Brief Definitive Report

METASTATIC POTENTIAL SEVERELY ALTERED BY CHANGES IN TUMOR CELL ADHESIVENESS AND CELL-SURFACE SIALYLATION

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It has been shown recently that high metastatic tumor lines can be converted to low metastatic ones by mutagenization and selection for resistance to toxic concentrations of the lectin wheat germ agglutinin (1-3). Lectin-resistant variants were found to differ from the parental lines and respective revertants in terminal cell-surface carbohydrates. Here we describe for the first time a drug-independent selection procedure for the isolation of a low metastatic variant type cell from a high metastatic tumor line. The method is based on the selection of plastic-adherent cells from a suspension culture of highly malignant cells. Similar to the above situation, plastic-adherent low-metastatic variant cells were found to differ from the parental cells and from metastatic revertants in terminal cell surface carbohydrates. The importance of terminal carbohydrates, in particular of sialic acid, for cell-surface adhesiveness and metastatic potential will be discussed.

Materials and Methods

Tumor Lines. Eb is the Heidelberg subline of the methylcholanthrene A-induced T cell lymphoma L5178Y of DBA/2 mice and ESb is a spontaneous high metastatic variant thereof (4). Details of these cells have been described elsewhere (for a review see ref. 5).

Selection of a Plastic-adherent Variant. Both tumor lines Eb and ESb grow in suspension culture (6). A strongly plastic-adherent variant (ESb-M) was isolated from the ESb cells by continuously selecting the adherent subpopulation from the suspension cultures over a period of 2 mo and >20 selection steps. ESb-M cells which were released into suspension from monolayer cultures grown to confluency were taken for analysis.

Flow Cytofluorography. Immunofluorescent staining was carried out by incubating 2×10^6 cells with appropriately diluted fluorescein isothiocyanate- (FITC) conjugated lectins for 45 min on ice. The reaction was carried out in saline containing 1 mM Mg and Ca salts. Flow cytofluorographic analysis was performed on an Ortho H-50 cell sorter (Ortho Instruments, Westwood, MA). Fluorescence gains were kept at constant setting for each lectin analyzed. Cell surface differentiation antigens were determined by indirect immunofluorescence and cytofluorography as described (7).

Isolation of Tumor Cells from Organs. Spleen and brain of ESb-M tumor bearing mice were removed 40 d after tumor cell inoculation and pressed through a fine nylon mesh. The cells were suspended and cultured in the appropriate tissue culture medium (4).

Results

When inoculated into syngeneic DBA/2 mice, the plastic-adherent ESb-M variant type cells were found to be tumorigenic but not metastatic. The survival curves of

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mice inoculated subcutaneously with Eb, ESb, or ESb-M tumor cells are shown in Fig. 1. ESb-tumor bearing mice died after 10–12 d with a small, just palpable tumor and widespread internal metastases. In contrast, ESb-M tumor-bearing mice lived more than four times as long, developed large locally growing tumors (2–4 cm diam), and died without overt metastases.

ESb-M cells were found to have lost also their capacity to form artificial metastases after intravenous inoculation. Animals survived inocula of 10^5 and even 10^7 ESb-M type cells, whereas they died from intravenous inocula of <10 ESb-type cells. This surprising in vivo finding raised the question as to whether ESb-M cells were still ESb-type cells. We therefore examined ESb-M cells for the expression of cell-surface markers that can discriminate between Eb and ESb type cells (4, 5, 7). ESb-M cells were found to be Thy-1^-, Lyt-1^+, Lyt-2^-, Fc receptor-positive and possessed the ESb-type tumor antigen ($TATA_{ESb}$) (Table I) thus showing a similar phenotype as ESb cells.

We then performed a biochemical analysis of cell-surface molecules to look for a structural basis of the altered biological behaviour of ESb-M cells. Cell-surface molecules labeled either by lactoperoxidase-catalyzed iodination or by the galactose oxidase/NaB^3H_4 technique and analyzed subsequently by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were found to be similar to those of ESb but not of Eb-type cells (data not shown). Differences between ESb and ESb-M cells were, however, noted in the expression of lectin-binding sites. This was assessed by staining the cells with various FITC-conjugated lectins and analysis by flow cytofluorography. The results are shown in Fig. 2. The low metastatic variant line ESb-M reacted with *Vicia villosa* (VV) and soybean agglutinin (SBA) lectin, which recognize preferentially terminal *N*-acetylgalactosamine (Gal-NAc) residues. ESb-M cells were also reactive with peanut agglutinin (PNA) possessing a strong affinity for terminal D-Galactose (D-Gal) residues. The binding of these lectins could be specifically inhibited by the respective free sugars.

In contrast, the high metastatic ESb-type cells were only poorly labeled by the above lectins. The staining with concanavalin A (Con A) which reacts with the core sugar mannose was, however, of similar degree as for Eb and ESb-M cells. When ESb

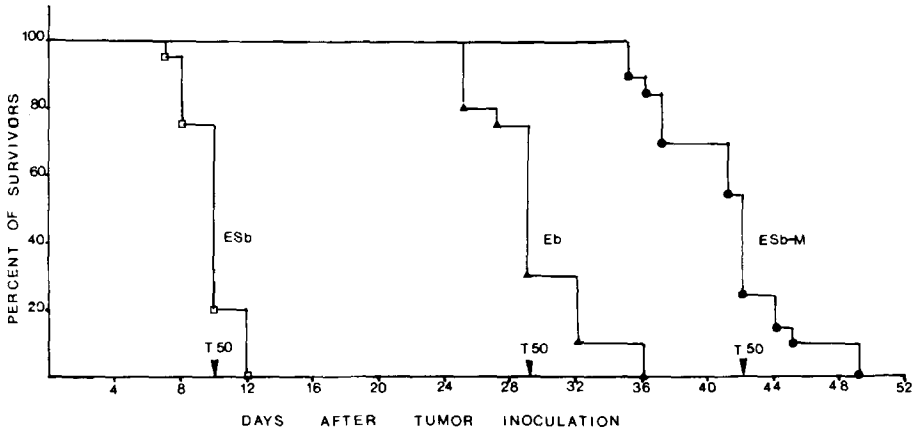

FIG. 1. Mortality curves of DBA/2 mice (10/group) inoculated subcutaneously with 10^6 Eb, ESb, or ESb-M cells.

TABLE I

Characteristics of ESb-M Variant Cells in Comparison with Eb and ESb-type Cells

	Eb	ESb	ESb-M
Induction	MCA	From Eb in vivo	From ESb in vitro
Growth in vitro	Suspension	Suspension	Adherent
Growth in vivo	Large primary (~2 cm ϕ)	Small primary (~0.5 cm ϕ)	Large primary (>2 cm ϕ)
Metastasis	±	+++ (liver, lung, spleen, etc.)	±
TATA expression:*			
Percent killed by anti-Eb	48.0	3.0	14.2
CTL	0	58.0	80.0
Percent killed by anti-ESb			
Expression of Fc receptor:‡			
Percent positive cells	0	65.0	86.0
Expression of differentiation antigens:§			
Thy-1	46.4	7.5	3.3
Lyt-1	0.2	28.9	82.5
Lyt-2	37.7	1.0	2.0

* Tumor-associated transplantation antigens detected by tumor-specific syngeneic cytotoxic T lymphocytes (CTL); values indicate percent specific ^{51}Cr release after 4 h incubation with a 50-fold excess of effector cells.

‡ Percent of cells forming rosettes with IgG antibody-coated erythrocytes.

§ Percent of cells stained with monoclonal antibodies against the indicated T lymphoid differentiation antigens; results from cytofluorographic analysis.

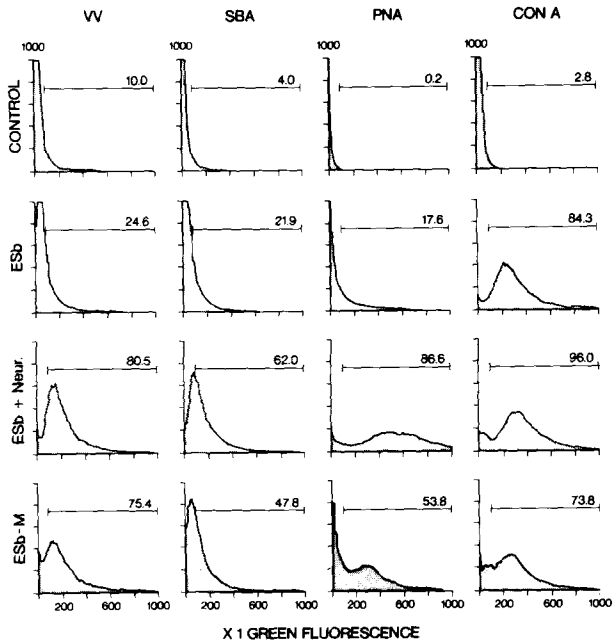

FIG. 2. Histograms from cytofluorographic analysis of tumor cells stained with the indicated FITC-conjugated lectins (Medac, Hamburg, Federal Republic of Germany). Background controls were done by incubating cells and lectins in the presence of 0.2 M inhibitory sugars (Gal-NAc for VV, SBA; D-Gal for PNA; α-methylmannoside for Con A). 3×10^4 cells were analyzed for each histogram, and the percentage of cells within a region (channel 100–1,000) is given. Biphasic curves (i.e., positive and negative cells within one population) indicate either heterogeneity or cell cycle dependence of lectin receptor sites.

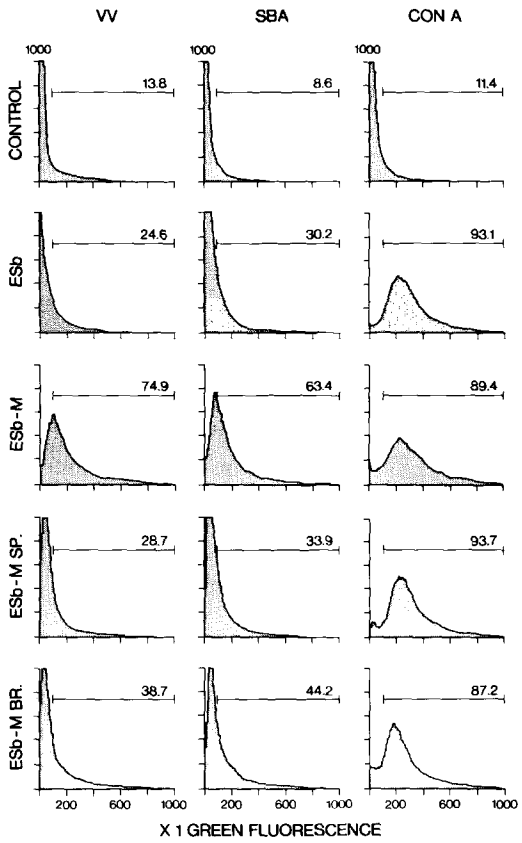

FIG. 3. Histograms of tumor cells isolated on day 40 after subcutaneous inoculation of 10^6 ESb-M tumor cells from brain (ESb-M-Br) and spleen (ESb-M-Sp). The assay was performed as described for Fig. 2.

cells were treated with neuraminidase before staining, they were found to react equally well with the lectins SBA, VV, and PNA (Fig. 2). These findings suggest that the failure to stain ESb cells was due to a masking of receptor sites by sialic acid.

The importance of the changes described in terminal carbohydrates between ESb and ESb-M was further substantiated by metastatic revertants from ESb-M type cells. Rare metastatic revertant tumor cells could be isolated from the spleen (ESbM-Sp) and brain (ESbM-Br) of ESb-M tumor-bearing mice 40 d after tumor inoculation. When these revertants were tested for lectin binding, they were found to have lost their ability to bind VV and SBA, whereas the binding of Con A was unchanged (Fig. 3). Neuraminidase treatment revealed that the lectin-binding sites were again masked by sialic acid (data not shown). These revertant lines were found to have regained the high metastatic capacity characteristic of the parental ESb line and had lost their ability to adhere to plastic.

Discussion

Previous observations in model systems for metastasis have emphasized the importance of cell-surface glycosylation (6, 8) and of the total amount of cell surface sialic acid for the metastatic capacity of certain tumor cells (9). Here we have demonstrated

that the selection of a plastic-adherent variant (ESb-M) from a highly metastatic mouse tumor line (ESb) has a greatly decreased metastatic potential. The isolation procedure is mild, does not require mutagenization, and exerts its selection pressure by simple in vitro culturing. The plastic-adherent variant, although not cloned, was stable over prolonged periods of in vitro and in vivo growth. Hochman et al. (10) recently reported on a plastic-adherent variant of a murine lymphoma that showed increased immunogenicity and impaired tumorigenicity. ESb-M cells were similar to ESb type cells in antigenicity (Table I) and immunogenicity (not shown), but showed loss of metastatic capacity while still being tumorigenic. The only major differences found between ESb and ESb-M cells were altered levels of terminal sialic acid at lectin sites for Gal-NAc specific lectins. These changes in sialylation were found to be reversed when rare revertants of ESb-M cells, isolated from two distant organs, were analyzed. Because uncloned tumor cell populations were studied, we are aware of the possibility that these revertants could theoretically represent derivatives from a few contaminating metastatic ESb cells within the ESb-M cells inoculated. This possibility is excluded, however, by the finding that even as many as 10^7 ESb-M cells inoculated intravenously did not give rise to metastases, while as few as 10 ESb cells killed the animals within 3 wk after intravenous inoculation (11).

In a recent study we have noted similar differences in sialylation as described here between ESb cells and their low metastatic parental line Eb.[1] Receptor sites for VV and SBA on Eb tumor cell were not blocked by sialic acid and could be directly stained with these lectins in a specific way. These findings were further corroborated in the MDAY-D2 tumor system consisting of a high metastatic parental line, low-metastatic, lectin-resistant variants, and high-metastatic revertants. In all cases there was a clear correlation between sialic acid content on VV and SBA receptor sites and metastatic potential.

In the Eb/ESb tumor system, the total amount of cell-surface sialic acid was not much different between the two lines. The results from this and other studies therefore suggest subtle qualitative changes in the positioning of sialic acid at the cell surface. Some sites, like those recognized by lectin-like hepatocyte receptors (12), seem to be sialylated on Eb cells and desialylated on ESb cells, whereas other sites, like those recognized by VV and SBA lectins, seem to be desialylated on Eb cells and sialylated on ESb-type cells. The possible transpositioning of sialic acid could thus have a double effect, a decrease of adhesive forces, for instance between tumor cells, and an increase of adhesive forces, for example to certain target organ structures.

We suggest that the expression of certain specific receptor sites by the removal of sialic acid alters the tumor cells' adhesive characteristics. The free receptor sites could be involved in the adhesiveness of the ESb-M type cells to plastic. Under physiological conditions in vivo, such sites could be involved in homotypic (tumor cell-tumor cell) adhesion or in the adhesion to host derived cells or their extracellular matrix.

Summary

A plastic adherent variant line (ESb-M) of a highly invasive and metastatic murine T cell lymphoma (ESb) was found to have lost its metastatic potential while still being tumorigenic in normal syngeneic hosts. The variant retained most of its ESb-

[1] P. Altevogt, M. Fogel, R. Cheingsong-Popov, J. Dennis, and V. Schirrmacher. Relayed high and low metastatic mouse tumor lines shows different patterns of lectin binding and cell surface sialylation. Manuscript submitted for publication.

derived antigenic and biochemical characteristics but differed at binding sites for certain lectins with specificity for terminal *N*-acetylgalactosamine residues. Whereas such sites were masked by sialic acid on metastatic ESb cells, they became unmasked on the adherent variant line. Metastatic revertants of ESb-M cells did not express the respective lectin receptor sites because these were again masked by sialic acid.

It is suggested that the masking of specific lectin receptor sites on the tumor cell surface is of crucial importance for metastatis. If freely exposed, these sites may change adherence characteristics of the cells possibly not only in vitro (to plastic) but also in vivo.

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References

1. Tao, T. W., and M. M. Burger. 1977. Nonmetastasizing variants selected from metastasizing melanoma cells. *Nature (Lond.)*. **270:**437.
2. Reading, C. L., P. N. Belloni, and G. L. Nicolson. 1980. Selection and in vivo properties of lectin-attachment variants of malignant lymphosarcoma cell lines. *J. Natl. Cancer Inst.* **64:**1241.
3. Dennis, J. W., and R. S. Kerbel. 1981. Characterization of a deficiency in Fucose metabolism in lectin-resistant variants of a murine tumor showing altered tumorigenic and metastatic capacities in vivo. *Cancer Res.* **41:**98.
4. Schirrmacher, V., and K. Bosslet. 1980. Tumor metastases and cell-mediated immunity in a model system in DBA/2 mice. X. Immunoselection of tumor variants differing in tumor antigen expression and metastatic capacity. *Int. J. Cancer.* **25:**781.
5. Schirrmacher, V., M. Fogel, E. Rußmann, K. Bosslet, P. Altevogt, and L. Beck. 1982. Antigenic variation in cancer metastasis. Immune escape versus immune control. Cancer Metastasis Reviews. **1:**241.
6. Burger, M. M. 1980. The cell surface and metastasis. *In* Biology of the Cancer Cell. K. Letnansky, editor. Kugler Medical Publications, Amsterdam. 193–207.
7. Altevogt, P., J. T. Kurnick, A. K. Kimura, K. Bosslet, and V. Schirrmacher. 1982. Different expression of Lyt differentiation antigens and cell surface glycoproteins by a murine T lymphoma line and its high metastatic variant. *Eur. J. Immunol.* **12:**300.
8. Fidler, I. J., and G. L. Nicolson. 1981. The immunobiology of experimental metastatic melanoma. *Cancer Biol. Rev.* **2:**1.
9. Yogeswaran, G., and P. L. Salk. 1981. Metastatic potential is positively correlated with cell surface syalylation of altered murine tumor cell lines. *Science (Wash. D. C.)*. **212:**1514.
10. Hochman, J., A. Katz, E. Levy, and S. Eshel. 1981. Substrate-adhering lymphoid cells show impaired tumorigenicity and increased immunogenicity. *Nature (Lond.)*. **290:**248.
11. Schirrmacher, V., D. Hübsch, and K. Clauer. 1980. Tumor metastases and cell-mediated immunity in a model system in DBA/2 mice. IX. Radioassay analysis of tumor cell spread from a local site to the blood and liver. *In* Metastatic Tumor Growth, Cancer Campaign. E. Grundmann, editor. Gustav Fischer Verlag, Berlin. 147–158.
12. Schirrmacher, V., R. Cheingsong-Popov, and H. Arnheiter. 1980. Hepatocyte tumor cell interaction in vitro. I. Conditions for rosette formation and inhibition by anti H-2 antibodies. *J. Exp. Med.* **151:**984.