

ESCAPE OF MOUSE MASTOCYTOMA P815 AFTER NEARLY COMPLETE REJECTION IS DUE TO ANTIGEN-LOSS VARIANTS RATHER THAN IMMUNOSUPPRESSION

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It is a common observation that many tumors of unquestionable immunogenicity grow progressively and eventually kill their syngeneic host. This is observed, for instance, with many methylcholanthrene-induced tumors that are able to elicit concomitant immunity (1-3) as well as a measurable cytolytic T lymphocyte (CTL)¹ response during the onset of tumor growth. It is possible that some tumors outpace the immune defenses of the host by their ability to multiply and metastasize very rapidly. However, this is certainly not a general explanation, since for some tumors an initial period of growth is followed by a period of stagnation or partial regression followed in turn by a resumption of tumor growth and death of the host. Mastocytoma P815 belongs to this category (4-6), where a partial immune rejection of the tumor by the syngeneic host is often observed but very rarely results in the complete elimination of the tumor cells.

Evidence suggesting that suppressor cells may be the cause of this phenomenon has been presented by Takei et al. (4, 7), who reported that suppressor T lymphocytes capable of inhibiting specifically the development of a CTL response against mastocytoma subline P815-X2 appeared in the spleen after a period of partial rejection. On the other hand, Biddison and Palmer (5) showed that tumor cells of subline P815-Y that were collected from the peritoneal cavity a few days after the partial rejection phase were much less sensitive than the original tumor cells to CTL found among peritoneal exudate cells of tumor-bearing animals. They suggested that this could be due either to the emergence of stable antigen-loss variants or to a modulation phenomenon.

We report here the results of experiments aimed at evaluating the contribution of immunosuppression and tumor cell modification in the escape of P815-X2 tumor cells after a nearly complete elimination by the immune defenses of the syngeneic host.

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, indifferently males or females, were between 10 and 14 wk old.

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¹ *Abbreviations used in this paper:* CTL, cytolytic T lymphocytes; DMF, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; i.p., intraperitoneally; HAT, 10^{-4} M hypoxanthine, 3.8×10^{-7} M aminopterin, 1.6×10^{-5} M 2-deoxythymidine; MLTC, mixed lymphocyte-tumor cell culture.

Culture and Cloning Conditions of Tumor Cells. Our P815 subline was P815-X2. The P815 cells were cultured in Petri dishes (Falcon 1001; Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA) in Dulbecco's modified Eagle's medium (DME) (1600; Grand Island Biological Co., Grand Island, NY) supplemented with 5–10% fetal calf serum (FCS) in an atmosphere containing 8% CO₂.

P815 clones were isolated by a limiting dilution procedure: diluted P815 cells resuspended in medium containing 10–20% FCS were distributed into microplates (96 round-bottom wells; Linbro Chemical Co., Hamden, CT). Plating efficiency was ~0.7–0.9.

Azaguanine-resistant Mutants. Azaguanine-resistant (*aza*^r) mutants were selected in limiting dilution conditions in culture medium supplemented with 10% FCS that contained 10 µg/ml of azaguanine (ICN Nutritional Biochemicals, Cleveland, OH). We used mutants that died in a culture medium supplemented with 10⁻⁴ M hypoxanthine (Merck AG, Darmstadt, Federal Republic of Germany), 3.8 × 10⁻⁷ M aminopterin (ICN Nutritional Biochemicals), and 1.6 × 10⁻⁵ M 2-deoxythymidine (Merck Ag) (HAT).

Injection of P815 Cells. P815 cells were injected intraperitoneally (i.p.) in DBA/2 mice in DME that contained no FCS. For the production of ascites cells, DBA/2 mice, given 700–750 rad of gamma radiation from a cesium source, were injected i.p. with 2 × 10⁶ P815 cells. Ascites cells were collected 3–6 d later.

Quantitation of Intraperitoneal P815 Cells by Colony Assay. Mice were injected i.p. with 3 ml of DME. A 0.3-ml sample of intraperitoneal fluid was immediately collected with a syringe and transferred to a tube that contained 6 µl of heparin (H-3125; Sigma Chemical Co., Sigma, St. Louis, MO). The cells were diluted in DME that contained 1% FCS. Aliquots of 0.2 ml were mixed in a petri dish (Falcon 1007) with 2 ml DME that contained 10% FCS and 0.4% Bactoagar (Difco Laboratories, Detroit, MI). After a 3- to 4-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 the cloning efficiency of in vitro growing P815, which was ~0.3. We verified with control animals that peritoneal exudate cells do not form colonies under those conditions.

To distinguish normal and azaguanine-resistant tumor cells growing in the same peritoneal cavity, the cells were plated in agar containing either 10 µg/ml of azaguanine or HAT medium. Normal P815 cell populations contained <10⁻⁵ cells capable of growing in azaguanine containing medium, while azaguanine resistant populations contained 10⁻⁶ cells capable of multiplying in HAT medium.

CTL Clones. The derivation of a CTL clone specific for P815 (CTL-P35:10) has been described (8). Other CTL clones were obtained by a similar procedure as follows. Spleen cells (3 × 10⁷) from mice immunized 4–15 wk previously with either irradiated (10,000 rad) P1 cells or living cells from P815 tum⁻ variant P89 were cultured in mixed leukocyte-tumor cell culture (MLTC) with 3 × 10⁵ stimulator cells. The origin of the stimulator cells is given below. The MLTC were established in 15 ml of DME supplemented with L-arginine HCl (116 mg/liter), L-asparagine (36 mg/liter), L-glutamine (216 mg/liter), glucose (4.5 g/liter final concentration), 10 mM Hepes, 5 × 10⁻⁵ M 2-mercaptoethanol, and 10% FCS (vol/vol). Cultures were maintained in tissue culture flasks (3013 Falcon) kept in an upright position at 37°C in an atmosphere of air with 8% CO₂. After 7 d of incubation, MLTC cells were cloned by limiting dilution. Limiting numbers of MLTC cells were plated with 7 × 10⁵ irradiated (2,000 rad) DBA/2 spleen cells and irradiated (5,000 rad) stimulator cells (3 × 10³) that were used for MLTC. Microcultures were prepared in round-bottomed microtiter plates (Titertek, cat. no. 76-213-05; Flow Laboratories, Rockville, MD) and contained 150 µl of DME supplemented as for MLTC, except that supernatant (25–50%) from secondary mixed leukocyte culture was added as a source of T cell growth factors (9). The plates were maintained for 6–8 d at 37°C in an atmosphere of air with 8% CO₂. After 7 d, aliquots from each microculture were tested for cytolytic activity against the appropriate target cells, as described (10).

CTL from selected microcultures were transferred to cultures containing 5 × 10⁶ irradiated (2,000 rad) DBA/2 spleen cells, 10⁵ irradiated (5,000 rad) P815 stimulator cells in 1 ml of supernatant-supplemented medium as described above. For subsequent cells transfers (every 3–5 d), 5 × 10⁴ cells were transferred to new, identical 1-ml cultures.

CTL clones are designated as CTL-X:Y, where X is the P815 clone used for immunization

of the mice taken as a source of immune spleen cells, and Y is the serial number of the clone. The stimulator cells used to isolate the new CTL clones described herein were as follows: for CTL-P1:3, P1; for CTL-P89:15, P89; for CTL-P89:20, cells of immunoselected clone P1-20-4 described in Results.

Measurement of the Lysis of P815 Cells by CTL Clones with a Colony Assay. P815 cells were collected from the peritoneal cavity, counted, resuspended in DME containing 10% FCS and directly mixed with CTL clones at various effector-to-target ratios in a volume of 200 μ l in 96-well round-bottomed microplates. After an incubation of 3 h at 37°C, 150 μ l of each well was mixed with 1 ml DME containing 10% FCS and 0.4% Bactoagar in a 9.6-cm² plate (Linbro 76-047-05; Flow Laboratories). After a 3-to-4-d incubation at 37°C the P815 colonies were counted.

Chromium Release Assays. Cytolytic assays were performed as described previously (10) with ⁵¹Cr-labeled target cells. All assays were performed in DME containing 5% FCS in conical microplates (Titertek, cat. no. 76-223-05; Flow Laboratories) with 2,000 targets per well.

Selection with CTL Clones. The selection of antigen-loss variants with CTL clones was described elsewhere (11). Briefly, 10⁶ P815 cells were mixed with the appropriate CTL clone (2–4 \times 10⁶ cells) in 2–5 ml of medium and incubated for 4 h. A portion of the selected cell suspension was put in culture to serve as a source of cells for a second selection. The selected P815 cells were also cloned in limiting dilution. Selected P815 clones were transferred to petri dishes for continuous culture.

Detection of Antigen Expression on P815 Clones by a Growth Inhibition Assay. The cells to be tested were cultured under limiting dilution conditions, and 7–10 d later, aliquots (20–50 μ l) from positive wells were transferred to round-bottomed microplates. A 10-fold dilution was also prepared in DME containing 5% FCS. The cells were incubated alone or together with 10⁴ cells from the appropriate CTL clone. Controls were prepared with cells from the original P1 clone. After 2–3 d, the wells were examined microscopically for the presence of a large number of growing tumor cells. Subsequent chromium release tests indicated that the growing cells were always resistant to the CTL clone used in the assay.

Results

Partial Rejection and Subsequent Escape of P815. To follow the fate of P815-X2 cells injected intraperitoneally in syngeneic DBA/2 mice, we repeatedly collected samples from the peritoneal cavity and estimated the number of viable tumor cells with an agar colony assay. Representative results obtained after the injection of 600 tumor cells are shown in Fig. 1. P815 cells multiplied exponentially to reach a number higher than 10⁸ around day 10 after injection. In some mice, the number of P815 cells continued to increase and the animals died around day 20. In others, a sudden drop in the number of tumor cells was observed between 10 and 17 d after injection together with a large increase in the number of lymphocytes and macrophages present in the peritoneal cavity. The decrease in the number of tumor cells was very variable from one mouse to another. For some mice, the number of P815 cells dropped from >10⁸ to <10⁴. This low level of intraperitoneal P815 cells was observed during periods varying from a few days to 3 wk. Eventually however, the P815 cells multiplied again and all the mice died.

The partial rejection phase was never observed for mice that had received an immunosuppressive dose of 600 rad of gamma radiation a few hours before the injection of tumor cells. Thus, it appears that, when P815 cells undergo a potent immune rejection by the host, a few surviving cells always escape and eventually multiply and kill the mice.

Rejection of Newly Injected P815 Cells at the Time of P815 Escape. To determine whether P815 tumor escape could be due to a suppression of the anti-P815 immune response, we tested whether, at the time of tumor cell escape, the mice were still

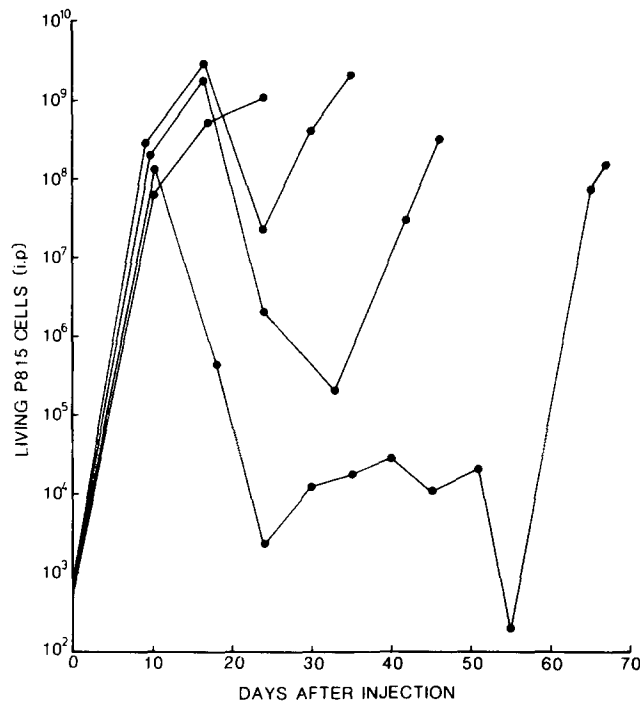


FIG. 1. Four DBA/2 mice were injected i.p. with 600 living cells of P1, a P815 clone. At various times thereafter, samples of the intraperitoneal fluid were collected and the number of living P815 cells was estimated in an agar colony test. Each curve represents an individual mouse and ends on the last sampling before the death of the animal.

capable of rejecting control P815 cells. For this experiment we used a P815 clone (P1) and an azaguanine-resistant mutant derived from it (P1.aza^r). P1 and P1.aza^r are equally tumorigenic. The number of each cell type present in the peritoneal cavity was estimated by plating the peritoneal content in agar with HAT medium, where only P1 formed colonies, or with azaguanine, where only P1.aza^r formed colonies.

A representative experiment is shown in Fig. 2A. A DBA/2 mouse was injected with 600 P1 cells. These cells were largely rejected around day 20. By day 31, they were again multiplying rapidly. P1.aza^r cells maintained *in vitro* were then injected in the same peritoneal cavity. While the P1 cells continued to grow rapidly and killed the host a few days later, the P1.aza^r cells were completely eliminated in a few days. This showed that the animal where tumor escape was occurring was still capable of rejecting P815 cells and the the escaping tumor cells were resistant to this anti-P815 response.

Three independent experiments of the kind described in Fig. 2A were performed. The P1.aza^r cells injected at the time of tumor escape were invariably rejected. Concordant results were obtained when mice were first injected with P1.aza^r cells and injected with P1 cells at the time of tumor escape. To exclude the possibility that our results might be due to problems of adaptation of *in vitro* growing cells to *in vivo* conditions, we injected mice in which P1 cells were escaping with P1.aza^r cells collected from ascites of irradiated animals. Similar results were obtained in five independent experiments (Fig. 2B). We also verified that P1.aza^r cells obtained either

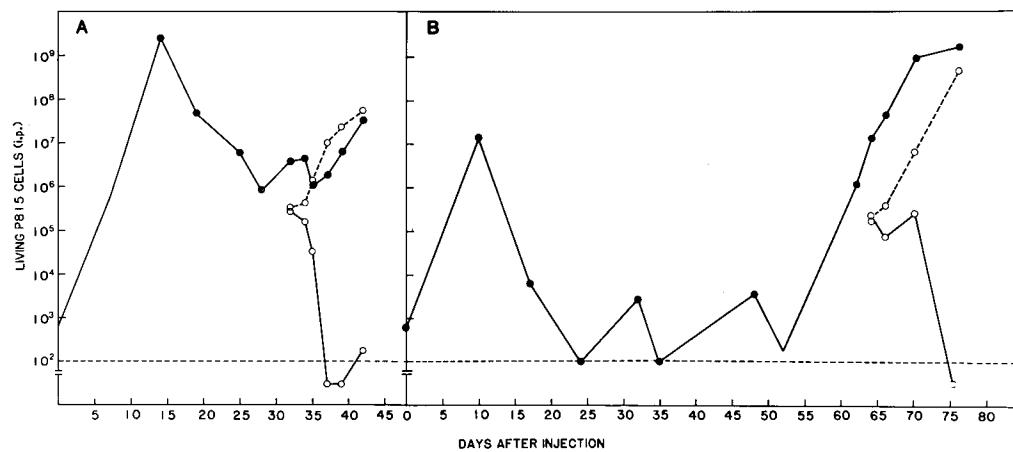


FIG. 2. Two DBA/2 mice were injected i.p. with 600 P1 cells (—●—). At various times thereafter, samples of the peritoneal fluid were collected and the number of P1 cells was estimated in an agar colony assay. On the day indicated on the graph, 3×10^5 P1.aza^r cells obtained from in vitro culture (A) or collected from the ascites of an irradiated DBA/2 mouse (B) were injected i.p. into the test animal (—○—) or into control DBA/2 mice (---○---). The number of viable P1 and P1.aza^r cells in the peritoneal cavity was estimated by plating samples in agar containing HAT or azaguanine, respectively.

from ascites or from long-term culture multiplied exponentially without significant latency in control mice and in mice that had been injected i.p. with 600 cells 10 d before and contained $\sim 10^7$ exponentially growing tumor cells.

Further evidence that escaping P815 cells were resistant to the rejection response directed against P815 was obtained as follows. We found a DBA/2 mouse that had rejected P1.aza^r to <100 cells, the detection threshold in our colony assay. This mouse was then injected i.p. with control P1.aza^r cells obtained from the ascites of an irradiated animal together with P1 cells from an animal showing tumor escape. The results are shown in Fig. 3. The control cells were completely rejected whereas the cells that had escaped previously multiplied rapidly.

Resistance of Escaping P815 Cells to an Anti-P815 CTL Clone. We have described long-term syngeneic CTL clones that specifically lyse tumor P815 (8). These CTL clones could be derived from mice that had rejected P815 tum⁻ variants, i.e., immunogenic variants obtained by mutagenesis that fail to form tumors in syngeneic mice. We used these clones to determine whether P815 antigens were still present on the escaping tumor cells.

Clone CTL-P35:10 was obtained by limiting dilution stimulation of spleen cells that were primed and restimulated with tum⁻ variant P35. This CTL clone lyses all P815-derived cells and is inactive on syngeneic tumor L1210. Three independent escaping tumor cell populations were tested with this CTL clone (Fig. 4). Their sensitivity was considerably lower than that of control ascitic cells obtained from irradiated mice injected with P815. The sensitivity of the three escaping populations to an anti-H-2^d CTL clone was very similar to that of the control population. Escaping population I, which showed a residual sensitivity to CTL-P35:10, was subcloned on day 6 after its collection. All the clones were found to be either completely resistant

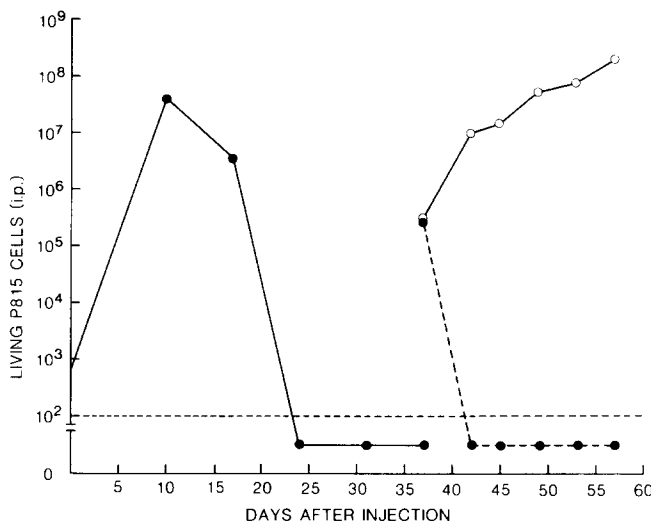


FIG. 3. A DBA/2 mouse was injected i.p. with 600 P1 cells (—●—). On day 36, the same mouse was injected with a mixture of 3×10^5 P1 cells collected from another DBA/2 mouse at the time of tumor escape (—○—) and 3×10^5 P1.aza^r cells collected from the ascites on an irradiated animal (---●---). The number of viable P1 and P1.aza^r cells in the peritoneal cavity was estimated by plating samples in agar containing HAT or azaguanine, respectively.

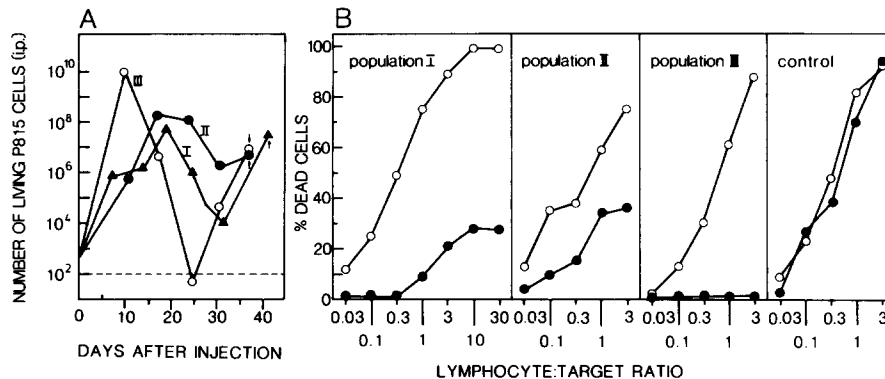


FIG. 4. Three DBA/2 mice were injected with 600 P1 cells. At various times thereafter, samples of the intraperitoneal fluid were collected and the number of living P1 cells was estimated in an agar colony test. Each curve in part A of the figure represents an individual mouse. At the times indicated by the arrows, the populations of P1 cells that escaped the immune rejection of these three mice were collected. The escaping P1 cells from mice I (▲), II (●), and III (○) were immediately mixed in vitro either with anti-P815 clone CTL-P35:10 (●) or with an anti-H-2^d CTL clone (○) at various effector-to-target ratios. After a 3-h incubation, the cells were plated in agar and the colonies counted 4 d later (part B: I-III). The same test was performed on control P1 cells collected from an irradiated DBA/2 mice (part B: control).

or completely sensitive (Fig. 5 A-C and Table I). For population III, which appeared to be completely resistant, no sensitive clone was found among the 24 tested.

The clones appeared to be stable in their resistance to CTL-P35:10. Four resistant clones from population I remained completely resistant when maintained in culture for >1 mo, as shown for one clone in Fig. 5 D. In contrast, the sensitivity of uncloned escaping populations increased gradually with time. For instance, population I, which

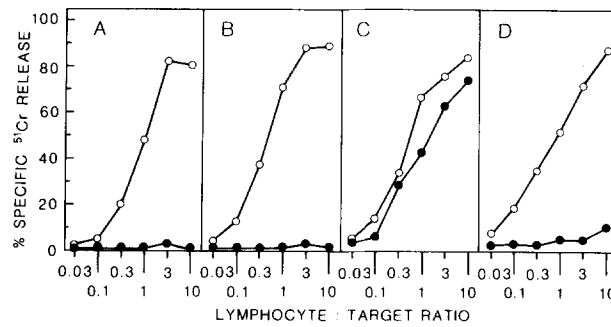


FIG. 5. Sensitivity of individual clones derived from escaping population I (see Fig. 4) to anti-P815 clone CTL-P35:10 (●) and to an anti-H-2^d CTL clone (○) was measured in a 4-h ⁵¹Cr release assay. The targets in parts A, B, and C were clones P1-20-1, P1-20-4, and P1-20-13, respectively. These clones were obtained by subcloning population I on day 6 after collection and were tested within 14 d. The target in panel D was clone A that had been maintained in culture for 3 mo.

TABLE I

Proportion of Resistant or Sensitive Clones in Escaping P1 Populations after Various Times of Culture

Escaping populations	Day after removal	Number of clones analyzed	Number of sensitive clones	Number of resistant clones
I	6	26	6	20
	17	27	14	13
	41	13	12	1
II	0	24	8	16
	17	35	25	10
III	0	24	0	24
	25	16	0	16

Escaping P1 populations collected from mice I, II, and III (see Fig. 4) were cultured in vitro and cloned at various times after their removal from the mice. The sensitivity of each tumor clones obtained to the clone CTL-P35:10 was tested by the growth inhibition method (see Materials and Methods).

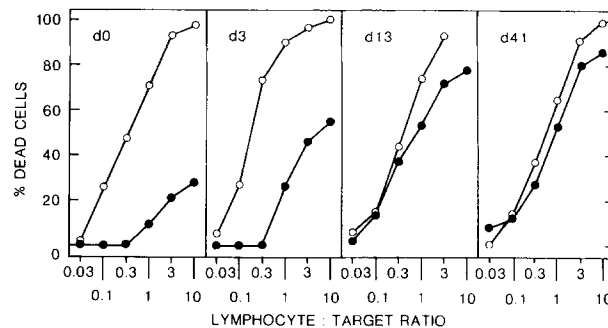


FIG. 6. Escaping P1 cells collected from mouse I (see Fig. 4) were cultured in vitro. At various times after their removal from the mice, the sensitivity of these escaping P1 cells to the CTL clone anti-P815 P35:10 (●) and to an anti-H-2^d CTL clone (○) was measured by mixing the tumor cells with the CTL clones at various effector to target ratios. After an incubation of 3 h, the surviving P1 cells were plated in agar and the colonies counted 4 d thereafter.

consisted predominantly of resistant cells on the day of its collection, was found to be completely sensitive after 41 d of culture (Fig. 6). Subcloning experiments indicated that the sensitive clones overgrew the resistant clones (Table I). We verified directly

with a resistant and a sensitive clone of population I that the former had a slightly longer generation time.

Presence of Multiple Antigenic Determinants on P815 and Related Antigen-loss Variants. The emergence of P815 cells resistant to an anti-P815 CTL clone provided an explanation for the escape of tumor cells in animals that were clearly not in a state of immunosuppression. However, it remained to be explained how sensitive cells could still be present in escaping populations at a time when newly injected P815 cells were promptly rejected. One possibility was that these sensitive cells would have lost another determinant present on P815 and thereby have a much decreased sensitivity to the effector population present in vivo.

We therefore tested other CTL clones specific for P815 and found that some of them failed to lyse escaping cell clones that were still sensitive to CTL-P35:10. For instance, clone CTL-P1:3, which was obtained after priming and restimulation with irradiated P815 cells, failed to lyse any clone obtained from populations I and III, not even those that were sensitive to CTL-P35:10. Since the specificity defined by CTL-P1:3 was clearly lost more readily, it was labeled P815A. That defined by CTL-P35:10 was labeled P815B. Escaping tumor cell population I consisted therefore of a mixture of P815A⁻B⁺ and P815A⁻B⁻ cells, whereas population III consisted exclusively of the latter type.

To continue the analysis of CTL-defined specificities expressed by P815 cells, we isolated a new CTL clone that recognized a residual specificity (P815C) on antigen-loss P815A⁻B⁻ cells (Fig. 7). This anti-P815C clone (CTL-P89:15) was obtained from lymphocytes primed and restimulated with tum⁻ variant P89. Also presented in Fig. 7 is the cytolytic activity of another CTL clone defining a fourth specificity, P815D. To detect the P815D determinant, we first isolated antigen-loss P815A⁻B⁻C⁻ cells by subjecting a P815A⁻B⁻ clone to in vitro immunoselection with anti-P815C clone P89:15. The anti-P815D clone (CTL-P89:20) was then obtained by using resistant P815A⁻B⁻C⁻ cells to stimulate spleen cells from a mouse immunized with tum⁻ variant P89. This clone lysed antigen-loss variants P815A⁻, P815A⁻B⁻, and P815A⁻B⁻C⁻ as effectively as P1 targets. None of the four anti-P815 CTL clones lysed the syngeneic target L1210. When tumor clones from escaping populations I and III were reanalyzed using all four anti-P815 CTL clones, none was found to have lost either the C or D determinant (Table II).

Additional antigen-loss variants were obtained by immunoselections with anti-P815 CTL clones to determine which of the P815 determinants could be expressed independently. Some of these variants are presented in Fig. 7, where antigen-loss consequent to tumor escape is indicated as *ist* and that resulting from in vitro selection as *isc*. The existence of P815 variants of antigenic types A⁻B⁺C⁺D⁺, A⁻B⁻C⁺D⁺, A⁺B⁺C⁻D⁺, and A⁺B⁺C⁺D⁻ demonstrates that, except for P815A and P815B, for all pairs of P815 determinants the two members can be expressed independently, since either one can be lost without the other. Even though A⁻B⁺ cells were found in escaping populations, only A⁻B⁻C⁺D⁺ cells were obtained after in vitro selection with an anti-P815A CTL clone. No completely resistant variant was obtained after repeated selections with an anti-P815B CTL clone.

Tumorigenicity of P815 Antigen-loss Variants. Cells (600) from P815A⁻B⁺C⁺D⁺ or P815A⁻B⁻C⁺D⁺ clones isolated from escaping population I were injected i.p. into normal DBA/2 mice. In all mice the cells grew progressively without a detectable

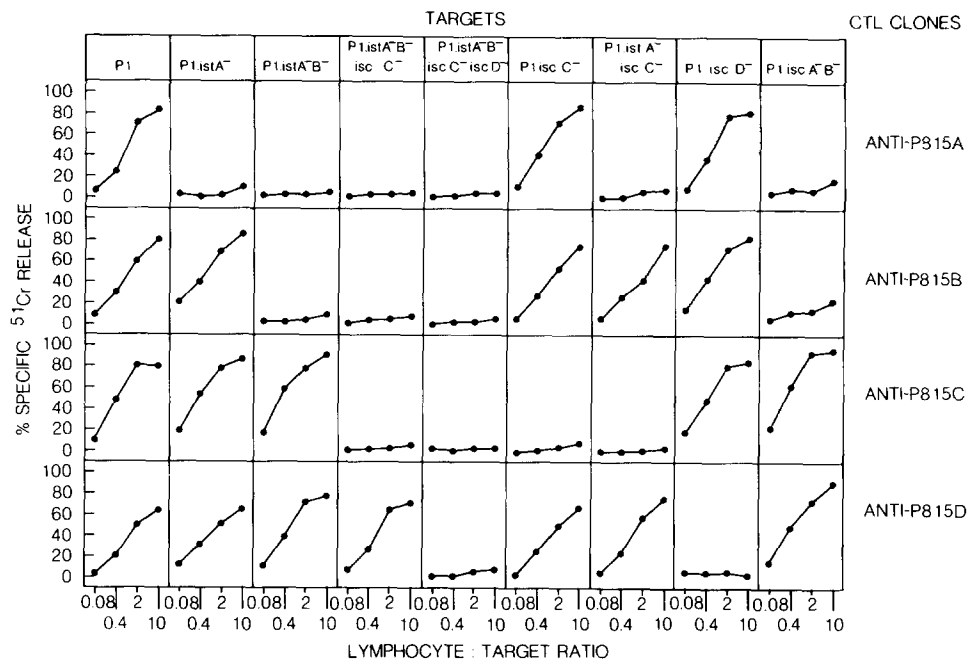


FIG. 7. The cytolytic activity of anti-P815A (CTL-P1:3), anti-P815B (CTL-P35:10), anti-P815C (CTL-P89:15), and anti-P815D (CTL-P89:20) CTL clones was measured in a 4-h ^{51}Cr release assay. The target cells were derived from the P1 clone of P815 after escape in vivo (ist), after in vitro selection with CTL clones (isc), or by a combination of both. All the cells used as targets were clones obtained from populations that were selected as follows: P1: original tumorigenic P815 clone; P1.istA⁻: clone P1-20-13 from escaping population I (Fig. 4); P1.istA⁻B⁻: clone P1-20-4 from escaping population I; P1.istA⁻B⁻iscC⁻: previous clone selected twice with anti-P815C CTL clone P89:15; P1.istA⁻B⁻iscC⁻iscD⁻: previous clone selected three times with anti-P815D CTL clone P89:20; P1.iscC⁻: P1 selected twice with anti-P815C CTL clone P89:15; P1.istA⁻iscC⁻: clone P1-20-13 selected twice with anti-P815C CTL clone P89:15; P1.iscD⁻: P1 selected twice with anti-P815D CTL clone P89:10; P1.iscA⁻B⁻: P1 selected once with anti-P815A CTL clone P1:3.

TABLE II
P815-specific Determinants on Escaping Cells

Escaping populations	Number of clones analyzed	Antigenic types
I	16	10: A ⁻ B ⁺ C ⁺ D ⁺ 6: A ⁻ B ⁻ C ⁺ D ⁺
III	16	16: A ⁻ B ⁻ C ⁺ D ⁺

Escaping P1 populations collected from mice I and III (see Fig. 4) were cultured in vitro and cloned. The sensitivity of each clone obtained to clone CTL-P1:3 (anti-P815A), clone CTL-P35:10 (anti-P815B), clone CTL-P89:15 clone (anti-P815C) and clone CTL-P89:20 (anti-P815D) was tested by the growth inhibition method (see Materials and Methods).

rejection phase, confirming the importance of antigen P815A in the rejection of the tumor. Representative data obtained with escaping populations or with antigen-loss variants is shown in Fig. 8.

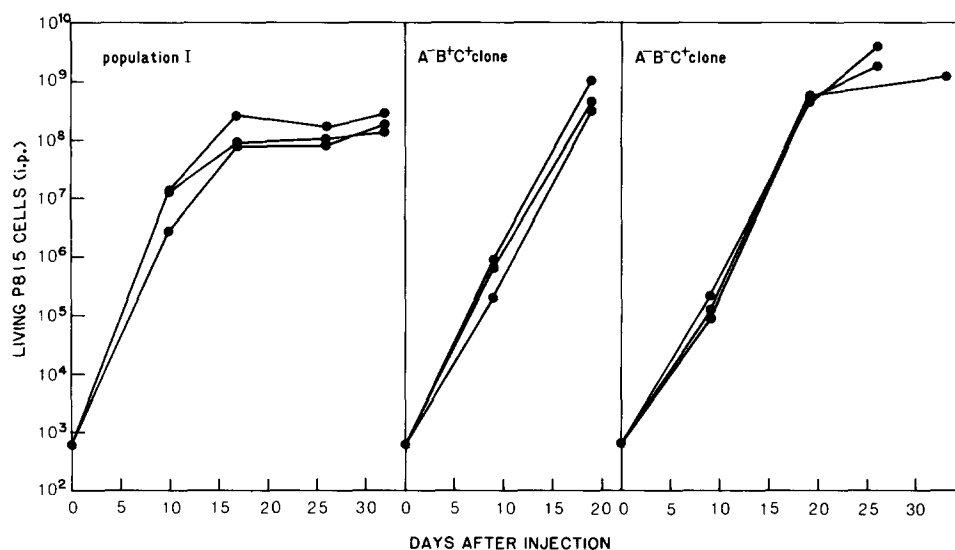


FIG. 8. DBA/2 mice were injected intraperitoneally with 600 cells obtained from escaping population I (see Fig. 4) after 6 d of culture or with 600 cells of P815A⁻B⁺C⁺D⁺ or P815A⁻B⁻C⁺D⁺ isolated from population I. At various times thereafter, samples of the intraperitoneal fluid were collected and the number of living P815 cells was estimated in an agar colony test. Each curve represents an individual mouse and ends on the last sampling before the death of the animal.

Discussion

Our results indicate that, when mastocytoma P815-X2 escapes from quasi-complete elimination due to syngeneic immune rejection, the tumor cells resume their expansion while the host is not in an immunosuppressed state, as shown by the rejection of newly injected P815 cells. We also find that the emergent tumor cells are less sensitive than the original cells to CTL directed against P815. A similar observation was made by Biddison and Palmer (5) with the P815-Y tumor subline. Our results indicate that the resistance is not due to modulation but to the presence of stable variants that have lost some of the tumor-associated antigenic specificities present on P815. The progressive reversal toward sensitivity to some anti-P815 CTL clones that is observed with escaping tumor cell populations maintained in culture appears to be due to a slight growth advantage of the clones that have retained the corresponding antigenic specificity.

Our findings are consistent with other reports indicating that for malignant cells of high immunogenicity, the cells found in progressive tumors or metastases are often largely resistant to CTL directed against the initial population (12-15). Our use of CTL clones and of both in vivo and in vitro selection procedures allowed us to distinguish on P815 at least four antigenic specificities capable of eliciting a T cell cytolytic response in the syngeneic host. From these, only two appear to be selected against in the course of the escape of the tumor cells, suggesting that P815 does not elicit an efficient response against the two other specificities.

Whereas our results exclude that suppressor T cells provide a sufficient explanation for the escape of P815, we do not think that they are incompatible with the existence of suppressor T cells affecting specifically the response against P815. Dye and North (16) conclusively demonstrated that specific suppressor T lymphocytes constitute a

major obstacle to adoptive immunotherapy directed against P815 present in mice bearing P815 tumors. Also, P815 tumor cells injected subcutaneously were observed to form tumors less rapidly in mice that had received an irradiation dosage reported to affect preferentially T suppressor cells (17). Finally, specific suppressor cells inhibiting the maturation of anti-P815 CTL have been reported to be present in the spleen of mice carrying advanced tumors (4, 7). However, we found that spleen cells of mice carrying advanced tumors were still capable of being stimulated *in vitro* to form active CTL populations, provided metastatic tumor cells were eliminated from the splenic population. This could be achieved by injecting mice with an azaguanine-resistant P815 variant and by treating the spleen cells with HAT (18).

In the assessment of the relative role of antigen-loss variants and suppressor T cells in the escape of P815, it may be useful to distinguish three types of situations encountered in the rejection of this tumor. In a first, the tumor cells multiply exponentially for ~10 d, the number of tumor cells then remains approximately stationary for a few days, and finally the tumor resumes its progressive growth. The stationary phase probably results from an equilibrium between the multiplication of the tumor cells and their destruction by the immune defenses. It is therefore plausible that suppressor T cells play a major role in resolving this equilibrium in favor of the tumor cells. In a second situation, the period of exponential growth is followed by a nearly complete elimination of the tumor cells followed in turn by a rapid multiplication of a surviving fraction of the tumor cells. On the basis of our results, we think that the emergence of these tumor cells can be explained entirely by the selection of antigen-loss variants. A third situation is sometimes observed where, after a nearly complete elimination of P815, a very low and stationary number of tumor cells is found in the peritoneal cavity for a period varying from a few days to a few weeks. Eventually the tumor cells resume their progressive growth. This situation corresponds to that referred by Weinhold (19) as the tumor dormant state. Our results indicate that during the dormant period and the secondary emergence of the tumor the mice are not in an immunosuppressed state and that the emergent tumor cells are antigen-loss variants. However, we have no explanation for the mechanism whereby residual tumor cells remain present in low numbers for weeks. It will be of great interest to examine the antigenic determinants present on these dormant cells.

Our analysis demonstrates that P815-X2 carries at least four antigenic determinants recognized by different CTL clones. Considering that P815 is used by a number of groups to study tumor rejection, it would be useful to type for the expression of these antigens P815 cells from different laboratories in order to facilitate comparison of results. Our antigen-loss variants may also become useful in determining whether tumor-associated specificities defined by antibodies coincide with specificities defined by CTL clones.

The recognition that tumor cells may carry multiple tumor-associated transplantation antigens and that they may escape the host defenses by the loss of only some of these antigens may be of significance for immunotherapy. It suggests that, as in some situations encountered in chemotherapy, it may be important to secure a simultaneous response against multiple targets, so as to minimize the emergence of resistant variants.

Summary

Even though mastocytoma P815 often undergoes a nearly complete rejection in syngeneic mice, the tumor cells almost always escape to form progressive tumors. We

found that this was not due to the establishment of an immunosuppressed state because genetically marked P815 cells, that were injected in mice where tumor escape was occurring, were readily rejected.

An analysis of escaping tumor cell populations with anti-P815 cytolytic T lymphocyte (CTL) clones showed the presence of stable resistant variants. Using antigen-loss variants found in escaping populations or selected in vitro with CTL clones, we were able to define four different tumor-associated antigenic specificities, each recognized by a specific CTL clone. One of these specificities was absent from all escaping tumor cells and another had been lost by some of them.

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