## *Brief Definitive Report*

# ESCAPE OF METASTASIZING CLONAL TUMOR CELL VARIANTS FROM TUMOR-SPECIFIC CYTOLYTIC T LYMPHOCYTES

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The mechanisms by which immunogenic tumors can avoid destruction by the immune system are only beginning to be understood. Some antigens on tumor cells such as the thymus-leukemia antigen (1) or the murine mammary tumor virus antigen (2) can be modulated upon interaction with the corresponding antibodies. This antigenic modulation makes the tumor cells resistant to complement-dependent immune cytolysis and is usually of short duration in the absence of antibody. It seems to be due to an epigenetic mechanism, such as lateral antigen displacement, shedding, and/or antigen internalization. The in vivo relevance of this escape from humoral anti-tumor immunity has not been shown yet (3).

Here we report on a new type of modulation of a tumor-associated transplantation antigen (TATA), which leads to the specific resistance of a chemically induced tumor to T cell-mediated immunity. These specifically immunoresistant tumor variants will be shown to develop during metastasis of immunosensitive cloned tumor lines in a normal immunocompetent host. In contrast to the above antigen modulation, this type of antigenic change is stable and inherited through over 100 subsequent tumor cell generations, even in the absence of immune T cells. The in vivo relevance of this type of tumor variant will be demonstrated by the results from specific immunotherapy experiments.

## Materials and Methods

*Tumor Lines.* ESb is a spontaneous highly metastatic variant of the chemically induced DBA/2 lymphoma L5178Y (Eb). It first arose in 1968 (4) and was found to differ from Eb by (*a*) increased invasive capacity in vitro (5); (*b*) increased shedding of membrane antigens (6); (*c*) selective binding to hepatocytes (7); (*d*) increased expression of Fcγ receptors (8); and (*e*) decreased expression of receptors for Semliki forest virus (9). In spite of functional, morphological, and antigenic differences, the ESb line could be shown recently to be closely related to Eb (10). Maintenance of the tumor lines has been described (10). Cloning was performed by growing single cells in suspension culture in microtiter plates.

*Typing of Tumor Antigens.* Tumor protection experiments revealed the presence of TATA on both Eb and ESb tumor cells. These TATA of Eb and ESb were shown to be distinct and non-cross-reactive and could be detected in vitro with the help of secondary tumor-specific syngeneic cytolytic T lymphocytes (CTL; 11). Cytotoxic activity was measured in a 4-h $^{51}Cr$ release assay as described in detail (11).

*Percoll Separation of Tumor Cells from Internal Organs.* Tumor cells from organ metastases were separated from host tissue cells by a newly devised technique based on isopycnic density gradient centrifugation in 70–20% Percoll.[1]

[1] Bosslet, K., R. Ruffmann, P. Altevogt, and V. Schirrmacher. 1981. A rapid method for the isolation of metastasizing tumor cells from internal organs with the help of isopycnic density gradient centrifugation in Percoll. *Br. J. Cancer.* In press.

## Results

*Development of Immunoresistant Tumor Variants during Metastasis in a Normal Syngeneic Host.* With the help of CTL typing, we followed the expression of the specific TATA on ESb tumor cells during the process of metastasis. 12 d after subcutaneous inoculation of $10^5$ ESb tumor cells into syngeneic DBA/2 mice, tumor cells were isolated either from the local tumor or from various internal organs. In the first experiments, the tumor cells were cleaned from host tissue by growing them out by two passages in tissue culture. In later experiments, the tumor cells were separated from host cells by isopycnic Percoll density gradient centrifugation and tested directly for expression of TATA. Identical results were obtained with both methods: whereas $^{51}$Cr-labeled tumor cells from the locally growing tumor and from most internal organs or tissues (lung, kidney, liver, bone marrow, and brain) were effectively lysed in a 4-h cytotoxicity test with tumor-specific CTL, the tumor cells isolated from the spleen could not be lysed.

The first experiments were performed with the uncloned ESb tumor cell population. This population could have contained immunoresistant tumor cells, although we did not detect a single anti-ESb CTL resistant clone among the 30 ESb clones that were isolated. The experiments were therefore repeated with individual TATA-positive cloned ESb cell lines. These lines were cloned twice by growing single cells in suspension culture in microtiter plates. The results from many experiments performed with the twice-cloned line ESb-Cl 18.1 are summarized in Table I. These cells were inoculated subcutaneously into normal syngeneic DBA/2 mice and 12 d later, the spleen-derived tumor cells were again immunoresistant to anti-ESb CTL (results from 10 individual experiments are shown), whereas the tumor cells from liver, lung, and the primary tumor were specifically lysed (i.e., lysed by anti-ESb but not by anti-Eb CTL). The spleen-derived clonal tumor cell variants were not generally resistant to lysis by CTL because they could always be killed by anti-H-$2^d$ CTL (Table I).

The inability of spleen-derived ESb tumor cells to be lysed by anti-ESb CTL was due to a reduced expression or even loss of TATA as shown by the following types of experiments. (*a*) These variants ($ESb_{Met\text{-}SPL}$) were not recognized as target cells by anti-ESb CTL, whether they were used directly as $^{51}$Cr-labeled target cells (Fig. 1 A) or as cold target competitors for TATA-positive ESb ($ESb_{ASC}$) target cells (Fig. 1 B). In the latter test, which measures binding rather than lysis, cold $ESb_{ASC}$ cells could completely inhibit the cytotoxic reactions, whereas cold $ESb_{Met\text{-}SPL}$ cells did not inhibit significantly more than the negative control of Eb ascites ($Eb_{ASC}$) tumor cells. (*b*) $ESb_{Met\text{-}SPL}$ variants could not be converted to an antigen-positive line by treatment with trypsin or neuraminidase, which suggests that the antigen was not covered up by components of the surface coat. (*c*) $ESb_{Met\text{-}SPL}$ variants remained stable and immunoresistant when grown and passaged in tissue culture for >100 cell generations. (*d*) In contrast to the antigen-positive ESb line, the $ESb_{Met\text{-}SPL}$ variant was not capable of inducing CTL activity, which suggests that it did not express a different tumor antigen. (*e*) To verify that the spleen-derived tumor line was a derivative of the inoculated ESb line, a genetic marker, azaguanine resistance (12), was introduced into the ESb-Cl 18.1 line. The spleen-derived variants were again specifically immunoresistant and carried the genetic marker (see Table I, footnote ‖).

*Specific Immune Escape in a Tumor-preimmunized Host.* The immune status of the host was found to influence considerably the process of generation of immunoresistant

TABLE I

*Sensitivity or Resistance of Organ Metastases to Specific Anti-Tumor CTL*

| Host* | Tumor cells isolated from‡ | Percent cytotoxicity with CTL§ | |
|---|---|---|---|
| | | Anti-ESb | Anti-H-$2^d$ |
| DBA/2 normal | Primary tumor | 51, 54, 47, 39, 42, 48, (60) ‖ | 78, 72, 69, 64, 60, 71, (48) ‖ |
| DBA/2 normal | Spleen | 6, 4, 8, 1, 0, 7, 10, 2, 5, (0) ‖ | 62, 64, 60, 58, 52, 61, 47, 55, 65, (53) ‖ |
| DBA/2 normal | Liver | 47, 49, 51, 53, 39, 42 | 71, 64, 78, 72, 62, 64 |
| DBA/2 normal | Lung | 54, 52, 49 | 72, 68, 64 |
| $DBA/2_{ESb}$ immune | Primary tumor | 7 ± 3.5 | 65 ± 4.0 |
| $DBA/2_{ESb}$ immune | Spleen | 0 ± 2.5 | 59 ± 2.7 |
| $DBA/2_{ESb}$ immune | Liver | 2 ± 3 | 73 ± 3.8 |
| BALB/c (*nu/nu*) | Primary tumor | 55, 62 | 65, 64 |
| BALB/c (*nu/nu*) | Spleen | 43, 46 | 58, 63 |
| BALB/c (*nu/nu*) | Liver | 52, 41 | 71, 63 |
| BALB/c (*nu/nu*) | Lung | 47, 44 | 84, 72 |

The metastases were derived from a twice-cloned, subcutaneously inoculated murine lymphoma, ESb-Cl 18.1.

* Hosts were 2–3-mo old syngeneic DBA/2 animals, which were either unimmunized (normal) or preimmunized (immune) with irradiated ESb tumor cells (11); a third group consisted of T cell-deficient nude mice.

‡ $10^5$ ESb-Cl 18.1 cells were inoculated subcutaneously; 12 d later, tumor cells were isolated from the primary tumor and from internal organs. Cell suspensions were prepared by mechanical disruption and tumor cells separated from host cells by isopycnic density gradient centrifugation in Percoll.[2] Alternatively, tumor cells were grown up intraperitoneally in vivo or in tissue culture. Results include data from all three separation procedures.

§ Percent specific $^{51}Cr$ release after 4-h coincubation of the isolated $^{51}Cr$-labeled tumor lines with tumor-specific (anti-ESb) or anti-H-$2^d$ (C57Bl/6 anti-DBA/2) specific CTL at an effector:target cell ratio of 40:1. The CTL were generated as described (11) and frozen in aliquots in liquid nitrogen. Values represent mean percent cytotoxicity of tumor cells from individual animals; SD were <5% and are mostly omitted. Specificity controls consisted of anti-Eb CTL and anti-H-$2^k$ CTL, both of which did not lyse the ESb tumor cells.

‖ Values in parentheses are data from an experiment with ESb-Cl 18.1 cells that had been made azaguanine resistant (12). The spleen-derived tumor cells were resistant to 1 μg/ml azaguanine in tissue culture and thus carried the genetic marker of the subcutaneously inoculated cells.

clonal tumor variants. DBA/2 mice immunized against inactivated ESb tumor cells were previously found to be only partially resistant to a challenge with live ESb tumor cells (11). When the tumor cells that eventually grew out from an immunized mouse were tested for sensitivity to anti-ESb CTL, they were always found to be resistant, regardless of whether the cells were isolated from various internal organs or from the local site (Table I). These results demonstrate the in vivo significance of the observed new immune escape mechanism. ESb-Cl 18.1-derived tumor cells that remained TATA-positive and immunosensitive obviously had no chance to grow in anti-ESb-immunized syngeneic mice. The decreased immunogenicity of ESb compared with Eb (11) thus seems to be due to this immune escape mechanism.

The induction of immunoresistant ESb tumor variants seemed to depend on the presence of mature T lymphocytes in the host. Spleen-derived tumor cells isolated from nude (*nu/nu*) mice that had been inoculated subcutaneously with $10^5$ ESb-Cl 18.1 cells remained immunosensitive (Table I).

*Evidence against the Preexistence of the Immunoresistant Tumor Variants.* The immunoresistant tumor variants were not preexistent in the original twice-cloned tumor cell population. When ESb-Cl 18.1 cells were subjected to stringent immunoselection procedures in vitro, no immunoresistant variants could be isolated. The cells were coincubated twice with a 500-fold excess of specific anti-ESb CTL for 4 h. In one experiment, no tumor cells survived, whereas in a second experiment, a small number of cells survived, which turned out to be immunosensitive after expansion in tissue culture. These cells were recloned and all of the 17 clones isolated were immunosensitive. In contrast, when the spleen-derived tumor variant from the same tumor line

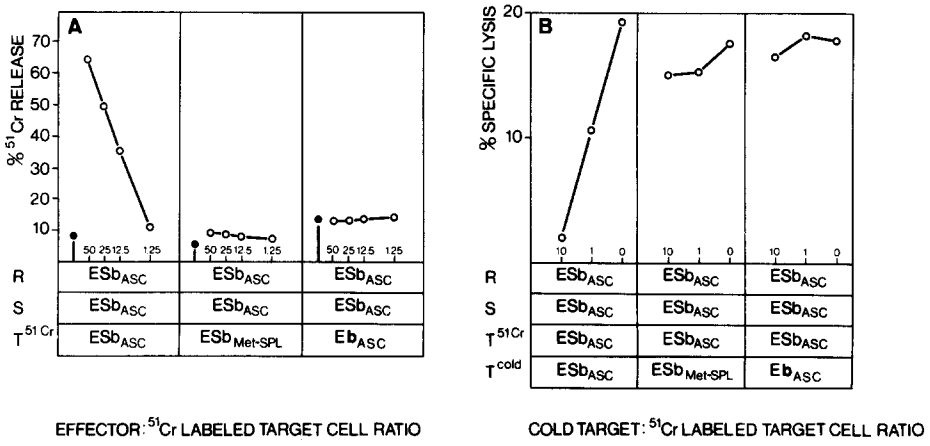


FIG. 1. Comparison of a spleen-derived tumor variant ($ESb_{Met\text{-}SPL}$) with the original ESb and Eb ascites tumor lines ($ESb_{ASC}$, $Eb_{ASC}$) in a direct cytotoxicity test (A) or a cold target competition test (B). Responder cells (R) were derived from spleens of mice immunized against $ESb_{ASC}$. These were cocultured in vitro for 5 d with mitomycin C-treated $ESb_{ASC}$ tumor cells as stimulator cells (S). The anti-ESb CTL effector cells thus generated were washed and tested in a 4-h cytotoxicity test against $10^4$ of the indicated $^{51}Cr$-labeled target cells ($T^{51Cr}$). (A) shows the percent $^{51}Cr$ release in dependency of the ratio of effector to target cells (○, from 50:1 to 1.25:1); (■) indicates the spontaneous $^{51}Cr$ release from the three targets. (B) shows the percent specific $^{51}Cr$ release (spontaneous release subtracted) from $^{51}Cr$ $ESb_{ASC}$ target cells in the presence of different amounts of unlabeled tumor cells ($T^{cold}$). The ratio of anti-ESb CTL effector cells to $T^{51Cr}$ cells was 5:1; the ratio of $T^{cold}$ to $T^{51Cr}$ is indicated.

was recloned, all of the 28 clones isolated were immunoresistant. These experiments show that under immunoselective conditions in vitro using excess anti-ESb CTL, no immunoresistant variants could be isolated from ESb-Cl 18.1. Furthermore, cells that were typed as either immunosensitive (from in vitro) or as immunoresistant (from in vivo) were homogeneous in this respect (results from testing 20–40 subclones of these lines).

## Discussion

Our experiments demonstrate a new type of tumor variant that can arise with a high frequency even from carefully cloned tumor lines. The immunoresistant phenotype remained stable for prolonged periods in tissue culture and has not reverted so far. In spite of this, we think it is unlikely that the variant represents a mutant within a structural gene for the TATA. If there was a high frequency of mutation for such a gene, we would not expect the $ESb_{TATA}$ to be stable for long periods of time. However, we recently reported that the same $ESb_{TATA}$ from the spontaneous variant of 1968 could be re-isolated in 1978 from the Eb line that had been transplanted in the meantime for many years.

The type of tumor variant described here differs from the organ-selective metastatic variants described by Fidler and Kripke (13) and Nicolson and Winkelhake (14) in several respects: (*a*) whereas the immunoresistant tumor variants were not preexisting, the organ-selective ones were suggested to be preexistent (13); and (*b*) whereas the immunoresistant variants developed during a single process of metastasis even when starting with cloned lines, organ-selective variants did not develop under such conditions even when starting with a noncloned tumor population (14). Repeated in

vivo selection procedures (up to 10 times) were usually necessary to obtain organ-selective metastatic tumor variants. These findings suggest either (*a*) that the immunoresistant variants arise with a much higher frequency than the organ-selective ones; or (*b*) that the negative-selection pressure in the case of the immunoresistant variants is much higher than the positive-selection pressure in the case of the organ-selective variants; or (*c*) a combination of both. The organ-selective variants may be the result of a random process of tumor variant generation and intensive selection by the experimentor. In contrast, the immunoresistant variants could be the result of a random variation process or of a specific induction process (15), followed by host selection. The exact mechanism of the development of tumor variants that are selectively resistant to tumor-specific CTL remains to be elucidated. A distinction between the above alternatives would be of great significance not only for mechanisms of immune escape but also for tumor heterogeneity, tumor progression, and metastasis in general.

## Summary

A metastasizing variant of a chemically induced lymphoma from a DBA/2 mouse is shown to carry a distinct tumor-associated transplantation antigen (TATA), which can be recognized by syngeneic secondary anti-tumor cytolytic T lymphocytes (CTL). During metastasis of twice-cloned cell lines of this tumor, variants develop that are specifically immunoresistant to lysis by anti-tumor CTL. The variants are detected in the spleen of normal syngeneic mice. They remain stable over long-term passage in tissue culture. The high frequency with which these immunoresistant metastatic variants develop was found to explain the relative ineffectiveness of specific immunization against this metastatic tumor.

Compared with organ-selective metastatic variants, the immunoresistant tumor variants seem to arise with a much higher frequency. The change in TATA expression described here differs from antibody-induced antigenic modulation in that it is more stable and genetically transmitted.

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