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Protection against a nonimmunogenic mouse leukemia by an immunogenic variant obtained by mutagenesis

(tumor immunology)

ALINE VAN PEL AND THIERRY BOON

Ludwig Institute for Cancer Research (Brussels Branch), U.C.L. 74.59, Avenue Hippocrate, 74, B-1200 Brussels, Belgium; and Cellular Genetics Unit, University of Louvain, Brussels, Belgium

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ABSTRACT **The nonimmunogenic thymic leukemia TH, obtained in mouse strain CBA/Ht, was adapted to culture. By *in vitro* treatment of a clonal TH cell line with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, stable variant cell clones (tum$^-$) were obtained that elicited a rejection response in syngeneic mice. Mice that had rejected a tum$^-$ variant were partially protected against a challenge with the original tumor. When spleen cells of these animals were restimulated *in vitro*, cytolytic T cells were obtained that were directed against an antigen present on the original tumor. The existence of these cytolytic effectors was confirmed by clonal analysis of the cytolytic response. No immune protection against TH was observed in mice that had been immunized with irradiated cells of the original TH tumor. These results confirm that tum$^-$ variants can elicit a syngeneic rejection response against tumors that are apparently devoid of transplantation immunogenicity. The experimental conditions and the results make it likely but not certain that the tumor-associated antigen detected on leukemia TH was present on the primary tumor.**

Little evidence is available to support the view that human tumors carry antigens constituting potential targets for a rejection response of the patient (1). In the mouse, the existence of tumor-associated transplantation antigens has been firmly established for most virus- or carcinogen-induced tumors by protection experiments involving immunization with irradiated tumor cells (2–4). However, for the majority of spontaneous and radiation-induced tumors, no evidence for antigenicity was obtained by this method (5, 6).

We reported previously that, by mutagen treatment of cell lines of three mouse tumors—namely, teratocarcinoma OTT6050, Lewis lung carcinoma, and mastocytoma P815-X2—we have obtained at high frequency stable tumor cell variants (tum$^-$) that fail to form tumors in syngeneic mice (7–9). These tum$^-$ variants undergo a process of immune rejection in the syngeneic host. Most tum$^-$ variants carry new singular transplantation antigens that can be demonstrated *in vivo* by cross-protection experiments (8–11). With the variants obtained from P815-X2 and Lewis lung carcinoma, these new antigens elicit the production of specific cytolytic T cells (10, 12).

In rejecting tum$^-$ variants, syngeneic mice often acquire a resistance against challenge with the original tumor cell line (tum$^+$). This is observed with tum$^-$ variants derived from two weakly immunogenic tumors, Lewis lung carcinoma and mastocytoma P815-X2 (8, 9). More surprisingly, protection is also observed with teratocarcinoma tum$^-$ variants, even though the tum$^+$ cell line appears completely devoid of transplantation immunogenicity (11, 13). This protection extends to the original transplantable teratocarcinoma OTT6050, indicating the presence on this tumor of an otherwise undetected tumor-associated transplantation antigen (14). Unfortunately, we have been unable to elicit a cytolytic T-cell response with teratocarcinoma tum$^-$ variants, so that this conclusion could not be strengthened by demonstration of specific effector cells directed against this tumor-associated antigen.

In view of the—admittedly remote—possibility of applying to human tumors the protection conferred by tum$^-$ variants, it was considered worthwhile to see whether our observations could be extended to other weakly or nonimmunogenic mouse tumors in conditions approaching those applying to primary tumors. This implied avoiding as far as possible artefactual antigenicity that can result from genetic differences between the primary tumor bearer and the experimental mice or from antigenic evolution of the tumor cells either *in vivo* or *in vitro*. For this purpose, we chose CBA thymic leukemia TH that had not been adapted to culture and had always been transplanted in a carefully maintained inbred colony (CBA/Ht). Leukemia TH was isolated by Hewitt from a female CBA/Ht mouse that had been repeatedly exposed to γ irradiation 6–3 months earlier so as to receive a cumulative dose of 1,800 rads (1 rad = 0.01 gray; ref. 5; H. Hewitt, personal communication). Hewitt observed no increase in the number of living tumor cells required to produce progressive tumors on addition of a preponderance of irradiated TH cells to the inoculum. He concluded that this tumor was not immunogenic (5).

We report here that we obtain protection against this thymic leukemia after immunization with an immunogenic tumor cell variant obtained by mutagen treatment. The immunized animals have precursors of cytolytic T cells directed specifically against the original tumor.

MATERIALS AND METHODS

Animals. We received CBA/Ht mice from the inbred colony of H. Hewitt. They were introduced in our animal house after sterile ceasarean delivery. CBA/Ht have a low tumor incidence (5). Male or female animals of 12–15 wk old were used.

Tumor Cells. Leukemia TH was received from H. Hewitt (5). Tumor TH was maintained by *in vivo* transfer in CBA/Ht mice. It was found to be positive for the Thy 1.2 antigen and negative for Rauscher murine leukemia virus antigens gp70 and p30. LEB and LEC are spontaneous CBA/Ht leukemias I (15) and IV obtained by H. Hewitt.

Culture Conditions. Tumor cells were cultured in Petri dishes in RPMI 1640 medium (GIBCO)/10 mM Hepes/10%

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Abbreviations: CTL, cytolytic T lymphocyte; MLTC, mixed lymphocyte/tumor cell culture; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; tum$^-$, variants having reduced ability to form progressive tumors in syngeneic mice; tum$^+$, variants capable of forming progressive tumors in syngeneic mice.

fetal calf serum. They were incubated at 37°C in 5% CO_2/95% air. The cells were cloned by a limiting-dilution assay in thymocyte-conditioned medium prepared by incubating CBA/Ht thymus cells (4×10^6/ml) for 24 hr at 37°C in RPMI medium/10 mM Hepes/1.5 mM L-glutamine/50 μM 2-mercaptoethanol/30% fetal calf serum.

Mutagenesis. Tumor cells were incubated with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and then cloned as described (7).

Immunization and Challenge. CBA/Ht mice were subcutaneously injected with cells suspended in culture medium/1% fetal calf serum. Prior to immunization, some cells were killed by 5,000 rads of γ irradiation from a cesium source. The challenge was carried out 1 to 2 months after the immunization, subcutaneously into the opposite flank. Mice found without tumor 2 months after the challenge were considered negative.

Mixed Lymphocyte/Tumor Cell Culture (MLTC). Immune spleen cells were cultured with stimulator cells as described (10, 16).

Limiting-Dilution Microcultures and Cytolytic T-Lymphocyte (CTL) Clones. Culture conditions were adapted from a described procedure (17). Briefly, immune peritoneal cells, immune spleen cells, or immune spleen cells stimulated in MLTC were plated in limiting numbers with 10^6 irradiated (2,000 rads) CBA/Ht spleen cells and 10^3 irradiated (5,000 rads) tum$^-$ or tum$^+$ TH cells. These cells were seeded in microwells (microtiter plates, 96 round-bottom wells) containing 0.1 ml of Dulbecco's modified Eagle's medium supplemented as for the MLTC and 0.1 ml of supernatant from a secondary mixed lymphocyte culture as a source of interleukin 2 (18). After 7–9 days of incubation, aliquots (40 μl) of the microcultures were mixed with 2×10^3 different ^{51}Cr-labeled target cells (in 160 μl) for 4 hr. Microcultures were considered to be positive when the specific ^{51}Cr release exceeded the mean spontaneous release obtained in the absence of responder cells by more than five SDs. The frequency of CTL precursors was calculated by the Poisson formula. Selected microcultures (CTL clones) were transferred and subcloned as described (16).

RESULTS

Isolation of Tum$^-$ Variants. Ascites cells of leukemia TH transplanted into syngeneic CBA/Ht mice were adapted to culture in RPMI medium. The tumor cells could be cloned in medium that had been conditioned with thymocytes. When injected into CBA/Ht mice, clone TH1 (tum$^+$) was found to have approximately the same tumorigenicity as TH cells that had always been transplanted *in vivo* (hereafter referred to as THt). The mice and all those used in the experiments described below were obtained from the original colony of Hewitt.

TH1 cells were incubated with MNNG and then cloned. Each clone was injected into CBA/Ht mice. Of 24 clones obtained from a population of TH1 cells treated with MNNG at 1 μg/ml for 60 min, one (TH18) was found to have a reduced tumorigenicity. Two additional tum$^-$ clones (TH43 and TH45) were found in a group of nine clones isolated from a TH1 population that had been treated with the same mutagen for 30 min. Tum$^-$ clones TH18 and TH45 were studied further. Whereas 300 TH1 cells injected subcutaneously almost always generated progressive tumors in syngeneic mice, 3,000 TH45 cells generated visible tumors almost all of which subsequently regressed. For clone TH18, only ≈20% of the tumors regressed after an injection of 3,000 cells. The experiments described below were carried out with TH45 and TH18 regressor mice. When tum$^-$ clones TH18 and TH45 were injected in mice that had received a sublethal immunosuppressive dose of 600 rads of whole-body γ irradiation, they showed the same tumorigenicity as TH1 cells.

Table 1. Protection induced by tum$^-$ variants against tumor TH

Exp.	Immunizing cells (dose)	Challenge	% mice with tumors
1	THt (2×10^6 irradiated)	THt	100 (15/15)
		THt	96 (11/12)
2	TH1 (2×10^6 irradiated)	TH1	85 (11/13)
		TH1	85 (11/13)
3	TH18 (3×10^3 living)	TH1	31 (5/16)*
	TH45 (3×10^3 living)	TH1	67 (16/24)†
		TH1	91 (31/34)
4	TH18 (3×10^3 living)	THt	43 (21/49)*
		THt	85 (44/52)

CBA/Ht mice were immunized by subcutaneous injection in the right flank with THt, TH1, TH18, or TH45 cells. The THt cells had always been transplanted *in vivo*. They were obtained from ascitic fluid of CBA/Ht mice injected intraperitoneally 8 days earlier with 2×10^6 cells. TH1, TH18, and TH45 cells were obtained from permanent cultures. THt and TH1 were killed by irradiation (5,000 rads) before immunization. TH18 and TH45 were injected as living cells. For the challenge, mice were injected subcutaneously in the left flank with 100 living THt or TH1 cells obtained as described for immunizations. In experiments 1 and 2, the challenge occurred 1 month after immunization. In experiments 3 and 4, the challenge occurred 45 days after immunization of mice that were observed to carry tumors that later regressed. Mice found without tumor 2 months after the challenge were considered negative. Controls died after an average of 18 days. Values in parentheses represent no. of mice with tumors/no. of mice injected.
*$P < 0.01$ (χ^2 test).
†$P < 0.05$ (χ^2 test).

Protection Against the Original Tumor. In agreement with Hewitt's findings (ref. 5; personal communication), we did not observe any protection against a THt challenge in mice that had been injected with irradiated THt cells (Table 1). The adaptation of leukemia TH to culture apparently did not confer any immunogenicity, as mice injected with irradiated TH1 cells remained as sensitive as control mice to a TH1 challenge. On the contrary, when we challenged TH18 and TH45 regressor mice with TH1 cells, protection was observed (Table 1). This protection was particularly significant in mice that had been injected with variant TH18. This tum$^-$ variant also conferred protection against the transplanted tumor THt, indicating that the relevant tumor-associated transplantation antigen is not an artefact induced by adaptation to culture.

CTL Activity. To find out whether the antigen detected *in vivo* on tumor TH could also be found *in vitro*, we examined the CTL response of spleen cells obtained from TH18 or TH45 regressor mice. As shown in Table 2, when such spleen cells were stimulated *in vitro* with the immunizing tum$^-$ variant in classical MLTC conditions, they showed a high cytolytic activity. This activity was preferentially directed against the immunizing variant, thereby indicating the presence of new individual antigens on variants TH18 and TH45. Moreover, an important cross-reactive activity was observed against all TH cells, demonstrating the presence of a common antigen. That this activity was specifically directed against a transplantation antigen of leukemia TH was suggested by the low level of lytic activity against spontaneous CBA/Ht leukemia LEB, which showed the same sensitivity as TH to anti-H-2^k CTL (Table 2, experiments 3 and 4). No lytic activity specific for TH was obtained after *in vitro* restimulation of splenocytes from mice immunized with irradiated TH1 cells (data not shown). Variant TH18, which conferred better protection than TH45 against the

Table 2. Cytolytic activity of spleen cells of regressor mice

Exp.	Spleen cells	Stimulator cells	E/T	% specific ^{51}Cr release from target cells: THt	TH1	TH18	TH45	LEB
1	a-TH18	TH18	10:1	—	45	72	62	—
			3:1	—	27	59	41	—
			1:1	—	12	32	19	—
	a-TH45	TH45	10:1	—	32	39	67	—
			3:1	—	6	20	73	—
			1:1	—	5	8	64	—
2	a-TH18	TH18	15:1	20	—	56	28	—
			5:1	8	—	35	12	—
	a-TH18	TH1	15:1	23	—	31	30	—
			5:1	11	—	12	16	—
3	a-TH18	TH18	3:1	—	43	65	—	11
			1:1	—	30	53	—	3
4	a-H-2^k	CBA	3:1	—	45	48	—	44
		spleen	1:1	—	25	29	—	21

Experiment 1: Spleen cells were obtained from regressor CBA/Ht mice injected 45 days earlier with 5×10^2 living TH18 (a-TH18) or TH45 (a-TH45) cells; 3×10^7 spleen cells were incubated with 10^5 irradiated (5,000 rads) tumor cells for 6 days in Dulbecco's modified Eagle's medium/10% fetal calf serum supplemented with other additions (16). The lytic activity of these effector cells was tested in a 4-hr ^{51}Cr release assay with 10^4 tumor target cells grown in RPMI medium/10% fetal calf serum as described (10). Experiment 2: Conditions were as in experiment 1 except that, to minimize fetal calf serum artefacts, the medium of the MLTC culture was removed after 5 days and replaced by medium containing 0.5% CBA/Ht mouse serum. The ^{51}Cr release assay was carried out 1 day later in medium containing 0.5% CBA/Ht serum with 10^4 target cells collected from ascites of irradiated mice injected with THt, TH18, or TH45. Experiment 3: Conditions were as in experiment 1 except that the regressor mice had been injected with 10^4 living TH18 cells. LEB is spontaneous CBA/Ht leukemia I obtained by H. Hewitt (15). Experiment 4: C57BL/6 mice were injected intraperitoneally with 10^7 living CBA/Ht spleen cells; 3×10^7 immune spleen cells were stimulated with 3×10^7 irradiated (2,000 rads) CBA/Ht spleen cells for 5 days. E/T, effector cell/target cell ratio.

tum$^+$ cells, also induced a higher level of lytic activity against the common antigen.

Significant lytic activity was found not only on TH1 cells but also on THt cells that were tested immediately after their removal from ascitic mice. To minimize the artefactual antigenicity that could result from the binding of foreign proteins to stimulator and target cells, we verified that anti-TH18 CTL lysed THt cells when the chromium release test was carried out in the absence of fetal calf serum (Table 2, experiment 2).

The specificity of the anti-TH cytolytic response was also analyzed at the clonal level. In recent reports, the feasibility of obtaining and expanding clonal populations of cytolytic T cells (19, 20) and using them to study tum$^-$ variants (16) has been demonstrated. Using limiting-dilution microcultures (17, 18), we estimated that, in spleen cell populations of TH18 regressor mice, the frequency of CTL precursors directed against a common TH antigen was $\approx 2 \times 10^{-5}$ whereas that of CTL precursors directed specifically against TH18 was 1×10^{-4}. A number of CTL clones showing anti-TH18 or anti-TH activity were expanded and maintained *in vitro* for ≈20 days. The activity of two TH18-specific clones and two TH-specific clones is shown in Table 3. The specificity of the anti-TH clones was suggested by their lack of activity against spontaneous syngeneic leukemia LEB. Other experiments indicated that these clones were equally inactive against spontaneous CBA/Ht leukemia LEC and against syngeneic lymphoblasts obtained by treating spleen cells with concanavalin A (data not shown).

Secondary Tum$^-$ Variants Obtained by Mutagenesis of TH18. Whereas TH18 confers significant protection against

Table 3. Activity of cytolytic T-cell clones obtained from TH18 regressor mice

Clone (specificity)	E/T	% specific ^{51}Cr release from target cells: THt	TH18	LEB
CTL-TH18:1	1:1	6	56	2
(anti-TH18)	0.3:1	4	29	0
	0.1:1	0	10	0
CTL-TH18:5	1:1	0	50	0
(anti-TH18)	0.3:1	0	31	0
	0.1:1	0	12	0
CTL-TH18:15	1:1	59	54	2
(anti-TH)	0.3:1	54	45	1
	0.1:1	29	24	0
CTL-TH18:14	1:1	54	50	7
(anti-TH)	0.3:1	44	40	2
	0.1:1	28	21	1

Spleen cells from TH18 regressor mice were stimulated *in vitro* as described in Table 2 with either TH18 or TH1 cells. The MLTC cells were restimulated under limiting-dilution conditions. CTL clones TH18:1 and TH18:5 (anti-TH18) were obtained from wells seeded with three responder cells and TH18 stimulator cells (10/48 positive wells; frequency, 8×10^{-2}). CTL clones TH18:15 and TH18:14 (anti-TH) were obtained from wells seeded with 10 responder cells and TH1 stimulator cells (12/48 positive wells; frequency, 3×10^{-2}). LEB is spontaneous CBA/Ht leukemia I obtained by H. Hewitt (15). E/T, effector cell/target cell ratio.

THt, it is a weak tum$^-$ variant since only ≈20% of the tumors regress. We examined whether mutagen treatment of TH18 would produce secondary variants that would have a stronger tum$^-$ character and still induce protection against tum$^+$ cells.

A TH18 cell population was treated with MNNG (3 μg/ml for 15 min). The surviving population, amounting to 0.5% of the initial cells, was cloned. Of 17 clones tested, 11 failed to form tumors after subcutaneous injection of 10^5 cells, a dose that, for TH18, produces 100% progressive tumors. Other cells of the surviving population were further treated with MNNG treatment (3 μg/ml for 20 min), and 27 of 30 clones obtained from the survivors (0.1% of input cells) failed to form tumors after subcutaneous injection of 10^5 cells. These results indicate that variants having increased tum$^-$ character can be obtained by further mutagen treatment of tum$^-$ variants.

Three clones isolated from the population that had been treated with mutagen twice and four clones isolated from the population that had been treated with mutagen three times

Table 4. Protection induced by TH18.21

Exp.	Immunizing cells (dose)	Challenge (dose)	% mice with tumors*
1	TH18.21 (10^7)	THt (10^2)	29 (4/14)
	TH18.21 (3×10^6)	THt (10^2)	28 (5/18)
		THt (10^2)	100 (15/15)
2	TH18.21 (10^4)	TH1 (3×10^2)	53 (8/15)
		TH1 (3×10^2)	92 (11/12)
	TH18.21 (10^4)	LEB (3×10^2)	93 (13/14)
		LEB (3×10^2)	100 (12/12)

CBA/Ht mice were immunized by subcutaneous injection of living cells of tum$^-$ TH18.21. Control mice received the same amount of medium. In experiment 1, 28 days after the first injection, mice were injected with 10^2 living cells of the original tumor THt subcutaneously at the opposite flank. In experiment 2, 32 days after the first injection, mice were injected subcutaneously with 3×10^2 living cells of clone TH1 or intraperitoneally with 3×10^2 living cells of spontaneous CBA/Ht leukemia LEB.

* Values in parentheses represent no. of mice with tumors/no. of mice injected.

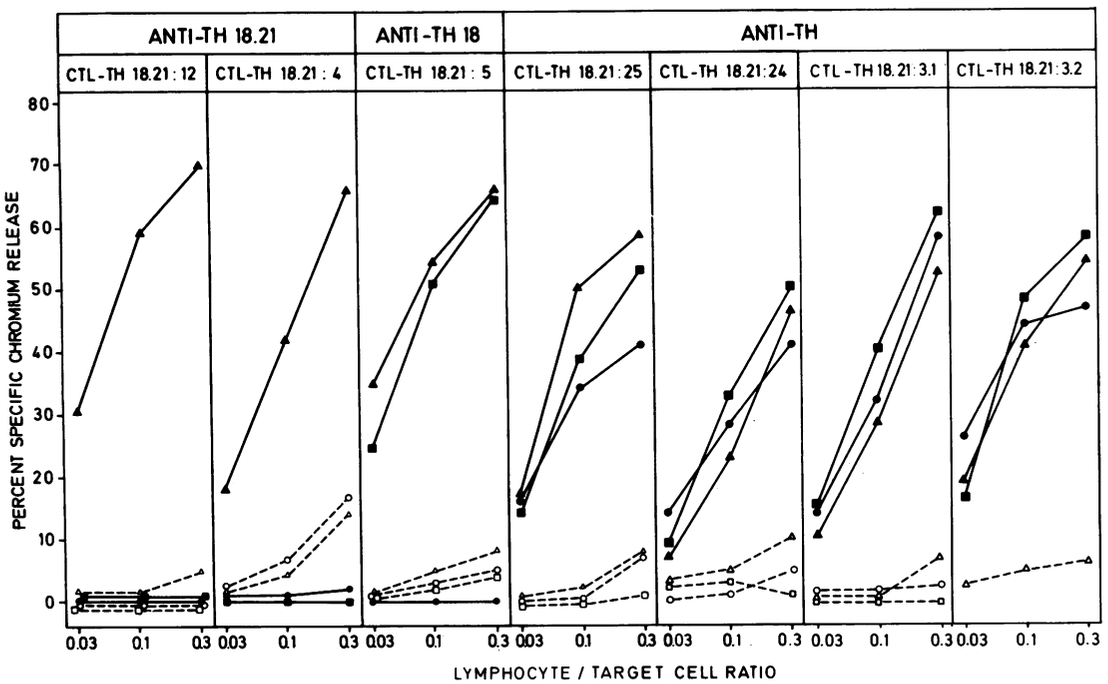

FIG. 1. Activity of CTL clones derived from peritoneal cells of mice immunized with tum⁻ TH18.21. CBA/Ht mice were immunized subcutaneously twice 43 days apart with 10^5 living cells of tum⁻ variant TH18.21. Forty days after the second immunization, 3×10^6 TH18.21 or TH1 cells killed by γ irradiation (5,000 rads) were injected intraperitoneally. Five days later, the immune peritoneal cells were stimulated in limiting-dilution microcultures with either irradiated TH18.21 cells or TH1 stimulator cells. CTL clones with different specificities were selected, maintained in culture, and subcloned as described (16). CTL clones CTL-TH18.21:12, CTL-TH18.21:4, CTL-TH18.21:5, and CTL-TH18.21:3 were obtained after intraperitoneal boosting and limiting-dilution stimulation with TH18.21. From CTL-TH18.21:3, subclones CTL-TH18.21:3.1 and CTL-TH18.21:3.2 were isolated. CTL clones CTL-TH18.21:24 and CTL-TH18.21:25 were obtained after intraperitoneal boosting and limiting-dilution stimulation with TH1. CTL clones were tested in a ^{51}Cr release assay at three effector cell/target cell ratios on six target cells: TH1 (●), TH18 (■), TH18.21 (▲), spontaneous CBA/Ht leukemias LEB (○) and LEC (□), and concanavalin A-stimulated syngeneic blasts (△). The blasts were used 2 days after treatment of CBA/Ht spleen cells with concanavalin A at 4 μg/ml.

were tested for ability to induce protection against leukemia TH. Only one of them, TH18.21 isolated from the twice-treated population, induced a level of protection that was at least as high as that obtained with TH18 (Table 4). The ability of clone TH18.21 to induce protection against the syngeneic spontaneous tumor LEB was tested. No protection was observed.

CTL clones were isolated after limiting-dilution restimulation of peritoneal cells obtained from mice that had rejected TH18.21. In agreement with the protection results, we obtained CTL clones that specifically lysed tumor TH. They lysed equally well TH1 (tum⁺), TH18, and TH18.21 and had little if any activity on syngeneic leukemias LEB, LEC, and syngeneic concanavalin A-induced lymphoblasts. We also obtained clones that lysed TH18 and TH18.21 and not TH1 (tum⁺) and appeared therefore to be directed against the tum⁻ antigen of TH18. Finally, clones were obtained that lysed exclusively TH18.21, thereby indicating the presence of a new antigen on this secondary variant. This new antigen probably accounts for the fact that TH18.21 has an enhanced tum⁻ character compared with TH18 (Fig. 1).

DISCUSSION

We have demonstrated that some tum⁻ variants obtained from leukemia TH can elicit against this tumor, which has no apparent immunogenicity, an immune protection and a specific T-lymphocyte cytolytic response. This confirms that *in vitro* mutagenesis can lead to improved responses against tumors after immunization with tumor cells to which new antigenic determinants have been added. Addition of new antigens to tumor cells, which has been named xenogenization (21), has also been achieved by viral infection (21–23), by chemical modification of the cell surface (24), and by *in vivo* treatment with an antineoplastic drug (25, 26). However, to the best of our knowledge, none of these other procedures has been reported to result in protection against tumors that did not already have some immunogenicity of their own.

The degree of protection conferred by TH tum⁻ variants differed from one variant to another, that obtained with TH18 being clearly superior to that obtained with TH45. Similar observations have been made with tum⁻ variants isolated from mastocytoma P815 (9). It is perhaps not a coincidence that, for both P815 and TH, the tum⁻ variants that elicit the best protection are those variants that have the weakest individual "tum⁻" antigen as judged from the response obtained either *in vivo* or *in vitro*.

Mutagen treatment of variant TH18 produced a high yield of secondary tum⁻ variants whose tumorigenicity was found to be further reduced. A new specific antigenic determinant not present on TH18 was detected on the secondary variant TH18.21 by T-cell cytolysis. Thus, it is possible that, by repeated mutagen treatment of tumor cells, populations will be obtained in which the majority of the cells are tum⁻ variants and most of these variants carry multiple new antigens.

Is it probable that the antigen recognized on TH was present on the primary tumor? This antigen certainly does not represent a tissue culture artefact since both the *in vivo* and *in vitro* responses are effective against tumor cells that had always been transplanted *in vivo*. It is more difficult to exclude the possibility that, after isolation of the tumor, there might have been some genetic change in the CBA/Ht line resulting, for instance, in the loss of a minor histocompatibility antigen that the mice would now recognize on the tumor. However, this is unlikely

because of the existence of a CTL activity directed specifically against leukemia TH and not against leukemia LEB, which was obtained by Hewitt in his CBA/Ht mouse colony before tumor TH. If the response was due to genetic evolution of the mice after the isolation of leukemia TH, it should affect both tumors. Finally, there is the possibility of antigenic evolution of the tumor during its passages *in vivo*. This type of artefactual antigenicity is practically impossible to rule out in experiments with transplantable tumors. It has often been noticed that tumors acquire murine leukemia virus antigens in the course of their passages *in vivo*. However, this does not appear to apply to leukemia TH since this tumor was negative for Rauscher murine leukemia virus antigens gp70 and p30. In conclusion, we consider that it is likely but not certain that the tumor-associated antigen recognized on TH was present on the primary tumor as a potential target for a response of the primary host.

Taken together with our previous observations on a mouse teratocarcinoma (11), the results obtained with leukemia TH suggest that there may be many tumors for which antigenicity that is not detectable by conventional immunization could become apparent in animals that have rejected tum$^-$ variants. If it is found that tum$^-$ variants elicit a significant response against other nonimmunogenic tumors under conditions that maximize the likelihood that the relevant antigen was present on the primary tumor, the prospect of applying to human tumors the protection conferred by tum$^-$ variants will appear less remote.

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