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Immunogenic Variants Obtained by Mutagenesis of Mouse Lewis Lung Carcinoma. Recognition of Variant-specific Antigens by Cytolytic T Lymphocytes

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Abstract—*By mutagenesis of a cell line derived from Lewis lung carcinoma (3LL), it is possible to obtain at high frequency stable tumor cell variants (tum⁻) that are rejected by syngeneic mice. The possibility of obtaining a cytolytic T cell (CTL) response directed specifically against these tum⁻ variants was examined. With the four variants that were analysed, a significant cytolytic activity was obtained with peritoneal cells from immune mice collected shortly after an intraperitoneal boost and also with spleen cells after a secondary stimulation* in vitro. *The CTL populations preferentially lysed the immunizing tum⁻ variant, while also showing a cross-reactive lysis against the other variants and the original 3LL cells. Highly active CTL clones could be isolated from limiting dilution microcultures of these CTL populations. The clonal analysis clearly showed the existence of two distinct CTL populations, one directed exclusively against the immunizing variant and another that lysed all 3LL targets equally. This CTL specificity analysis therefore demonstrates directly the presence of new antigens on the 3LL tum⁻ cell variants.*

INTRODUCTION

VARIOUS treatments resulting in artificial antigenicity have been applied to tumor cells, often with the aim of eliciting an immune rejection response against the original tumor. Increased immunogenicity has been described for tumor cells infected with viruses [1–3], for tumor cells whose surfaces have been modified with chemicals [4, 5] and for tumor cells treated *in vivo* with antineoplastic drugs [6]. We reported previously that by *in vivo* treatment of a Lewis lung carcinoma cell line with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, it was possible to obtain at high frequency tumor cell variants (tum⁻) that were rejected by syngeneic mice [7]. Cross-immunization patterns observed *in vivo* suggested that these tum⁻ variants express new individual transplantation antigens. Similar results have been obtained with a mouse teratocarcinoma cell line, with mouse mastocytoma P815 [8–10], with radiation-induced leukemia TH and with a number of spontaneous mouse tumors (Van Pel and Boon, in preparation). A remarkable property of some tum⁻ variants is their ability to confer partial immune protection against the original tumor (tum⁺), even when this tumor appears to be completely deprived of transplantation immunogenicity [9, 11].

Progress in understanding the nature of tum⁻ variants and the mechanism of their protective action will undoubtedly depend on a better characterization of the specific antigens present on these variants. This in turn depends on the possibility of analysing *in vitro* the response against these antigens. For mastocytoma P815 it has been possible to obtain a cytolytic T

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Abbreviations: CTL: Cytolytic T lymphocytes; CTL-P: CTL precursor cell; DMEM: Dulbecco's modified Eagle's medium; FCS: fetal calf serum; HEPES: *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MLC: mixed leukocyte culture; MLTC: mixed leukocyte–tumor cell culture; 3LL: Lewis lung carcinoma; tum⁻: incapable of forming progressive tumors in syngeneic mice; tum⁺: tumorigenic in syngeneic mice.

cell (CTL) response showing specificity for the immunizing tum$^-$ variant. The results indicate that most P815 tum$^-$ variants carry new singular antigens in addition to a common antigen that is present on the original tumor cell line [12]. Our initial attempts to obtain a CTL response against Lewis lung carcinoma using the same immunization and stimulation conditons as those used for P815 uniformly failed. We report here, however, that within a narrow range of stimulation conditions it is possible to obtain a significant and specific CTL activity directed against 3LL tum$^-$ variants. Highly active CTL clones directed against these variants can be obtained and maintained in long-term culture.

MATERIALS AND METHODS

Mice and cell lines

The C57BL/6 mice used in these experiments were derived from breeder animals obtained from J. L. Guénet (Institut Pasteur, Paris). The mice were between 12 and 16 weeks old at the time of the experiments.

The isolation from Lewis lung carcinoma of clonal tum$^+$ cell line L1 and of tum$^-$ variants obtained after treatment of L1 with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, as well as the culture conditions of these cells, were described earlier [7].

Peritoneal cytolytic T lymphocytes

Mice were injected subcutaneously with 2×10^5 living tum$^-$ 3LL cells suspended in culture medium containing 1% fetal calf serum (FCS). After three to six weeks they were injected intraperitoneally with 2×10^6 cells of the immunizing clone that had received 4500 rad of gamma irradiation from a cesium source. Six days later the mice were killed and their peritoneal cavity was rinsed with 4 ml of RPMI-1640 medium (Grand Island Biological Co, Grand Island, NY, U.S.A.) supplemented with 10% FCS, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and 10 U/ml heparine (Roche, Basel, Switzerland). The peritoneal cells were washed once and resuspended in Dulbecco's modified Eagle's medium (DMEM) (cat. No. 1600, Grand Island Biological Co.) 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 HEPES, 5×10^{-5} M 2-mercaptoethanol and 5% FCS. The cells were then tested directly in a chromium release assay.

Mixed leukocyte–tumor cell cultures (MLTC)

Spleens from mice immunized with 2×10^5 living tum$^-$ cells 3–12 weeks earlier were teased in Hank's balanced salt solution supplemented with 5% FCS. Cell suspensions were filtered through a nylon mesh gauze (80 micron, Nylon Swiss, Staniar, Manchester, U.K.). Spleen cells (3×10^7) were cultured with 1×10^5 irradiated (10,000 rad) cells of the immunizing variant in 10 ml of DMEM supplemented as described above except that 10% FCS was added. Cultures were maintained in 50-ml tissue culture flasks (Falcon 3013, Div. of Becton Dickinson Co., Oxnard, CA, U.S.A.) standing upright. After 6 days of incubation at 37°C in a humidified atmosphere of air containing 8% CO_2, the stimulated splenocytes were centrifuged, resuspended in the same medium containing 5% FCS and tested in a chromium release assay.

Limiting dilution MLTC

Culture conditions were similar to those described by Brunner *et al.* [13]. Responder cells (either immune spleen cells or day 5–7 MLTC cells) were plated in limiting dilution numbers with 10^6 irradiated (2000 rad) C57BL/6 spleen cells and 3×10^3 irradiated (10,000 rad) tumor cells of the immunizing clone. Microcultures were prepared in round-bottom microtiter plates (96 wells Titertek, Flow Laboratories, U.S.A.) and contained 0.1 ml DMEM supplemented as described for MLTC and 0.1 ml supernatant from a secondary allogeneic mixed leukocyte culture (MLC) (C57BL/6 anti-DBA/2) as a source of interleukin-2 [14]. The plates were maintained at 37°C in air with 8% CO_2.

Cultures of cytolytic T lymphocytes (CTL clones)

The procedure was adapted from that described by Weiss *et al.* [15]. CTL clones were taken from selected microcultures and transferred to flat-bottom wells (24 wells, Linbro Chemical Co., Hamden, CT, U.S.A.) containing 5×10^6 syngeneic irradiated (2000 rad) splenocytes, 1×10^4 irradiated (10,000 rad) tumor cells from the immunizing clone in 1 ml of culture medium supplemented with 5% FCS and 50% secondary MLC supernatant. The clones were passaged every 4–6 days by transferring 5×10^4 cells to fresh cultures of irradiated splenocytes and tumor cells supplemented with supernatant.

CTL clones are designated as CTL-*X* : *Y*, where *X* is the variant used for immunization of the mice taken as a source of immune spleen cells and *Y* is the serial number of the clone (e.g. CTL-L62:1).

Chromium release assay

Target cells (3×10^6) grown *in vitro* were suspended in 0.3 ml of Hanks medium supplemented with 10% FCS and incubated at 37°C with 150 μCi of sodium 51 chromate (Institut des radioéléments, Fleurus, Belgium). After 1 hr, the labeled cells were washed twice with 50 ml of Hanks medium supplemented with 5% FCS. They were then resuspended in DMEM supplemented as described above and 50 μl aliquots containing 5×10^3 cells were distributed into conical microplates (96 wells Titertek). Stimulated effector cells were added in 100 μl of the same medium. Assays were made in triplicate. The plates were centrifuged for 5 min at 100 *g* and incubated for 8 hr unless otherwise mentioned at 37°C in air with 8% CO_2. They were then centrifuged again and 75-μl aliquots of supernatant were collected and counted.

The percentage of [^{51}Cr]-specific release was calculated as follows: % specific release = (ER-SR) × 100/(MR-SR), where ER was the observed experimental [^{51}Cr] release, SR the spontaneous release measured by incubation of 5×10^3 labeled cells in 150 μl of DMEM medium alone and MR the maximum release obtained by adding to the target cells 100μl of 0.3% Triton X-100 (Sigma Chemical Co., St. Louis, MO). The spontaneous release was never higher than 25% of the maximum release. One lytic unit is defined here as the number of spleen cells that lyse 50% of 5×10^3 target cells in 8 hr. This number was estimated from the specific release obtained at various effector to target ratios by means of the regression $(1 - e^{-kx})$. The results are expressed in number of lytic units/10^6 cells.

For limiting dilution microcultures the assays were performed similarly, except that 60-μl aliquots of cells from individual microcultures were added to 2×10^3 target cells in a final volume of 200 μl. After 4 or 8 hr incubation at 37°C the plates were centrifuged and 100 μl of supernatant was removed for counting. Microcultures were considered positive for cytolytic activity when the mean spontaneous release obtained in the absence of responder cells was exceeded by at least five standard deviations. Minimal estimates of CTL-P frequency were then calculated from the zero-order term of the Poisson distribution by the statistical method of chi-square minimization, as described by Taswell [16]. Only frequency determinations for which the Poisson hypothesis was not rejected ($P > 0.05$) are reported.

Purification of T cell and non-T cell populations

The T cells were purified according to the technique of Julius *et al.* [17]. To purify the non-T cell population an aliquot of 10^7 effector cells was incubated on ice for 20 min with monoclonal antibodies directed against the Thy 1.2 antigen (NEI-001, New England Nuclear, Dreieich, W. Germany) at a final dilution of 1/1000. Rabbit complement (CL3051 Cedarlane, Canada) was then added at a final dilution of 1/30. The suspension was incubated for 1 hr at 37°C. The cells were then washed in 50 ml Hank's medium supplemented with 2% FCS. The purity of the final populations was tested by immunofluorescence using fluorescein-labeled monoclonal antibodies directed against the Thy 1.2 antigen (cat. No. 1333, Becton Dickinson, Sunnyvale, CA, U.S.A.).

RESULTS

As reported previously, tum⁻ variants L20, L48, L50 and L62 were obtained after mutagenesis of malignant clone L1 (tum⁺) derived from a Lewis lung carcinoma cell line [7]. Syngeneic C57BL/6 mice injected with 3×10^5 living cells of these variants routinely reject them. Upon subsequent irradiation and challenge with various 3LL cells these mice show a significant protection, with definite specificity for the immunizing variant. Representative data are summarized in Table 1.

Cytolytic activity of immune peritoneal T lymphocytes

Mice that had rejected tum⁻ variants were boosted intraperitoneally with the same variant. Six days later the peritoneal cells were tested for cytolytic activity against different tum⁻ variants and against L1. A significant cytolysis directed against the immunizing variant was obtained (Table 2). The effector cells were T lymphocytes, as shown by their presence in

Table 1.

Immunizing clone*	No. mice with tumors/No. mice injected Challenging clone†			
	L20	L50	L62	L48
L20	1/19	12/18	12/22	20/28
L50	17/18	1/20	7/14	14/22
L62	17/18	18/19	0/24	16/18
L48	19/20	14/17	10/18	1/20
—	20/20	18/20	20/24	21/23

*C57Bl/6 mice were injected subcutaneously with 3×10^5 living cells of tum⁻ clones L20, L50, L48 or L62. Controls were injected with the same amount of injection medium.

†After 22 days, immunized and control mice received 600 rad of gamma radiation and were injected subcutaneously with 1×10^5 (L62) or 5×10^4 (L20, L50, L48) living tum⁻ cells (for details see ref. [7]).

Table 2. Cytolysis of tum⁻ variants by peritoneal cells

Experiment No.	Peritoneal* effector cells	Effector: target ratio	Percentage of specific [^{51}Cr] release from target cells					
			L20	L48	L50	L62	L1	Blasts†
1	anti-L20	60:1	33	1	3	4	3	<1
		20:1	11	<1	2	<1	<1	<1
	anti-L48	60:1	7	37	7	6	8	<1
		20:1	3	9	4	2	3	<1
	anti-L50	60:1	6	4	24	4	8	<1
		20:1	2	2	8	1	3	<1
2	anti-L62	30:1	<1	<1	<1	35	.5	<1
		10:1	<1	<1	<1	13	<1	<1
	anti-L62 T cells‡	30:1	<1	<1	<1	38	<1	<1
	anti-L62 T cells depleted§	30:1	<1	<1	<1	<1	<1	<1

*C57Bl/6 mice were injected subcutaneously with 2×10^5 living L20, L48, L50 or L62. After 23 (experiment 1) or 60 (experiment 2) days they received an intraperitoneal boost with 2×10^6 irradiated cells (4500 rad) of the immunizing clone. The peritoneal cells were collected 6 days later and assayed immediately in an 8-hr [^{51}Cr] release test.

†C57Bl/6 spleen cells (10^6/ml) were stimulated *in vitro* for 48 hr with concanavalin A (4 μg/ml).

‡Filtered once on nylon wool, the control with fluorescein-labeled anti-Thy 1.2 antibodies showed an enrichment from 20 to 83% of T cells.

§Treated with anti-Thy 1.2 (monoclonal antibodies) and complement. Controls with fluorescein-labeled anti-Thy 1.2 antibodies showed no more T cells.

nylon-filtered populations and by their inactivation with anti-Thy 1.2 antibodies. In agreement with the specificity of the protection observed *in vivo*, the cytolytic activity was preferentially directed against the immunizing variant. No lysis was observed against syngeneic lymphocyte blasts obtained with concanavalin A.

Cytolytic activity of immune spleen cells stimulated in vitro

When spleen cells of animals that had been immunized and boosted so as to contain active peritoneal cells were collected and tested immediately, no measurable cytolytic activity was obtained. However, a cytolytic response was observed when immune spleen cells were submitted *in vitro* to a secondary immunization in a mixed leukocyte–tumor cell culture (MLTC). The magnitude of the cytolytic response was found to be critically dependent on the duration of the stimulation and on the dose of stimulatory cells. A significant activity was obtained only when spleen cells were stimulated for five or six days at ratios of responder to stimulator cells ranging from 150 to 500 (Fig. 1). The cytolytic activity was completely abolished by treatment of responder cells with anti-Thy 1.2 antibodies and complement. No specific cytolytic activity was obtained after stimulation of spleen cells from naïve animals (data not shown).

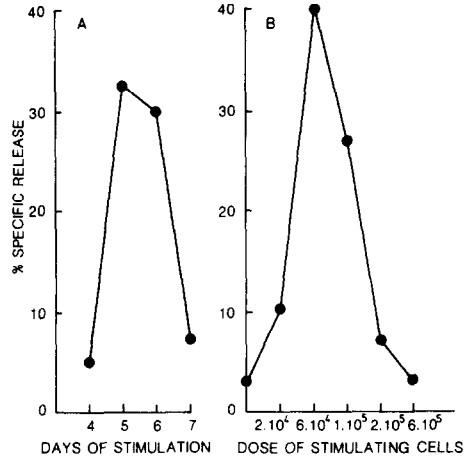

Fig. 1. Effect of duration of MLTC and dose of stimulatory cells on the level of immune CTL lysis against tum⁻ variant L62. Spleen cells (3×10^7) from mice immunized against L62 were incubated in 10 ml total volume with either 10^5 irradiated L62 cells for 4–7 days (A), or with 2×10^4–6×10^5 irradiated L62 cells for 6 days (B). The lytic activity against L62 was measured in a 4-hr chromium release assay at an effector to target ratio of 30 to 1.

As shown in Table 3, the spleen CTL obtained after MLTC preferentially lysed the immunizing variant. This confirms the results obtained with peritoneal CTL and indicates clearly that each of the four tum⁻ variants carries a different new antigen. The most specific CTL response was obtained with variant L62, which also confers the least amount of cross-protection *in vivo*.

Table 3. Specificities defined by immune spleen cell cytolysis

Experiment No.	Spleen* effector cells	Effector: target cells ratio	Percentage of specific [^{51}Cr] release from target cells					
			L1	L20	L48	L50	L62	Bl†
1	anti-L20	30:1	24	54	28	22	25	—
		10:1	11	30	13	10	8	—
		3:1	4	13	6	3	3	—
			(1.4)‡	(4.2)	(1.7)	(1.2)	(1.3)	
	anti-L48	30:1	30	26	47	27	26	—
		10:1	15	13	27	11	12	—
		3:1	5	6	14	5	4	—
			(1.8)	(1.6)	(3.5)	(1.6)	(1.6)	
	anti-L50	30:1	35	34	32	61	33	—
		10:1	16	17	15	38	13	—
		3:1	7	6	7	22	6	—
			(2.2)	(2.1)	(2)	(7.9)	(2)	
	anti-L62	30:1	18	21	19	12	70	—
		10:1	9	8	8	6	61	—
		3:1	3	2	3	2	37	—
			(1)	(1.1)	(1.1)	(.7)	(16.5)	
2	anti-L20	30:1	16	32	21	15	21	<1
	anti-L48	30:1	9	15	24	13	14	<1
	anti-L50	30:1	11	10	6	48	11	<1
	anti-L62	30:1	<1	3	<1	2	52	<1

*C57Bl/6 mice were immunized with 2×10^5 living tum⁻ variants. After 21 (experiment 2) or 28 (experiment 1) days their spleen cells were restimulated *in vitro* with the irradiated immunizing variant for 6 days, then assayed in an 8-hr [^{51}Cr] release test.

†Obtained by stimulating normal syngeneic lymphocytes (10^6/ml) with 4 μg/ml concanavalin A for 48 hr.

‡Lytic units/10^6 cells.

A significant level of cross-reactive lysis was observed on all 3LL targets, indicating the existence of a common antigen already present on the tum⁺ cells. No lysis was observed against syngeneic concanavalin A blasts.

Clonal analysis of the cytolytic response

Although the results obtained with mass culture populations clearly demonstrated the existence of tum⁻ specific antigens, the cytolytic activities obtained with such populations were rather low and contained an important component directed against the tum⁺ antigen. We therefore attempted to isolate individual clones directed exclusively against the tum⁻ antigen. These clones were isolated by limiting dilution in the presence of interleukin-2, irradiated feeder cells and stimulating cells under conditions similar to those described by Brunner *et al.* [13]. The anti-L62 immune spleen cells were first stimulated in MLTC and then stimulated again in limiting dilution microcultures with variant L62. In three experiments the frequency of CTL-P ranged from 1×10^{-3} to 2.3×10^{-3}. This was approximately 500-fold higher than the frequency of CTL-P in anti-L62 immune spleen cells directly stimulated in limiting dilution microcultures.

The specificity of anti-L62 CTL clones obtained in limiting dilution microcultures was examined by assaying these clones on two different 3LL targets. As shown in Fig. 2, most of the positive microcultures had a lytic activity directed against the immunizing variant and not against other 3LL cells. Similar results were obtained with CTL clones derived from mice immunized with tum⁻ variant L50. Other CTL clones were obtained that lysed all 3LL cells.

A number of the CTL clones shown in Fig. 2 were transferred for further expansion to 1-ml cultures with irradiated C57BL/6 spleen cells, tum⁻ stimulator cells and interleukin-2. The cells were subsequently transferred every 3–4 days under the same conditions. Transfers of 5×10^4 cells routinely produced 1×10^5–3×10^5 cells. These long-term CTL clones showed a high level of activity and conserved their specificity during more than 30 days of continuous culture. Table 4 shows results obtained with several clones that were specific for variant L50 or L62 and one clone that lysed equally well all the 3LL targets but not syngeneic blasts.

DISCUSSION

The results presented here demonstrate the existence of a syngeneic CTL response directed

Table 4. Antigenic specificities recognized by immune CTL clones

Experiment No.	CTL clones* (specificity)	Effector: target ratio	Percentage of specific [^{51}Cr] release from target cells L1	L50	L62	Blasts
1	CTL-L50:4†	3:1	4	56	9	<1
	(anti-L50)	1:1	2	32	5	<1
		.3:1	1	22	4	<1
	CTL-L50:9	3:1	9	72	17	<1
	(anti-L50)	1:1	3	61	13	<1
		.3:1	<1	56	5	<1
	CTL-L50:8	3:1	52	38	41	<1
	(anti-3LL)	1:1	34	33	35	<1
		.3:1	33	26	28	<1
2	CTL-L62:8	3:1	5	3	76	<1
	(anti-L62)	1:1	4	2	59	<1
		.3:1	2	2	35	<1
	CTL-L62:4	3:1	14	9	61	<1
	(anti-L62)	1:1	8	6	47	<1
		.3:1	4	4	28	<1

*The CTL clones were maintained in culture for 30 days (CTL-L50) or 50 days (CTL-L62) before being tested.

†CTL-L50: CTL obtained from mice immunized against L50; CTL-L62: CTL obtained from mice immunized against L62.

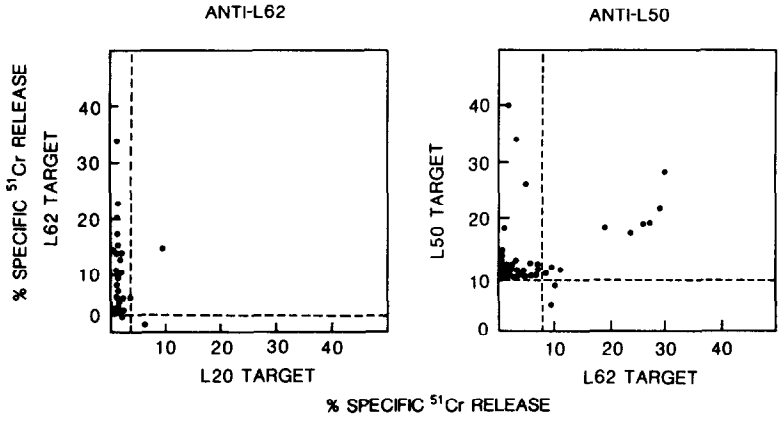

Fig. 2. Cytolytic specificity of microcultures prepared by stimulating anti-L62 or anti-L50 immune spleen cells with the immunizing variant. Each point represents the lysis obtained with an individual positive microculture on the two relevant targets. Spleen cells from mice immunized with 2×10^5 living L50 or L62 cells 45 days before were stimulated in mass MLTC with the immunizing variant. After 6 days, anti-L62 (60 cells per culture) or anti-L50 (300 cells per culture) MLTC responder cells were restimulated in microcultures with the homologous (L62 or L50) variant. After 8 days, aliquots from each microculture were assayed in a 4-hr chromium release test against L62 and L20 (anti-L62 effectors) or in a 8-hr test against L50 and L62 (anti-L50 effectors). The clone probability was 92% for the anti-L62 response (27 positive cultures out of 192) and 96% for the anti-L50 response (45 positives out of 576). The broken line indicates the mean spontaneous release plus five standard deviations, which defines the minimum value for positive cultures.

against four different tum$^-$ variants obtained from Lewis lung carcinoma. The pattern of CTL activity clearly indicates that each of these tum$^-$ variants carries a specific new 'tum$^-$' antigen. This is concordant with the results obtained *in vivo* in protection experiments [7]. We also observed a weaker CTL response against a common antigen present on all the tum$^-$ variants and on the original tum$^+$ cells. Previous reports have already suggested the presence of a syngeneic cytolytic response against a specific 3LL antigen [18, 19]. This weak antigen probably accounts for the protection conferred by tum$^-$ variants against the original Lewis lung carcinoma cells [7]. All instances of CTL cross-reaction between different tum$^-$ variants could be explained by the presence of this common antigen. The existence of an unique and a common antigen on each variant is further indicated by the clonal analysis of the CTL response. This analysis shows that tum$^-$ variants elicit the proliferation

of two distinct classes of CTL: one class directed against the specific tum⁻ antigen and another directed against the common antigen.

These results are in complete agreement with those reported previously for the CTL response directed against tum⁻ variants derived from mastocytoma P815 [12]. However, the lytic activities obtained against 3LL variants with peritoneal exudate cells or spleen cells restimulated in MLTC are markedly lower than those obtained against P815 variants. This is not due to intrinsic weaker lytic activity of the CTL on 3LL cells as the CTL clones directed against 3LL variants have approximately the same activity as those directed against P815 variants [20]. Rather, it can be explained by the different frequencies of CTL precursors found in immune spleens. For 3LL variants, stimulation of immune spleen cells in limiting dilution conditions indicated a frequency of CTL-P in immune C57BL/6 spleen of the order of 10^{-6}, while the corresponding frequency obtained with DBA/2 mice immunized with P815 variants was of the order of 10^{-4} [20]. For comparison, the corresponding values obtained with C57BL/6 mice immunized either against allogeneic H-2^d cells or a syngeneic murine sarcoma virus-induced tumor are 1/330 [21] and 1/443 [13] respectively.

The specificity pattern obtained with CTL appears to reflect closely that observed *in vivo*. Tum⁻ variant L62, that confers little if any cross-protective immunity *in vivo*, elicits the CTL response that shows the least amount of cross-reactive activity. While this is compatible with the notion that CTL are effective in the rejection of 3LL variants, more direct evidence will be required to establish the role of CTL in the rejection of 3LL tum⁻ variants.

Our attempts to generalize the *in vitro* analysis of tum⁻ variants have two main justifications. First, the *in vitro* analysis, particularly with CTL clones, allows a precise characterization of the tum⁻ antigens. For instance, we have observed recently that partial antigen-loss variants can be obtained for P815 tum⁻ variants by *in vitro* selection with CTL clones [22]. This allows us to separate complex tum⁻ antigens into simpler components, a prerequisite for a genetic analysis of the tum⁻ phenomenon. Hopefully, a better characterization of the tum⁻ antigen will also help us to find out why some tum⁻ variants confer a better protection than others against tum⁺ cells. A second justification for the *in vitro* analysis is as follows. It is now clear that immunogenic variants with remarkably similar properties have been obtained with four different mouse tumors. It appears likely, therefore, that this phenomenon is generally applicable to all mouse tumor cells, and it is also tempting to consider the possibility that similar variants can be obtained with human tumor cells. However, it is obvious that with human cells the mutagenized clones will have to be tested *in vitro* for the presence of new antigens. The analysis with immune CTL can be considered as a first step in developing methods whereby tum⁻ variants can be detected *in vitro* with normal syngeneic lymphocytes.

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