19)	37% (7/19)	21% (4/19)
8	17	94% (16/17)	6% 1/17)	0% (0/17)

*Each clone (2×10^5 living cells) was injected i p. to 4 normal syngeneic DBA/2 mice.

†The frequency of clones given in the table is expressed as a % of the number of clones that formed progressive tumors in the irradiated mice. The actual numbers are given in parentheses. For populations obtained after 1, 3 or 5 mutagen treatments all the clones formed tumors in irradiated mice. For that obtained after 8 treatments only 17/20 clones did so.

assayed a few days later for cytolytic activity. For several of these variants we observed a cytolytic response that showed a clear preference for the immunizing variant (Table 3), indicating the presence of variant-specific antigens.

The presence of specific new antigens on tum$^-$ clones P1509 and P1517 that had been isolated after 5 mutagen treatments was confirmed by the obtention of CTL-clones that lysed these variants exclusively. Immune spleen cells were stimulated in MLTC conditions with the immunizing variant before being cloned in limiting dilution conditions in microcultures containing the appropriate variant killed by irradiation as stimulator cells, irradiated DBA/2 spleen cells as feeder cells and supernatant from an allogeneic mixed lymphocyte culture as a source of interleukin 2. After 7–8 days each microculture was tested for cytolytic activity on both the immunizing variant and the tum$^+$ clone P1.

Table 2. Tumorigenicity of P815 cells after repeated mutagen treatments

No. of mutagen treatments	No. of cells injected*	Normal mice No. of tumors/No. of mice†	M.S.T.‡	Irradiated mice No. of tumors/No. of mice	M.S.T.
0	10^5	8/8	34	5/5	16
	3×10^3	8/8	37	5/5	16
1	10^5	8/8	30	5/5	15
	3×10^3	8/8	37	4/4	18
2	10^5	8/8	37	5/5	16
	3×10^3	8/8	41	5/5	20
3	10^5	8/8	41	5/5	18
	3×10^3	8/8	49	5/5	22
5	10^5	8/8	60	4/4	18
	3×10^3	7/8	63	5/5	24
7	10^5	3/8	94	4/4	21
	3×10^3	1/8	64	5/5	28
8	10^5	1/8	118	5/5	25
	3×10^3	1/8	82	5/5	51
9	10^5	0/8	—	5/5	34
	3×10^3	1/8	108	5/5	47

*10^5 or 3×10^3 living cells of P815 populations obtained after repeated mutagen treatments were injected i.p. in normal mice and in sublethally irradiated mice (750 rad). The initial P1 population was injected as a control.

†No. of mice that developed a progressive tumor/No. of mice injected.

‡Mean survival time of the mice that formed a tumor (days).

Table 3. CTL lysis of P815 tum$^-$ variants obtained after 5 mutagen treatments

Effector cells*	E/T†	Percentage of specific ^{51}Cr-release from target cells‡ P1509	P1517	P1530	P1	L1210
Anti-P1509	30/1	46	24	27	22	1
	10/1	23	13	13	11	<1
	3/1	5	6	3	5	<1
Anti-P1517	10/1	14	62	30	17	<1
	3/1	3	33	10	7	<1
	1/1	<1	13	3	<1	<1
Anti-P1530	10/1	16	28	62	22	<1
	3/1	5	15	29	9	<1
	1/1	1	6	8	3	<1
Anti-H-2^d CTL clone	3/1	46	59	51	74	50
	1/1	19	32	22	39	25
	0.3/1	7	8	6	13	7

*Spleen cells were obtained from DBA/2 mice immunized 2 months earlier with 2×10^5 living P1509, P1517 or P1530 cells and stimulated in MLTC conditions with irradiated cells of the immunizing clone for 5 days. To assess the general sensitivity to lysis of these different targets we also compared their lysis by an anti-H-2^d CTL clone obtained by stimulating *in vivo* primed C57BL/6 lymphocytes with DBA/2 spleen cells.

†The lytic activity of the MLTC cultures was tested in a 4-hr ^{51}Cr-release assay. Cells from syngeneic tumor L1210 were used as a specificity control.

‡E/T: effector/target cells ratio.

Microcultures were obtained that showed specific activity for P1509 and P1517, at frequencies of 1.2×10^{-2} and 2.9×10^{-2} respectively. These frequencies were similar to those previously reported for other tum$^-$ variants obtained after a single mutagenesis [9]. The microcultures were then transferred for further clonal expansion and maintained in culture as decribed earlier [9]. The cytolytic activity of many of these clones was very high and remained stable for at least 70 days of culture. In addition, most of these CTL clones proved strictly specific for the immunizing variant (Fig. 2).

Presence of 3 independent new antigenic determinants on tum$^-$ variant P1517

Previous reports from this laboratory have described the possibility of obtaining antigen-loss secondary variants from tum$^-$ variants either by submitting them to *in vitro* selection with an appropriate CTL clone or by collecting tumor cells from the occasional mice that develop a progressive tumor after being injected with tum$^-$ variants [15, 16].

One million cells of tum$^-$ variant P1517 were incubated for 6 hr with 3×10^6 cells from a CTL clone that lysed P1517 specifically (CTL anti-P1517A). The survivor P1517 cells, which amounted to 2×10^{-3} of the initial cells, were subcloned and all of the clones (12/12) proved to be completely resistant to CTL anti-P1517A. However, these immunoselected clones (P1517.iscA$^-$) were still lysed by other CTL clones that were specific for P1517 (Fig. 3). By submitting P1517 to immunoselection by one of these CTL clones (anti-P1517B) we obtained P1517.iscB clones that were resistant to CTL anti-P1517B but were still lysed by CTL anti-P1517A. These results indicated the presence on P1517 of at least 2 independent variant-specific antigenic determinants. Moreover, we observed that both P1517.iscA$^-$ and iscB$^-$ clones were lysed by yet another CTL clone that showed specificity for P1517 (anti-P1517C). An ascitic tumor obtained in a mouse that had been injected with P1517 was found to be resistant to CTL anti-P1517C while sensitive to CTL anti-P1517A and B. Taken together, these results indicate that the P1517 tum$^-$ antigen consists of 3 determinants that can be lost independently. By repeated *in vitro* selections with different CTL clones it was possible to obtain an antigen-loss variant of the type P1517A$^-$B$^-$C$^-$ which was resistant to all 3 types of P1517-specific CTL clones. Mixed lymphocyte–tumor cell cultures involving as responder cells spleen cells from mice immunized with living P1517 cells and as stimulator cells P1517A$^-$B$^-$C$^-$ failed to produce any cytolytic activity that was specific for P1517, suggesting that there were no additional tum$^-$ specific antigenic determinants on P1517 (data not shown).

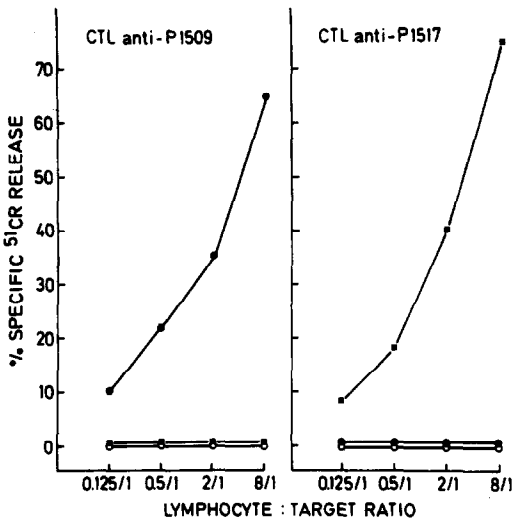

Fig. 2. Lytic activity of CTL clone anti-P1509:19 and CTL clone anti-P1517:22 on target cells P1509 (●), P1517 (■) and P1 (○). Spleen cells from mice immunized with one of these tum$^-$ variants were restimulated in vitro with the same variant in MLTC conditions. They were then restimulated in limiting dilution in the presence of interleukin 2. After 8 days microcultures that specifically lysed the immunizing variant were transferred for further clonal expansion and maintained in culture for 70 days before the test shown here was carried out.

Decreased sensitivity to mitomycin C of tum$^-$ clones obtained after 8 mutagenesis steps

In the course of MLTC experiments where the stimulator tum$^-$ cells had been treated with mitomycin we noticed that many cultures involving tum$^-$ clones obtained after 8 mutagenesis steps contained a large number of living tum$^-$ cells at the end of the stimulation period. We found that several of these tum$^-$ clones had a sensitivity to mitomycin that was significantly lower than that of control P815 cells. For instance, after a treatment of tum$^-$ clone P1814 with 150 μg/ml of mitomycin C for 120 min approximately 1.5×10^{-4} of the initial cells survived whereas with tum$^-$ variant P198 obtained after a single mutagenic treatment less than 10^{-6} survivors were obtained (Table 4).

DISCUSSION

The results described here indicate that by repeated *in vitro* treatments of mastocytoma P815 with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, it is possible to obtain cell populations containing more than 90% tum$^-$ variants. The gradual increase from a few percent tum$^-$ variants after 1 mutagenesis step to 95% after 8 steps demonstrates further the role of the

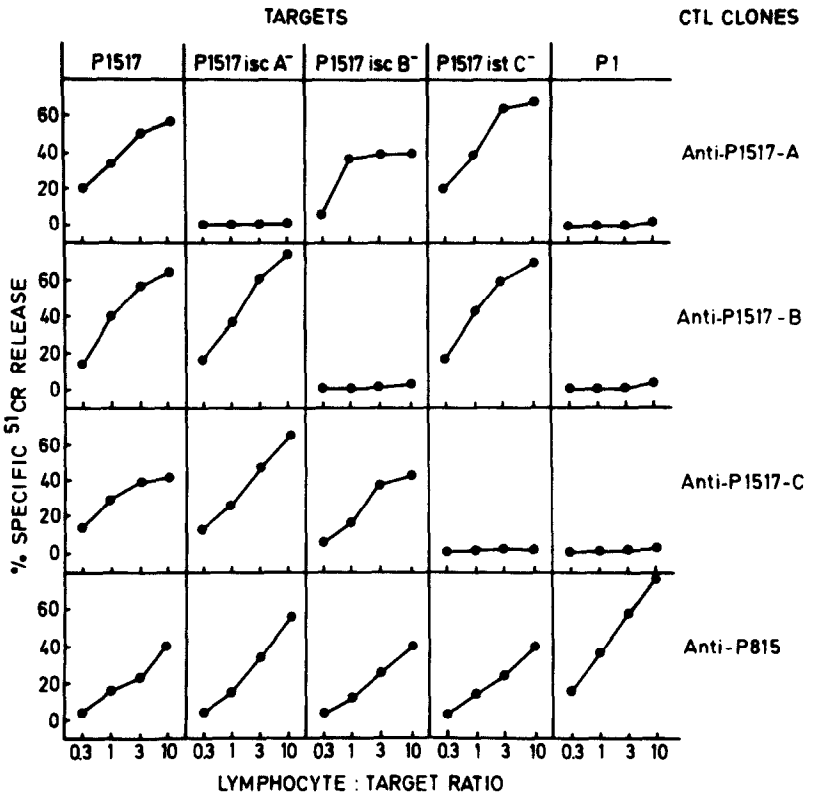

Fig. 3. Lytic activity of CTL clones: CTL-P1517:11 (anti-P1517A), CTL-P1517:28 (anti-P1517B), CTL-P1517:21 (anti-P1517C) and CTL-P198:31 (anti-P815). The first three CTL clones were obtained by immunization and restimulation with P1517 and lysed this tum⁻ variant specifically. The last clone was obtained by immunization and restimulation with P198 and lysed all P815 cells but not L1210 cells [9]. Among the target cells, antigen-loss variant P1517isc.A⁻ and P1517isc.B⁻ were obtained by in vitro *selection with CTL-P1517:11 and CTL-P1517:28 respectively. Antigen-loss variant P1517ist.C⁻ was obtained from a tumor arising after injection of P1517. Four-hours ⁵¹Cr-release tests were performed on 2000 target cells.*

mutagen treatment in the production of the tum⁻ variants. Similar results have recently been reported by Frost *et al.* [6], who obtained a higher frequency of tum⁻ variants in a population of mouse tumor MDAY-D2 treated twice with MNNG than in a population treated once. What may limit this procedure of repeated mutagenesis is the gradual increase in resistance to the mutagen that is observed in the course of the multiple mutagenesis steps. Since we also observed a decreased sensitivity to mitomycin C, it would be interesting to find out whether it is correlated to the MNNG resistance. Perhaps cells are selected that have an increase in the activity of a system of enzymes that repair DNA alterations caused by agents like MNNG and mitomycin C.

It may be noted that the frequency of tum⁻ variants obtained after a single mutagenesis step

Table 4. Reduced sensitivity to mitomycin C of tum⁻ variant P1814 obtained after 8 MNNG treatments

No. of cells per well	Fraction of positive wells obtained with: mitomycin-treated cells P1814	mitomycin-treated cells P198	untreated cells P1814	untreated cells P198
10^5	48/48	0/48	—	—
10^4	47/48	0/48	—	—
3×10^3	22/48	0/48	—	—
10^3	7/48	0/48	—	—
3×10^2	0/48	0/48	—	—
3	—	—	48/48	48/48
0.3	—	—	11/48	14/48

10^7 cells from tum⁻ variant P1814 and control tum⁻ variant P198 (obtained after 1 mutagen treatment) were treated with mitomycin C (150 μg/ml) for 120 min at 37°C in 1 ml of DMEM medium containing 5% FCS. After the treatment the cells were washed by centrifugation and cloned by limiting dilution in culture medium without mitomycin.

reported here (1/78) is lower than that reported previously (11/76) [3]. Besides variability in the mutagenesis conditions, this might be explained by the fact that here the mutagenized clones were tested by injecting 200,000 cells whereas in the previously reported experiment they were tested by injecting 600 cells. Some clones with weak tum$^-$ antigens may produce tumors at the higher number and not at the lower.

We observed that when the proportion of tum$^-$ variants in the whole mutagenized population reaches 90% the population becomes incapable of forming progressive tumors. These results are in agreement with those of Bonmassar and his associates, who reported that by treating L1210 cells with the mutagenic antineoplastic triazenyl-imidazole derivative DTIC either *in vivo* or *in vitro*, populations are obtained that gradually lose their tumorigenicity [17, 18]. It is not surprising that this is not observed with populations containing a lesser proportion of tum$^-$ variants since reconstitution experiments indicate that equal mixtures of tum$^+$ and tum$^-$ cells produce tumors. This is presumably due to the fact that by the time (approximately 14 days) the tum$^-$ variant has produced an immune rejection reponse the tum$^+$ cells have become so numerous that the rejection response fails to eliminate all of them.

Whereas we previously observed that some tum$^-$ variants express two independent variant-specific antigenic determinants, we had not found any variant such as P1517 that expressed three. It is probably not a coincidence that such a variant was found in a population that was mutagenized repeatedly since we observed with a T cell leukemia that when a tum$^-$ variant carrying a tum$^-$ antigen detectable by CTL was submitted again to mutagen treatment a new variant was obtained that carried an additional antigenic specificity [4].

To detect mouse tum$^-$ variants there is no procedure presently available that involves only *in vitro* operations as opposed to injection of mutagenized tumor cells in the syngeneic animal. Up to now it has not been possible to develop reliable *in vitro* stimulation conditions of unprimed lymphocytes that produce a cytolytic T cell response showing specificity for the stimulating variants (Uyttenhove *et al.*, unpublished results). However, it appears that it may be possible to detect tum$^-$ variants of P815 by injecting irradiated cells twice and collecting immunized lymphocytes for mixed lymphocyte tumor cell cultures followed by assessment of cytolytic activity (Marchand *et al.*, unpublished results). While the latter procedure might be applicable to some human situations, it is clear that it will not be possible to screen more than a few putative tum$^-$ clones by this procedure. It may therefore be useful to start from a population that is enriched for tum$^-$ clones by repeated mutagenesis, as described here.

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