

# PROTECTION AGAINST TWO SPONTANEOUS MOUSE LEUKEMIAS CONFERRED BY IMMUNOGENIC VARIANTS OBTAINED BY MUTAGENESIS

BY ALINE VAN PEL, FRANÇOISE VESSIÈRE, AND THIERRY BOON

*From the Ludwig Institute for Cancer Research, UCL 7459, B-1200 Brussels; and the Cellular Genetics Unit, Université Catholique de Louvain, Belgium*

It is well established that the experimental tumors induced in the mouse by oncogenic viruses and the majority of those induced by polycyclic hydrocarbons such as methylcholanthrene carry tumor-specific transplantation antigens (1-4). In most experiments, transplantation immunogenicity could be demonstrated on the basis of increased resistance against tumor cell challenge by animals that had previously received cells of the same tumor, either as irradiated cells, as living cells at subthreshold doses, or as living cells that were later eliminated by ligation or resection of the tumor. On the contrary, no immune rejection response appeared to be elicited by spontaneous mouse tumors, i.e. tumors arising without experimental intervention (2, 5). Similar results have been obtained with spontaneous rat tumors (6).

We have reported that, by treating a variety of mouse tumor cell lines with a mutagen, it is possible to obtain at high frequency stable tumor cell variants ( $\text{tum}^-$ )<sup>1</sup> that fail to form tumors in syngeneic mice. These  $\text{tum}^-$  variants elicit a strong immune rejection response (7). They have been obtained from teratocarcinoma OTT6050, Lewis lung carcinoma, mastocytoma P815, and a thymic leukemia (8-10). Most  $\text{tum}^-$  variants carry new antigens that are specific for each variant. These antigens can be detected either in vivo by protection experiments or in vitro with cytolytic T lymphocytes (CTL) (11-14). In rejecting  $\text{tum}^-$  variants, syngeneic mice often acquire a resistance against challenge with the original tumor cell line ( $\text{tum}^+$ ). This has been observed with weakly immunogenic tumors like Lewis lung carcinoma or P815 mastocytoma. More remarkably, it has also been observed with a teratocarcinoma cell line, even though no protection was conferred by injection of irradiated  $\text{tum}^+$  cells or by subcutaneous injection of living cells followed by surgical removal of the tumor (11, 15). Recently, a similar observation was made with a radioinduced thymic leukemia for which no protection was observed after immunization with irradiated cells (10). For both the teratocarcinoma and the thymic leukemia, protection after immunization with  $\text{tum}^-$  variants was observed not only against a challenge with the  $\text{tum}^+$  cell line but also against the original transplantable tumor, indicating that the target antigens do not represent tissue-culture artefacts.

Since the use of  $\text{tum}^-$  variants appears to extend the class of tumors on which

<sup>1</sup> Abbreviations used in this paper: CTL, cytolytic T lymphocyte; CTL-P, cytolytic T lymphocyte precursor; FCS, fetal calf serum; i.p., intraperitoneally; LEB, leukemia I; LEC, leukemia IV; MLTC, mixed lymphocyte-tumor cell culture;  $\text{tum}^-$ , with reduced ability to produce tumors;  $\text{tum}^+$ , forming progressive tumors.

specific transplantation antigens can be detected, it is important both from a fundamental point of view and from that of tumor immunotherapy to find out whether such antigens can be detected by this method on spontaneous tumors, which on the basis of previous experimentation appear least likely to carry them. This should be done in conditions that minimize the possibility of artefactual antigenicity. We choose to examine two spontaneous leukemias obtained by H. Hewitt in CBA/Ht mice (reference 16 and personal communication). These leukemias had not been adapted to culture and had always been transplanted in a carefully maintained inbred colony. We show here that tum<sup>-</sup> variants could be obtained from both leukemias. Immunization of CBA/Ht mice with these variants conferred protection against the parental tumor and ability to generate cytolytic T cells specific for that tumor.

### Materials and Methods

**Mice.** CBA/Ht mice were received from the inbred colony of H. Hewitt. Male or female animals of 12–15 wk were used.

**Tumor Cells.** Spontaneous leukemias I (16) and IV were obtained and generously given to us by Dr. H. Hewitt. Both leukemias were maintained by in vivo transfer of tumor cells found in the liver in CBA/Ht mice. Leukemias I and IV had been transplanted in CBA/Ht mice 340 and 205 times respectively before being adapted to culture. We redefined leukemia I as LEB and leukemia IV as LEC. Thymic leukemia TH was obtained by H. Hewitt in a mouse that had previously received gamma irradiation (5, 10).

**Culture Conditions.** LEB cells were cultured in petri dishes, in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10 mM Hepes, 1.5 mM L-glutamine, 10% fetal calf serum, and 50  $\mu$ M 2-mercaptoethanol. LEC cells were cultured in tissue culture dishes, in DME medium (n°1600; Gibco Laboratories) supplemented with 10 mM Hepes, L-glutamine (216 mg/l), L-asparagine (36 mg/l), L-arginine-HCl (116 mg/l), glucose (4.5 mg/l final concentration), 50  $\mu$ M 2-mercaptoethanol, and 10% fetal calf serum. Both cells were cultured in 5% CO<sub>2</sub>.

**Mutagenesis and Cloning.** Tumor cells were incubated with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine at 37°C in Earle's medium (7). After removal of the mutagen, the cells were incubated in normal medium for at least 8 d before they were cloned or treated a second time with the mutagen. The number of survivor cells was estimated by comparing the number of cells present in these cultures 8 d after mutagenesis to that of control cultures, that had received parallel treatment without mutagen. At that time the cells were cloned by distributing them in limiting dilution conditions in 96-well microplates. LEB cells, which grow in suspension, were distributed in round bottom microplates (NUNC 1-63320) in thymocyte-conditioned medium prepared by incubating CBA/Ht thymus cells ( $4 \times 10^6$ /ml) for 24 h at 37°C in RPMI medium containing 10 mM Hepes, 1.5 mM L-glutamine, 50  $\mu$ M 2-mercaptoethanol, 30% fetal calf serum. LEC cells, which adhere to tissue culture plastic, were distributed in flat bottom microplates (Falcon 3040; Falcon Labware, Oxnard, CA) in the normal culture medium except that 30% fetal calf serum was added. Clones were collected from dilutions yielding <20% positive wells.

**Immunization and Challenge.** Cells were resuspended in medium containing 1% fetal calf serum and were injected intraperitoneally into CBA/Ht mice. The challenge was carried out intraperitoneally 1–2 mo after immunization. Mice still alive 70 d after the challenge were considered negative.

**Mixed Lymphocyte Tumor Cell Cultures.** Spleen cells ( $3 \times 10^7$ ) from immune CBA/Ht mice were stimulated with  $3 \times 10^5$  irradiated LEB or LEC cells (5,000 rads gamma irradiation). The stimulation was performed in 10 ml of the medium used for the culture of LEC described above, except that fetal calf serum was replaced by 0.2% CBA/Ht serum. Cytolytic activity was measured after 6 d by chromium release as described before (12). Percent specific release =  $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$ . The spontaneous release was lower than 20% of the label incorporated into the cells.

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*Limiting Dilution Stimulation and Expansion of CTL Clone.* CBA/Ht mice were injected intraperitoneally (i.p.) with  $10^6$  living LEC30 cells. 35 d later, these mice were boosted i.p. with  $3 \times 10^6$  irradiated (5,000 rads) LEC30 or LEC1 cells. 5 d later, the peritoneal exudate cells were collected and limiting numbers (10–100) of these cells were stimulated with  $10^3$  irradiated (5,000 rads) LEC30 or LEC1 cells in the presence of  $10^6$  irradiated syngeneic spleen cells as feeder cells in 200  $\mu$ l of medium containing secondary allogeneic mixed lymphocyte culture supernatant as a source of T cell growth factor (13). Aliquots of 150  $\mu$ l of microcultures lysing specifically LEC30 or LEC cells were transferred to 1 ml of culture containing  $5 \times 10^6$  irradiated syngeneic spleen cells,  $10^5$  irradiated LEC30 or LEC1 cells and secondary allogeneic supernatant (13). For subsequent passages (every 5–8 d),  $5 \times 10^4$  cells were transferred to similar cultures.

## Results

*Isolation of tum<sup>-</sup> Variants.* Spontaneous transplantable leukemias LEB and LEC were adapted to culture and clonal cell lines LEB1 and LEC1 were obtained. LEB1 produced progressive tumors in >95% of the CBA/Ht mice that received an intraperitoneal injection of 300 cells. It was found to express the Thy1.2 antigen, indicating that LEB is a T cell leukemia. LEC appears to be a B cell leukemia, since surface IgM was found on clonal line LEC1. 100 cells of this clone regularly produce tumors in >95% of the syngeneic mice.

LEB1 and LEC1 cultures were treated with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) at a concentration of 3  $\mu$ g/ml for a time varying from 20 to 40 min so as to obtain mutagenized populations in which cell survival was approximately equal to 0.5%. After a period of cell multiplication of 15 d, these populations were submitted to a second round of mutagenesis under the same conditions and 8 d later populations with a survival rate of  $\sim$ 0.5% were cloned. From 49 mutagenized clones derived from LEB1, 5 failed to form tumors in most CBA/Ht mice even when injected at a dose of  $10^5$  cells, that is, >300 times the tumorigenic dose of LEB1. No such tum<sup>-</sup> variant had been found among 27 clones isolated from the LEB1 population mutagenized only once. For LEC1, 41 clones were isolated after two rounds of mutagenesis. Four tum<sup>-</sup> clones were found that formed progressive tumors in fewer than 10% of the mice after injection of  $10^5$  cells. Representative results obtained with tum<sup>+</sup> and tum<sup>-</sup> cells derived from LEB and LEC are shown in Table I.

As observed previously in other tumor systems (7–9), LEB and LEC tum<sup>-</sup> variants formed tumors in mice that had received 580 rads of gamma radiation, a sublethal immunosuppressive dose (Table I). In vitro growth kinetics of the tum<sup>-</sup> variants were very similar to those of the parental LEB1 and LEC1 lines.

*Protection Against the Original Tumors.* In agreement with the observations of Hewitt on spontaneous mouse tumors (5), we did not observe any resistance against a challenge with LEB1 or LEC1 in mice that had received 1 mo earlier a large number of the corresponding irradiated cells (Table II). However, mice that had been injected with living cells from tum<sup>-</sup> variants derived from LEB or LEC and had rejected these cells, were found to be largely resistant to a challenge with the corresponding tum<sup>+</sup> cell line. The results shown in Table II were confirmed in other experiments. The protection by variant LEC37 shown in Table II was rather weak since 67% of the mice formed tumors. However, tumor formation in those mice was considerably retarded with respect to the controls and in another experiment involving animals immunized with LEC37 and challenged with LEC1 only 36% (5/14) of the mice formed tumors versus 100% (19/19) in the controls.

TABLE I  
*Tumors Produced by tum<sup>+</sup> and tum<sup>-</sup> LEB and LEC Cells in CBA/Ht Mice*

| Clone*                   | Cell dose       | No. mice with tumors/No. mice injected |                              |
|--------------------------|-----------------|----------------------------------------|------------------------------|
|                          |                 | Unirradiated mice                      | Irradiated mice <sup>‡</sup> |
| LEB1 (tum <sup>+</sup> ) | 300             | 20/20 (31 d) <sup>§</sup>              | ND                           |
| LEB26                    | 10 <sup>5</sup> | 0/37                                   | 3/3 (26 d)                   |
| LEB28                    | 10 <sup>5</sup> | 0/16                                   | ND                           |
|                          | 10 <sup>4</sup> | 4/17 (32 d)                            | 3/3 (20 d)                   |
| LEC1 (tum <sup>+</sup> ) | 100             | 19/19 (13 d)                           | ND                           |
| LEC30                    | 10 <sup>5</sup> | 1/38 (42 d)                            | 2/2 (15 d)                   |
| LEC37                    | 10 <sup>5</sup> | 5/32 (39 d)                            | 3/3 (15 d)                   |

\* Injected i.p.

<sup>‡</sup> 580 rads of gamma irradiation from a cesium source, given 2 h before injection.

<sup>§</sup> Mean survival time.

ND, not determined.

TABLE II  
*In Vivo Protection Against the Original Tumor Cell Line*

| Immunizing cell*            |       | % Mice with tumor after challenge with tum <sup>+</sup> clone: <sup>‡</sup><br>(No. mice with tumors/No. mice injected) |                  |
|-----------------------------|-------|-------------------------------------------------------------------------------------------------------------------------|------------------|
|                             |       | LEB1                                                                                                                    | LEC1             |
| Irradiated tum <sup>+</sup> | LEB1  | 95 (19/20), d34 <sup>§</sup>                                                                                            | ND               |
| Living tum <sup>-</sup>     | LEB15 | 14 (3/21), d39                                                                                                          | 95 (20/21), d15  |
|                             | LEB26 | 29 (6/21), d40                                                                                                          | 100 (19/19), d16 |
|                             | LEB28 | 37 (7/19), d41                                                                                                          | 89 (17/19), d16  |
| Irradiated tum <sup>+</sup> | LEC1  | ND                                                                                                                      | 100 (20/20), d16 |
| Living tum <sup>-</sup>     | LEC30 | 86 (12/14), d40                                                                                                         | 6 (1/18), d18    |
|                             | LEC37 | 100 (10/10), d41                                                                                                        | 67 (12/18), d36  |
| None                        | —     | 100 (30/30), d34                                                                                                        | 100 (20/20), d15 |

\* CBA/Ht mice were immunized i.p. either with  $5 \times 10^6$  tum<sup>+</sup> cells killed by gamma irradiation (5,000 rads) or with  $10^6$  living cells of various tum<sup>-</sup> variants. Control mice were injected with the same amount of medium. 27-32 d later, they were injected i.p. with cells from tum<sup>+</sup> clone LEB1 ( $3 \times 10^3$ ) or LEC1 ( $10^5$ ).

<sup>‡</sup> Animals found without tumor 70 d after the challenge were considered negative.

<sup>§</sup> Mean survival time.

The protection conferred by the LEB and LEC tum<sup>-</sup> variants appears specific for the parental tumor cell line. No protection was observed when mice immunized with LEB tum<sup>-</sup> variants were challenged with LEC1 or conversely (Table II). Other experiments showed that LEB or LEC tum<sup>-</sup> variants did not induce any protection against syngeneic T cell leukemia TH or vice versa (reference 10 and unpublished results).

The protection conferred by LEB and LEC tum<sup>-</sup> variants was not limited to tum<sup>+</sup> cells grown in culture. Mice immunized with LEB26 showed a significant resistance against a challenge with LEB cells that had been exclusively transplanted in vivo.

Mice immunized with LEC30 were similarly protected against a LEC challenge (Fig. 1). However, LEC30 did not induce a protection against LEB nor did LEB26 protect against LEC (data not shown).

*Cytolytic T Lymphocyte Response.* Spleen cells from mice that had rejected LEB or LEC tum<sup>-</sup> variants were stimulated in vitro with irradiated cells of the immunizing variant. To avoid nonspecific stimulation, the fetal calf serum often used in such tests was replaced by a low concentration of syngeneic mouse serum. After 6 d, these mixed lymphocyte-tumor cell cultures (MLTC) were assayed for cytolytic activity on the immunizing tum<sup>-</sup> clone, on other clones derived from the same tumor line and on other syngeneic tumor cells (Tables III and IV). As had been observed with tum<sup>-</sup> variants derived from other tumors (12, 14), the cytolytic activity was higher on the

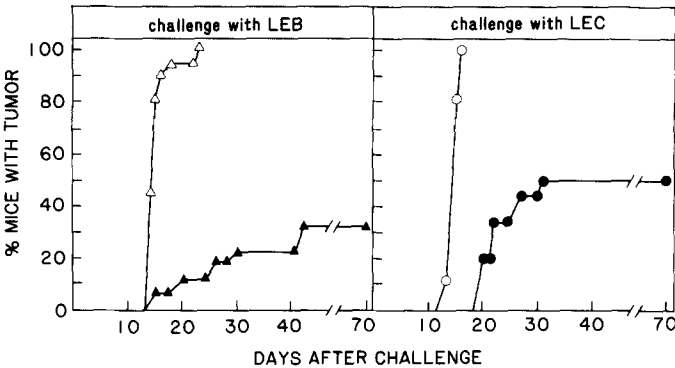


FIG. 1. Protection against transplantable tumors LEB and LEC. CBA/Ht mice were immunized intraperitoneally with 10<sup>6</sup> living cells of tum<sup>-</sup> variants LEB26 (▲) and LEC30 (●). Control mice were injected with medium (Δ and ○). After 32 d, the mice were challenged with 60 living LEB cells (left) or 40 living LEC cells (right). The challenging cells were obtained from the liver of CBA/Ht mice injected 12 d earlier with tumor cells that had been transferred exclusively in vivo. 20 mice were used in each group.

TABLE III  
*Cytolytic Activity after In Vitro Stimulation of Spleen Cells from Mice Immunized with LEB tum<sup>-</sup> Variants*

| Spleen cells* | Stimulator cells | E/T† | Percent specific Cr <sup>51</sup> release from target cells‡ |       |       |      |      |
|---------------|------------------|------|--------------------------------------------------------------|-------|-------|------|------|
|               |                  |      | LEB1                                                         | LEB26 | LEB28 | LEC1 | TH1‡ |
| anti-LEB26    | LEB26            | 3:1  | 26                                                           | 51    | 34    | 3    | 0    |
|               |                  | 1:1  | 14                                                           | 31    | 19    | 1    | 0    |
|               | LEB1             | 10:1 | 28                                                           | 39    | 37    | 5    | 0    |
|               |                  | 3:1  | 17                                                           | 22    | 23    | 1    | 0    |
| anti-LEB28    | LEB28            | 10:1 | 7                                                            | 16    | 72    | 2    | 0    |
|               |                  | 3:1  | 4                                                            | 8     | 56    | 0    | 0    |
|               | LEB1             | 10:1 | 14                                                           | 20    | 25    | 5    | 0    |
|               |                  | 3:1  | 8                                                            | 12    | 12    | 1    | 0    |

\* CBA/Ht mice were immunized i.p. with 10<sup>6</sup> living cells of tum<sup>-</sup> variants LEB26 and LEB28. After 72 d (LEB26) or 61 d (LEB28), spleen cells were stimulated with irradiated LEB cells, for 6 d in medium containing 0.2% CBA/Ht serum.

† E/T, effector to target cell ratio.

‡ The lytic activity of these effector cells was tested in a 6-h Cr<sup>51</sup> release assay performed with 5 × 10<sup>3</sup> target cells in medium containing 10% FCS.

‡ TH1 is a clonal line derived from thymic leukemia TH.

TABLE IV  
Cytolytic Activity after In Vitro Stimulation of Spleen Cells from Mice Immunized with LEC tum<sup>-</sup> Variants

| Spleen cells* | Stimulator cells | E/T <sup>†</sup> | Percent specific Cr <sup>51</sup> release from target cells <sup>‡</sup> |       |       |      |     |
|---------------|------------------|------------------|--------------------------------------------------------------------------|-------|-------|------|-----|
|               |                  |                  | LEC1                                                                     | LEC30 | LEC37 | LEB1 | TH1 |
| Anti-LEC30    | LEC30            | 90:1             | 17                                                                       | 47    | 20    | 0    | 0   |
|               |                  | 30:1             | 5                                                                        | 29    | 5     | 0    | 0   |
|               |                  | 10:1             | 1                                                                        | 18    | 2     | 0    | 0   |
|               | LEC1             | 90:1             | 48                                                                       | 46    | 51    | 1    | 0   |
|               |                  | 30:1             | 36                                                                       | 32    | 34    | 0    | 0   |
|               |                  | 10:1             | 18                                                                       | 20    | 18    | 0    | 0   |
| Anti-LEC37    | LEC37            | 30:1             | 36                                                                       | 32    | 90    | 2    | 2   |
|               |                  | 10:1             | 19                                                                       | 13    | 75    | 0    | 2   |
|               | LEC1             | 30:1             | 46                                                                       | 43    | 48    | 2    | 0   |
|               |                  | 10:1             | 26                                                                       | 24    | 25    | 1    | 0   |

\* CBA/Ht mice were immunized intraperitoneally with 10<sup>5</sup> living cells of tum<sup>-</sup> variants LEC30 and LEC37. After 30 d, spleen cells were stimulated with irradiated LEC cells for 6 d in medium containing 0.2% CBA/Ht serum.

<sup>†</sup> E/T, effector to target cell ratio.

<sup>‡</sup> The lytic activity of these effector cells was tested in a 4-h Cr<sup>51</sup> release assay carried out with 5 × 10<sup>3</sup> tumor target cells in medium containing 10% FCS.

immunizing variants than on other tum<sup>-</sup> clones or on tum<sup>+</sup> cells derived from the same tumor, indicating the presence of a new antigen specific for the immunizing tum<sup>-</sup> variant. This was found with the three LEB and the three LEC tum<sup>-</sup> variants that were tested. In addition, a significant cross-reactive lysis was always observed on all the targets derived from the same parental tumor as the immunizing cells. When the immune spleen cells were restimulated with the corresponding tum<sup>+</sup> cells, the levels of lytic activity observed on all the targets derived from the parental tumor were approximately equal. These results confirm those obtained in vivo to indicate that syngeneic mice recognize an antigenic determinant on leukemias LEB and LEC. These antigens appear to be specific for each tumor since spleen cells immunized and restimulated with LEB variants showed very little activity on LEC or TH targets. Likewise, spleen cells immunized with LEC variants do not lyse LEB or TH targets.

The absence of protection observed in vivo in mice that had received irradiated LEB or LEC cells was paralleled by the observation that no significant lytic activity could be demonstrated after in vitro restimulation of the lymphocytes of these animals (data not shown).

**Cytolytic T Cell Clones Directed Against LEC.** The specificity of CTL directed against LEC cells was also analyzed at the clonal level. Mice immunized with living LEC30 cells were boosted i.p. with irradiated LEC30 or LEC1 cells. 5 d later, lymphocytes from the peritoneal cavity were collected and restimulated in limiting dilution microcultures with irradiated LEC30 or LEC1 cells in medium containing T cell growth factor. After 8 d, aliquots from each microculture were assayed for cytotoxicity against LEC30 and LEC1. Only microcultures prepared with numbers of responder cells resulting in clonal conditions were considered. When the boost and the in vitro stimulation had been performed with LEC30, the majority of the CTL clones were LEC30-specific. The frequency of CTL precursors (CTL-P) present in the peritoneal cell populations, that were specific for LEC30 was estimated to be 7.5 × 10<sup>-3</sup> and

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that of CTL-P directed against LEC was  $2.5 \times 10^{-3}$ . When LEC1 cells were used as booster and stimulator cells, almost all of the positive wells lysed both LEC30 and LEC1, and the anti-LEC CTL-P frequency was  $2 \times 10^{-3}$ .

A number of CTL clones that lysed either LEC30 only or both LEC30 and LEC1 were transferred for further clonal expansion in cultures containing irradiated LEC30 or LEC1 cells as stimulator cells and T cell growth factor. The cells were subsequently transferred every 5–8 d under the same conditions. It was possible to expand and maintain for more than 1 mo without loss of specificity or activity CTL clones that lysed LEC30 exclusively and others that lysed LEC30 and LEC1 equally well but not LEB, TH, or syngeneic blasts. Results obtained with two representative clones are shown in Fig. 2.

## Discussion

By mutagen treatment we have been able to obtain tum<sup>-</sup> variants from two spontaneous leukemias that were adapted to culture. Three LEB and three LEC tum<sup>-</sup> variants were found to carry new variant-specific antigens that elicited a specific CTL response. The frequency of tum<sup>-</sup> variants obtained with LEB and LEC was somewhat

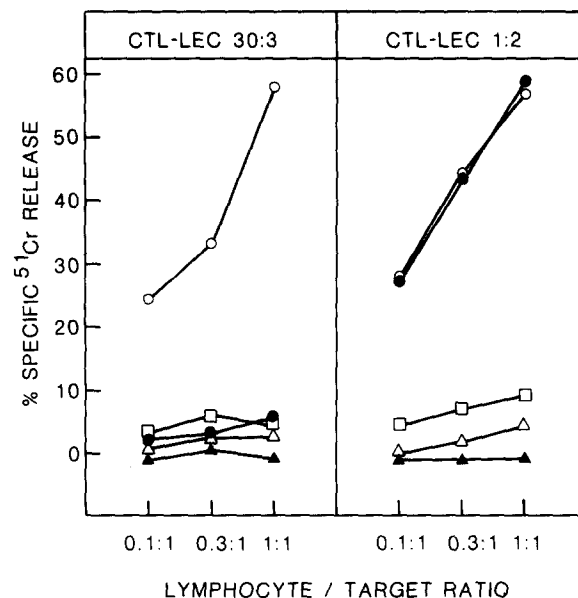


FIG. 2. Cytolytic activity of long-term CTL clone CTL-LEC30:3, which is specific for the tum<sup>-</sup> antigen of LEC30, and CTL-LEC1:2 directed against a tumor-associated antigen of tumor LEC. For CTL-LEC30:3, mice were immunized intraperitoneally with living LEC30 cells, boosted intraperitoneally with irradiated LEC30 cells, and 5 d later the peritoneal lymphocytes were collected and stimulated in limiting dilution conditions with LEC30. A microculture showing specificity for LEC30 was maintained and expanded in culture with LEC30 stimulator cells (Materials and Methods). For CTL-LEC1:2, the mice were immunized with LEC30, boosted with LEC1, and the peritoneal lymphocytes were stimulated with LEC1. A microculture showing specificity for LEC was expanded with LEC1 as stimulator cells. The assay was performed after the clones had been maintained in culture for more than 30 d following initial transfer from limiting dilution microculture. The cytolytic activity shown here was measured using 2,000 <sup>51</sup>Cr-labeled target cells as follows. LEC30 (○), LEC1 (●), LEB (△), TH (▲), and syngeneic concanavalin A-stimulated lymphoblasts (□) in a 6-h chromium release assay.

lower than that obtained by similar treatment of other mouse tumors such as teratocarcinoma, Lewis lung carcinoma, or mastocytoma P815. This may be due to the highly malignant and metastatic character of these tumors, which are able to kill animals rapidly after injection of a very small number of cells. It is likely that such tumor cells must acquire extremely immunogenic determinants to lose their tumorigenicity. Yet, together with our previous observations these results suggest that the obtainment at high frequency of immunogenic variants after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment will be generally applicable to mouse tumors. Recent observations by Frost, Kerbel, and their associates (17) indicate that the mutagen ethyl methane sulfonate is also capable of generating in vitro immunogenic mouse tumor cell variants.

The protection conferred by LEB or LEC-derived tum<sup>-</sup> cells against the corresponding tum<sup>+</sup> cell line demonstrates that these spontaneous tumors carry tumor-associated transplantation antigens that are the targets of a syngeneic rejection response. The absence of cross-protection between LEB and LEC shows that each of these tumors carries a different antigen. We also reported that mice immunized with tum<sup>-</sup> variants derived from syngeneic leukemia TH were not protected against a challenge with LEB cells (10). The patterns of T cell cytolytic activity observed in animals that have rejected tum<sup>-</sup> variants confirm the view that LEB, LEC, and TH each carry a different tumor-associated antigen.

Since a protection was observed against the transplantable LEB and LEC tumors that had never been passaged in vitro, the relevant tumor-associated antigens are certainly not tissue-culture artefacts. It is also improbable that these antigens represent minor histocompatibility differences between the primary tumor-bearers and the presently available CBA/Ht mice as such antigens would be expected to be common to these tumors. Even though it is impossible to exclude that these antigens arose during evolution of the tumors in the course of their multiple transplantations, our observations raise the possibility that at least some primary spontaneous mouse tumors carry tumor-specific transplantation antigens. These results by no means contradict the well-established contrast between the immunogenicity observed on virus- or carcinogen-induced tumors and the lack of it on spontaneous tumors. However, they indicate that this antigenic difference may not be an absolute one and that the immunogenicity of weak determinants can be notably increased by interaction with artificially added new determinants, a possibility that has previously been suggested and demonstrated by others (18–20).

Since the use of tum<sup>-</sup> variants appears to extend notably the class of mouse tumors in which transplantation antigens can be detected, our results provide a strong incentive to examine whether similar variants, carrying new antigens detectable by autologous CTL, can be obtained from human tumor cells. Such variants may permit the demonstration of tumor-associated transplantation antigens on human tumors. Then, the possibility of using tum<sup>-</sup> variants to induce a rejection response against human tumors could be cautiously considered.

### Summary

Two spontaneous mouse leukemias were adapted to culture. In agreement with most reported observations on spontaneous tumors, injection of irradiated cells of the malignant culture cell lines failed to protect mice against these leukemias. These cell

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lines were treated in vitro with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and stable immunogenic variants (tum<sup>-</sup>) were obtained, that failed to form progressive tumors in syngeneic CBA/Ht mice. Mice that had rejected tum<sup>-</sup> variants showed a significant degree of resistance to challenge not only with the original malignant cell line but also with the original transplantable tumor. No protection was observed against syngeneic tumor cells other than those of the parental tumor. These results indicate that these two spontaneous leukemias carry a specific transplantation antigen that can be the target of a rejection response by syngeneic mice. In confirmation of this, we found that lymphocytes of CBA/Ht mice that had rejected tum<sup>-</sup> variants could be restimulated in vitro so as to develop a cytolytic activity directed against an antigen that was specific for the original tumor cell line.

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