Selective Events in the Metastatic Process Defined by Analysis of the Sequential Dissemination of Subpopulations of a Mouse Mammary Tumor

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ABSTRACT

To identify selective steps in metastasis, those that eliminate nonmetastatic tumor cells more efficiently than metastatic cells, we have evaluated the sequential dissemination of tumor cells from a mammary fatpad, using both metastatic (4T1 and 66cl4) and nonmetastatic (67NR, 168FARN, and 4TO7) subpopulations of a single mouse mammary tumor. Each of these variant subpopulations is resistant to one or more selective drugs so they could be quantitatively identified by colony formation in selective media. We found that the 2 metastatic cell lines metastasized by different routes and that the nonmetastatic tumor cell lines failed at different points in dissemination. Line 67NR did not leave the primary site; clonogenic tumor cells were not detected in the nodes, blood, or lungs during the experiment (7 weeks). Tumor line 168FARN disseminated from the primary tumor because clonogenic cells were cultured from the draining lymph nodes throughout the experiment. However, dissemination essentially stopped in the node as cells were rarely isolated from blood, lungs, or livers. Whether 168FARN cells failed to reach these tissues or were killed very rapidly after traversing the lymph node is unknown. Line 4TO7 cells disseminated via the blood and were consistently recovered from lungs by day 19 but failed to proliferate. This panel of 5 subpopulations thus identifies different points of selective failure in tumor cell dissemination and should be valuable in the assessment of antitumor therapies.

INTRODUCTION

The metastatic process is a sequence of steps (invasion, intravasation, transport, arrest, extravasation, and growth) that must be accomplished by cancer cells before distant metastases are established (1, 2). Metastasis of even highly metastatic cells is normally a very inefficient process; most tumor cells that enter the bloodstream do not develop into metastatic nodules (3, 4). Both random and selective events may be responsible for this metastatic inefficiency (5). Metastasis is selective because: (a) cells isolated from spontaneous metastases may be more metastatic than the original parent tumor (6); and (b) genetically stable metastatic and nonmetastatic sublines have been characterized (6–8). Nonmetastatic cell lines are unable to complete one or more steps in the metastatic cascade, whereas metastatic cell lines must be able to complete all steps involved in the metastatic process (2). Although random events in the metastatic process eliminate the majority of tumor cells irrespective of metastatic phenotype (9), one can define any step that more efficiently eliminates cells of nonmetastatic lines than metastatic lines as a selective step. If host immune functions are important for the elimination of potentially metastatic cells, the selective events may be the best targets for therapeutic intervention with biological response modifiers.

We have developed methods to quantitate clonogenic tumor cells in host tissues using tumor cells with drug-resistance markers and selective media (10–12). In this report, we have identified selective steps in metastasis by evaluating the sequential dissemination of tumor cells from a mammary fatpad using both metastatic and nonmetastatic subpopulations of a single mouse mammary tumor.

MATERIALS AND METHODS

Mice. Female BALB/c mice, 8–12 weeks old, were produced in our animal colony from a BALB/c breeding colony established by cesarean derivation of a litter of mice from BALB/cfC3H parents obtained from the Cancer Research Laboratory, Berkeley, CA. The Michigan Cancer Foundation Animal Facility is fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Tumor Cell Lines. Tumor subpopulation lines 66, 67, 168, and 410.4 were isolated from a single spontaneously arising mammary tumor from a BALB/cfC3H mouse (8, 13). Line 66cl4 is a thio guanine-, ouabain-resistant variant of line 66 (14). Line 168FARN is a diaminopurine-, genet i cin-resistant variant clone of line 168 obtained by transfecting the bacterial pSV2 plasmid containing the neomycin resistance gene into 168FAR (15). Similarly, the genet i cin-resistant 67NR was obtained by transfecting line 67. Line 4TO7 is a thioguanine-, ouabain-resistant variant of 410.4 (16). Line 4T1 is a thioguanine-resistant variant that was selected from 410.4 without mutagen treatment. Line 4T1 spontaneously metastasizes to both the lung and liver as evidenced by the formation of visible nodules in these organs in mice bearing line 4T1 primary tumors. Line 66cl4 spontaneously metastasizes to the lung. Lines 67NR, 168FARN, and 4TO7 are highly tumorigenic but very rarely metastasize spontaneously.

Media. Cells were grown in DMEM (10), Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum, 5% newborn calf serum, 1 mM mixed nonessential amino acids, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). To prepare cells for injection, flasks were rinsed with 0.25% trypsin in 0.05% EDTA, washed once, and suspended in DMEM.

Detection of Dissemination. Tumor cells were suspended to a final concentration of 1 x 107 cells/ml in DMEM-10. Mice were anesthetized with sodium pentobarbital (65 mg/kg body weight), and 1 x 107 tumor cells in 0.01 ml were injected into a no. 4 mammary fatpad (17). At various times, groups of 4–5 mice were anesthetized and bled from the right ventricle with a syringe rinsed with heparin (6.29 mg/10 ml), and draining lymph nodes, lungs, and livers were removed.

Whole heparinized blood was washed twice with DMEM-10 and plated in 100-mm tissue culture dishes. Lymph nodes from the brachial region and the no. 4 mammary fatpad were removed aseptically with sterile forceps and tease apart into individual wells of a 6-well plate (18). At later time points, tumors often enveloped the mammary lymph node so that this node was unavailable for analysis. Lungs were minced into 1-mm3 pieces and presoaked for 60 min in 5 ml of an enzyme cocktail containing 1 mg/ml collagenase type IV (Sigma Chemical Company, St. Louis, MO) and 36 units of elastase (ICN Biomedicals, Inc., Costa Mesa, CA) at 4°C. The samples were mechanically dispersed with 4 sequential 30-s and 3 sequential 1-min periods in a Stomacher blender (Tekmar Company, Cincinnati, OH). Following each dispersion period,
a portion of the cell suspension was removed and an equal volume of DME containing 10% calf serum (DME-10) added (10).

Livers were minced and an enzyme solution containing collagenase type I (Sigma), hyaluronidase (Sigma), each at a concentration of 1 mg/ml, in a final volume of 5 ml was added. After a 15-min incubation at 37°C, 5 ml of DME-10 were added and samples were mechanically dispersed for 5 sequential 1-min periods. Between bursts, a portion of the cell suspension was removed and an equivalent volume of DME-10 added (10).

All cell suspensions were plated in DME-10 containing the appropriate selective drug and incubated for 10 to 14 days in 10% CO2-air atmosphere at 37°C. The colonies were fixed with Carnoy's solution, stained with crystal violet, and counted, and total colony-forming cells per organ calculated.

RESULTS

Sequential dissemination of tumor cells from a mammary fatpad was evaluated using both metastatic (4T1 and 66cl4; Fig. 1) and nonmetastatic (67NR, 168FARN, and 4TO7; Fig. 2) subpopulations of a mouse mammary tumor. Line 4T1 appeared to metastasize primarily, but not exclusively, by a hematogenous route because clonogenic tumor cells were recovered significantly more frequently (P < 0.001, χ² analysis) from the blood (38 of 58 mice) than from draining lymph nodes (12 of 58 mice). Figure 1A depicts 1 of 2 experiments in which the sequential spread of 4T1 from a mammary fatpad was monitored. Lungs were seeded early (clonogenic 4T1 cells were first detected at day 7, and 5 of 5 mice had clonogenic 4T1 cells in the lung by day 14) and the lung tumor burden increased on subsequent assay days with an apparent doubling time of 30 h. (The apparent doubling time is an underestimate because it not only measures the growth rate of occult metastases but also reflects continuous seeding of tumor cells from the primary tumor.) Livers were seeded later, in that 4T1 cells were first detected at day 21 and incidence did not reach 100% until day 28. Seven mice were necropsied at day 31; all had visible lung nodules (43 ± 7; mean ± SEM) and 5 had visible liver nodules (2.0 ± 0.8). In a second experiment, lungs were again seeded early (66% incidence by day 9) and the lung tumor burden increased on subsequent assay days. Clonogenic 4T1 cells could be detected in the liver within days after detection in the lungs. Ten mice from the second experiment were sacrificed for necropsy between days 34 and 40. All 10 had visible nodules on the surface of the lungs (49 ± 6.5) and 5 mice had a visible nodule on the surface of the liver.

Clonogenic tumor cells were recovered from both the blood (27 of 82 mice) and lymph nodes (31 of 59 mice) of mice bearing line 66cl4 tumors in a mammary gland. χ² analysis suggests that line 4T1 more often metastasized hematogenously than did line 66cl4 (P < 0.02) and that line 66cl4 more often metastasized via the draining lymph node than did line 4T1 (P < 0.01). Figure 1B depicts 1 of 2 experiments in which the sequential spread of 66cl4 from a mammary fatpad was monitored. Clonogenic cells were detected in lungs of 66cl4 tumor bearing mice on day 14 and all mice had detectable tumor cells in the lungs by day 28. After day 28, expansion of the tumor cell population in the lung proceeded at an exponential rate with an apparent doubling time of 39 h. In addition, clonogenic cells were recovered from livers on day 35 in this experiment, but all 8 mice sacrificed for necropsy at day 50 had visible nodules in the lung (26 ± 3) but not liver nodules. Metastatic nodules have never been observed in livers of mice with 66cl4 primary tumors. In a second experiment with 66cl4, clonogenic cells were again detected in lungs on day 14, and the apparent doubling time in the lungs after day 28 was 36 h. Seven mice from this experiment were sacrificed for necropsy at day 50. All 7 had visible nodules in the lungs (6 ± 2); no liver nodules were found.

The nonmetastatic line 67NR appeared to be unable to intravasate. Clonogenic cells were not recovered from any of the tissues (blood, lymph nodes, lungs, or liver) sampled from a total of 44 mice over a period of 7 weeks for 2 experiments, with one exception in which 3 clonogenic cells were recovered from a draining lymph node of one animal on day 7 in one experiment (Fig. 2A). Livers were dissociated and plated in selective media in one experiment (no clonogenic cells were detected) but not in the other. None of the mice sacrificed at days 49–50 for necropsy in the 2 experiments had visible metastases or detectable clonogenic cells in any organ. The size
of the primary 67NR tumors at the time of necropsy (6.6 ± 1.5 g and 11.2 ± 4.6 g in the 2 experiments) far exceeded the size of either 66cl4 (2.6 ± 0.3 g and 2.3 ± 0.8 g at necropsy) or 4T1 (1.9 ± 0.2 g and 1.7 ± 0.5 g at necropsy) primary tumors, when metastatic nodules were detectable in animals with the latter 2 tumor lines.

The nonmetastatic line 168FARN cells spread through the lymphatics, since clonogenic cells were recovered from draining lymph nodes in the mammary gland (Fig. 2B). In a second experiment, low numbers of clonogenic 168FARN cells were occasionally recovered from lungs (1 animal of 6 at necropsy on day 51 had 43 clonogenic cells) and livers (none of the 6 animals at necropsy had detectable cells in the liver, but 2 of 5 mice on day 21 had 13 and 65 clonogenic cells, and 2 of 5 mice on day 28 had 8 and 15 clonogenic cells in the liver). Although clonogenic 168FARN cells could be recovered from draining lymph nodes, clonogenic cells were not recovered in either experiment from the blood of any of the 61 mice bearing line 168FARN tumors. Visible nodules were not observed on lungs at necropsy, and clonogenic cells were recovered in only one animal as noted above despite the presence of very large primary tumors (9.3 ± 1.1 g and 8.6 ± 2.2 g at necropsy for the 2 experiments).

The nonmetastatic line 4TO7 appeared to be able to complete all steps of metastasis except the final one. Line 4TO7 spread via the blood to lungs and occasionally to livers, but did not establish progressively growing metastatic nodules. Fig. 2C depicts one of 2 experiments in which the sequential spread of 4TO7 from a mammary fatpad was monitored. Clonogenic cells were recovered from the lungs at day 19. At necropsy, visible nodules were absent but clonogenic 4TO7 cells were recovered from lungs in 4 of 5 mice (primary tumor weight of 1.35 ± 0.32 g on day 28) with a geometric mean of 32 clonogenic cells detected.

In a second experiment with 4TO7, clonogenic cells were recovered from the lungs on day 7 after tumor cells were
implanted into a mammary fatpad. The discrepancy between the two 4TO7 experiments is most likely due to the fact that the primary 4TO7 tumors appeared earlier in the second experiment and, thus, tumors were larger on the various assay days. In the second experiment, necropsies were done on day 38 when primary tumor weights were 2.85 ± 0.42 g. Visible nodules were absent, but clonogenic 4TO7 cells were recovered from lungs of all 8 mice with a geometric mean of 1086 clonogenic cells. However, if primary 4TO7 tumors are surgically removed, clonogenic cells disappear from the lung within 1 week (data not shown), indicating that the presence of clonogenic 4TO7 cells in the lungs is dependent upon continuous seeding from primary tumors rather than on proliferation of tumor cells in the lung.

The growth properties of all 5 subpopulations in monolayer culture in vitro were similar. The population doubling times ranged from 16 h for 67NR and 66cl4 to 21 h for 168FARN. The clonogenic potential (no. of colonies formed/no. of cells plated) ranged from 0.54 for 4T1 and 4TO7 to 0.68 for 67NR. The addition of heparinized blood had little effect on the clonogenic potential of the subpopulations. As compared to clonogenic potential in medium alone, recovery in the presence of heparinized blood ranged from 86% for 168FARN to 97% for 67NR.

Line 67NR cells (100 cells) were plated with lymph node cells (2 × 10^6) to determine if the failure to detect 67NR in draining lymph nodes was due to lymph node cell mediated suppression of colony formation by 67NR in vitro. Neither lymph node cells from normal mice (0.63 clonogenic potential) nor lymph node cells from mice bearing 67NR tumors (0.57 clonogenic potential) markedly affected growth of 67NR in vitro.

Clonogenic potential in selective media was not reduced by the addition of 1 × 10^7 lung cells in a 100-mm dish. In fact, when fewer than 10 clonogenic tumor cells were mixed with 1 × 10^7 lung cells, more tumor cell colonies were recovered than in controls without added lung cells. Thus, the sensitivity of the method for detecting occult clonogenic tumor cells seems to be limited only by the ability to recover tumor cells during the tissue dissociation procedure.

The relative resistance of the 5 subpopulations to the method used to dissociate lungs was determined (Fig. 3). Line 168FARN was the most sensitive and 4TO7 was the least sensitive. However, the data indicate only a 2-fold differential recovery between these extremes. It is not likely that this differential sensitivity to organ disruption was responsible for the differential recovery of sublines from the lung.

**DISCUSSION**

It is not uncommon for tumor sublines to display heterogeneous metastatic potential. Mouse melanomas (19, 20), lymphomas (21, 22), mammary carcinomas (8, 23, 24), lung carcinomas (25, 26), a fibrosarcoma (27), a colon carcinoma (28), rat mammary carcinoma (29), rat prostate carcinoma (30), human renal cell carcinoma (31), and human melanoma (32) have all been used to derive sublines with high and low metastatic potential. Further characterization of such subpopulations frequently uncovers differences in phenotypes of apparent importance in the metastatic sequence such as the production of enzymes for degradation of extracellular matrix and basement membrane (33–36), reduced production of tissue inhibition of metalloproteinases (37), migration (38–42), response to autotomy factor (43), laminin expression (40, 44), laminin binding (45), invasion in vitro (46, 47), lectin expression (48), homotypic (49) and heterotypic aggregation (50), anchorage independence (51), expression of g-protein (52), gap junctions (53), response to growth factors (54, 55), antigenicity (56, 57), and differential susceptibility to tumoricidal macrophages (58), natural killer cells (59, 60), or T-cells (61, 62).

It may not be prudent to assume that properties expressed in vitro, despite their correlation with the end result, i.e., formation of visible metastatic nodules, reflect similar properties mechanistically responsible for selection in vivo during the course of metastasis. Thus, for example, a metastatic line that produces some proteolytic enzyme and a nonmetastatic line that does not produce that enzyme might invade equally well but differ in their ability to respond to some growth factor or have a differential sensitivity to immune resistance in the involved organ. On the other hand, 2 cell lines, 1 metastatic and 1 nonmetastatic, may both produce some proteolytic enzyme that enables both cell lines to invade, but the nonmetastatic line fails to survive some subsequent step. Thus, one can conclude neither that a property is important in metastasis because it correlates with metastasis nor that a property is unimportant because it does not correlate with metastasis. Analysis of specific steps in the metastatic cascade would advance the understanding of the basic biology of metastasis and allow a more critical evaluation of the roles of the many factors implicated in metastasis. Furthermore, if host immune functions are important for the elimination of potentially metastatic cells, then it may be the selective events that are likely to be the best targets for therapeutic intervention. Random events in the metastatic process eliminate the vast majority of tumor cells irrespective of metastatic phenotype. Weiss (9) argues that, if 99.9% of cells of a metastatic line injected i.v. fail to form metastatic nodules (e.g., 100 colonies are formed after injecting 10^5 cells) and 99.999% of cells of a nonmetastatic line injected i.v. fail to form metastatic nodules (e.g., 1 colony is formed after injecting 10^5 cells), the metastatic process is essentially random. Although this may be true on a statistical basis, each individual metastasis may have profound clinical consequences. Weiss (9) suggests that “if any population of cancer cells entering the metastatic cascade goes sequentially through five randomly traumatic steps associated with invasion, etc., each of which kills 90% of the cells, then only 0.001% of the initial cellular input from the primary cancer will form metastases.” However, to further develop Weiss’s example, if we assume that, in addition to the random trauma that eliminates cells of both metastatic and nonmetastatic lines equally, the occurrence of a selective process at any of the steps accounts for an additional log kill of the nonmetastatic cells, the occurrence of 2 selective steps in the cascade would decrease the likelihood of developing metastatic disease by 100-fold. Although these calculations of log kills are hypothetical, they illustrate the impact that even weak selective events might have on the final outcome in an otherwise random metastatic process. To date, knowledge of which steps in the metastatic cascade are selective has been limited by deficiencies in existing methodology to analyze quantitatively the clonogenicity of potentially metastatic cells at various points in the metastatic cascade.

Bioassays have been used to estimate the number of tumor cells with replicative potential present in tissues of tumor-bearing experimental animals, but the methods were insensitive and not quantitative (63–65). By mixing lethally irradiated cells
with blood or minced lungs, Mayhew and Glaves (66) were able to increase the sensitivity of their bioassay for B16 melanoma or Lewis lung carcinoma cells, but a threshold of 10–100 cells still existed. Another undesirable feature of a bioassay is the requirement for multiple animals to test for the presence of metastatic tumor cells in 1 experimental tumor-bearing animal.

Susuki (67) enzymatically dissociated lungs with protease type IX and then plated the cell suspensions from tumor-bearing mice onto irradiated feeder layers. After 2 weeks, colonies were fixed, stained, and counted (67, 68). In our own studies, the background lawn of normal tissue cells, even without using a feeder layer, made it impossible to quantitate colonies formed by some of our tumor cell lines that do not form dense colonies. Furthermore, we have occasionally observed distinct colonies in wells of cell suspensions prepared from the lungs of normal mice when plated in nonselective media. By using tumor cells with drug resistance markers, it is possible to simultaneously quantitate and unambiguously identify the tumor cell colonies developing in the presence of selective media (10–12, 18, 69, 70). Since first demonstrating our ability to follow the sequential spread of mammary tumor cells from a mammary fatpad (18), we have significantly improved our methods for dissociating the lung and liver so that greater than 50% of the tumor cells (identified by \( ^{125}\text{I} \)IUrd labeling) are recovered (10).

All 5 mouse mammary tumor subpopulations used in these experiments colonize the lung if a sufficient number of cells are injected i.v. However, only 2 are metastatic based on their ability to metastasize spontaneously from a mammary fatpad; the other 3 obviously are unable to complete some step in the metastatic process. We found a significant difference in the route of dissemination by the metastatic subpopulations 66cl4 and 4TO7. Because venolymphatic anastomoses may allow tumor cells to move readily from one compartment to the other (blood/lymphatics) (71), one might expect that lymph node metastases act as a generalizing site for blood-borne metastases. This may be the case for subpopulation 66cl4, which was detected in the blood of 33% of tumor-bearing mice and in the lymph nodes of 53%. However, subpopulation 4TO7 appears to enter the blood stream (66% of mice) without lymph node involvement [21% of mice \( \times 0.001 \)]. Our results with these 2 subpopulations are consistent with those of Alterman et al. (72) and Weiss and Ward (73, 74). Although bloods from tumor-bearing mice were not evaluated, Alterman et al. (72) concluded that 2 metastatic clones of the B16 melanoma must metastasize hematogenously to the lung because cells were recovered from the lung by bioassay before they could be detected in regional draining lymph nodes. Both B16 melanoma (72) and Lewis lung carcinoma (73) appear to metastasize independently via both lymphogenous and hematogenous routes. Weiss and Ward (74) also found that the rat mammary carcinoma MT-100-TC metastasized by both lymphogenous and hematogenous routes, but hematogenous transport was apparently due to entry of cells into blood vessels within the primary tumor rather than via venolymphatic anastomoses because tumor cells were rarely found in blood vessels associated with regional lymph nodes.

Previous studies have compared different tumor models and subpopulations to elucidate mechanisms of metastatic inefficiency. Clones of K1735 murine melanoma were able to colonize lungs in syngeneic euthymic mice, able to colonize lungs in athymic but not euthymic mice, or unable to colonize lungs in either athymic or euthymic mice (75). The authors classified these clones as metastatic, metastatic antigenic, and nonmetastatic, respectively. Aggregation and initial arrest of \( ^{125}\text{I} \)IUrd-labeled cells was similar for metastatic and nonmetastatic clones, but clearance within 24 h of arrest was much more rapid for a nonmetastatic clone than for a metastatic clone (75). Similarly, we have described that arrest was similar, but 3 of our subpopulation panel had different rates of clearance post-arrest (11); 168FARN was cleared more rapidly than 66cl4 or 4TO7.

Death of arrested cells, as reflected by \( ^{125}\text{I} \)IUrd clearance rates, is not the only postarrest selective event in metastasis. Clearance rates do not necessarily correlate with lung colony forming efficiency of tumor cells injected i.v. Reeve and Twymann (76) found that the more metastatic of 2 murine sarcoma clones was more rapidly killed (faster clearance of labeled cells), but the clonogenic population expanded more rapidly in the lungs for the highly metastatic clone than for the poorly metastatic clone. If injected i.v., our nonmetastatic subpopulation 4TO7 is better able to colonize the lungs than the metastatic line 66cl4 or the nonmetastatic line 168FARN, and these 3 subpopulations expand in the lung in the same rank order of 4TO7 > 66cl4 > 168FARN (11). Because 4TO7 does reach the lungs from primary tumors growing in mammary fatpads but does not form metastatic nodules, perhaps it should be classified as metastatic antigenic (75). However, in the study of Aukerman et al. (75), line 4TO7 would have been classified as metastatic rather than metastatic antigenic because the criterion used for classification was lung colony formation following i.v. injection rather than spontaneous metastasis.

Intravasation appears to be an important selective step in metastasis. Our line 67NR appears unable to leave the primary site. Glaves (77), comparing B16 melanoma and Lewis lung carcinoma, and Weiss et al. (78), comparing B16 melanoma variants, found that cells more highly efficient at colonizing lungs following i.v. injection were similar in abilities to spontaneously metastasize from i.m. tumors. Bioassays for tumorigenic cells in the bloods from tumor-bearing mice indicated that low colony-forming efficiency lines compensated by releasing more tumorigenic cells into the blood.

By defining the selective step in metastasis at which our nonmetastatic subpopulations fail, we have begun to establish an experimental model that will allow the validation of many phenotypes implicated in metastasis. A battery of subpopulations with defined deficiencies will facilitate the verification of the role in metastasis played by a given cell characteristic. This panel of subpopulations also has obvious value in the analysis of tumor cell interactions. Various pairs have been used to demonstrate clonal dominance (16, 79) and interactions affecting responses to chemotherapeutic drugs (80, 81). In our studies, clonal dominance does not correlate with metastatic phenotype as has been reported by Kerbel et al. (82) and Waghorne et al. (83). In addition, nonmetastatic subpopulations derived from the mouse mammary tumor used in our studies may metastasize in the presence of some metastatic subpopulations (84). This phenomenon has also been observed by Waghorne et al. (83). One reason for developing drug-resistant metastatic and nonmetastatic lines with defined metastatic deficiencies is to investigate mechanisms by which cell interactions alter metastatic phenotypes. With the methodology described, we continue to isolate and characterize new variants for these purposes.
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