Endocytosis and Degradation of Murine Anti-Human CD3 Monoclonal Antibodies by Normal and Malignant T-Lymphocytes

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ABSTRACT

Treatment of lymphoid malignancies with monoclonal antibodies (mAbs) and immunotoxins is promising new immunotherapeutic approach. However, few published studies have examined in detail the subcellular fate of antibodies following binding to lymphocyte cell surface antigens. In this study, we have investigated the disposition of monoclonal anti-CD3 antibody 64.1 following binding to normal and malignant T-lymphocytes by using cellular radioimmunoassays and immunoperoxidase and immunogold electron microscopy. Anti-CD3 mAbs were predominantly cleared from the cell membrane at 37°C by receptor-mediated endocytosis, although passive shedding of antibody was also observed. Internalized antibody was sequentially transferred from coated pits to endosomes and eventually to lysosomes. Intralysosomal degradation appeared to be the ultimate fate of internalized radiolabeled mAbs and was followed by exocytosis of free 125I to the culture medium. Ammonium chloride and monensin were potent inhibitors of lysosomal degradation of 125I-anti-CD3 mAbs and caused intracellular trapping of radiolabeled antibodies. The rapid endocytosis, degradation, and exocytosis of antibody observed in these studies elucidate the mechanism of the improved efficacy of anti-CD3 immunotoxins as compared with inhibitors of lysosomal action. CD3 trimolecular complex from the cell surface, and also causes concomodulation of the noncovalently associated T-cell receptor (13). Disappearance of these molecules from the T-cell surface membrane results in the loss of antigen-specific proliferative and cytolytic responses. The precise mechanism of CD3 modulation induced by anti-CD3 antibodies is controversial, with some studies suggesting shedding of the complex from the cell surface (14) while others demonstrate endocytosis of antibody-receptor complexes (24, 25). We have used a cellular radioimmunoassay and immunoelectron microscopy to further investigate the fate of murine anti-CD3 mAbs following binding to normal and malignant human T-cells. Our findings suggest that both antibody shedding and endocytosis occur, but that the latter process predominates. Furthermore, degradation of antibody in lysosomes appears to ensue shortly after mAb internalization. These results have important implications for therapy with anti-CD3 immunoconjugates (4, 26), and may yield insights into the normal process of T-cell activation.

INTRODUCTION

Specific immunotherapy of malignant and autoimmune diseases with monoclonal antibodies, radioimmunoconjugates, and immunotoxins has yielded encouraging preliminary results (1, 2). However, efficacy appears to be compromised in some instances by rapid intracellular metabolism of immunotoxins following internalization into cells (3, 4). Although the subcellular details of endocytosis of many biologically important ligands are well understood (e.g., low density lipoprotein (5), epidermal growth factor (6), transferrin (6), immune complexes (7, 8), and interleukin 2 (9, 10)), few monoclonal antibody-lymphoid antigen pairs (11, 12) have been fully investigated using the types of assays conventionally utilized for other ligands.

In this paper we investigate the endocytosis and intracellular fate of mAbs recognizing the human CD3 ("T3") T-lymphocyte antigen. These studies are particularly pertinent since the CD3 antigen plays a crucial role in T-cell activation (13–17), and since anti-CD3 mAbs have been used clinically for the treatment of T-cell malignancies (23). Exposure of T-cells to anti-CD3 antibodies results in modulation of the cell surface (14) while others demonstrate endocytosis of antibody-receptor complexes (24, 25). We have used a cellular radioimmunoassay and immunoelectron microscopy to further investigate the fate of murine anti-CD3 mAbs following binding to normal and malignant human T-cells. Our findings suggest that both antibody shedding and endocytosis occur, but that the latter process predominates. Furthermore, degradation of antibody in lysosomes appears to ensue shortly after mAb internalization. These results have important implications for therapy with anti-CD3 immunoconjugates (4, 26), and may yield insights into the normal process of T-cell activation.
cells were used as a control cell line to assess nonspecific binding. Cells were incubated with $^{125}$I-mAbs on ice for 1 h and then washed twice with 10 ml of ice-cold RPMI. Aliquots containing 1 x 10$^6$ cells were plated in 200 μl of RPMI in microtiter test plates (Flow Laboratories) and either warmed to 37°C (in a humidified CO$_2$ incubator) or maintained at 4°C. At various time intervals from 0 to 24 h, cell cultures were assayed for supernatant radioactivity, surface membrane-bound radioactivity, and intracellular radioactivity as described below.

At 0, 1, 2, 4, 10, and 24 h, cell suspensions were pelleted in a refrigerated centrifuge (4°C) at 300 x g for 3 min. Supernatants were aspirated and γ counted (Beckman Gamma 7000 Counter; Beckman Instruments, Palo Alto, CA). Surface-bound antibody was then stripped from cell membranes by a modification of previously described methods (9, 10, 33) using 2 consecutive 15-min acid/papain washes. The wash solution was prepared by titrating RPMI 1640 medium to pH 2.5 with 1N HCl and adding 2.5 mg/ml of papain (Sigma, St. Louis, MO). Cells were sedimented by centrifugation (300 x g) and subjected to a second acid/papain wash. The two acid washes were pooled, and the eluted, "acid-releaseable" $^{125}$I-mAb was determined by γ counting. This technique was capable of releasing ≥95% of cell-associated radioactivity from NWT and HPB-ALL cells labeled with $^{125}$I-64.1 and from Daudi cells labeled with $^{125}$I-1F5 (data not shown) when supernatants were maintained at 0–4°C to inhibit endocytosis. In some experiments, cells were cultured with 20 mM NH$_4$Cl or 50 μM monensin (Sigma, St. Louis, MO) to inhibit lysosomal degradation of internalized ligands (4, 34). Nonspecific binding to cells lacking the target antigen was consistently <1% of specific binding. After stripping of surface $^{125}$I-mAbs and harvesting of the acid wash, cell pellets were harvested from microplate wells with cotton swabs and assessed for internalized $^{125}$I-mAbs ("acid-resistant") by γ counting.

Determination of Antibody Degradation. Estimation of the extent of $^{125}$I-mAb degradation was performed as described (7, 10). Culture supernatants (0.2 ml) were mixed with 0.2 ml of 25% TCA to precipitate protein-bound $^{125}$I shed from the cell surface. Precipitates were washed with an additional 0.2 ml of 25% TCA, and then pellets and pooled non-TCA-precipitable washes were γ counted separately.

Immunoelectron Microscopy. Immunoperoxidase electron microscopy was used to provide morphological corroboration of the events transpiring during endocytosis of anti-CD3 antibodies. HRP (RZ = 3.0; Sigma) was conjugated to mAb 64.1 or to purified, monovalent Fab' fragments of GAM Ig by the metaperiodate method as previously described (4, 35). Direct immunoperoxidase labeling was performed by incubating 5 x 10$^6$ T-cells with an optimal dilution of 64.1-HRP for 30 min at 4°C, washing 3 times with RPMI, and then warming cells to 37°C. After incubating for various time intervals (0 to 24 h), cells were washed, fixed for 30 min with half strength Karnovsky's fixative, washed twice with 0.1 M NH$_4$Cl or 50 μM monensin (Sigma, St. Louis, MO) to inhibit lysosomal degradation of internalized ligands (4, 34). Nonspecific binding to cells lacking the target antigen was consistently <1% of specific binding. After stripping of surface $^{125}$I-mAbs and harvesting of the acid wash, cell pellets were harvested from microplate wells with cotton swabs and assessed for internalized $^{125}$I-mAbs ("acid-resistant") by γ counting.

Indirect immunoperoxidase labeling was accomplished by incubating T-cells with 64.1 for 30 min at 4°C, washing 3 times, incubating with HRP-sub-GAM Ig for 30 min at 4°C, and then proceeding as described above. No differences were discovered between the direct and indirect methods except that immunolabeling was more intense with the indirect method. Some grids were stained with uranyl acetate and lead citrate to enhance contrast.

Colloidal gold (20 nm; SPI Supplies, West Chester, PA)-adsorbed antibody was prepared by the method of DeMey (36). Gold-64.1 conjugates were incubated with T-cells at 4°C for 30 to 60 min, washed 3 times, warmed to 37°C for various time intervals (0 to 24 h), and then fixed and sectioned as described above. Some fixed cell suspensions were treated with the Gomori method for demonstration of acid phosphatase activity (a lysosomal marker) by the method of Barka and Anderson (37) before embedding in Epon 812.

Colloidal gold (10 nm)-adsorbed transferrin was purchased from E-
tivity accumulated in the culture supernatant was consistently faster for NWT than HPB-ALL, with 80% of total cpm being unassociated with cells at 24 h for NWT versus 55 to 60% for HPB-ALL.

Degradation of $^{125}$I-labeled Anti-CD3 mAbs. There were TCA-precipitable and TCA-nonprecipitable components in the radioactivity which accumulated in the culture supernatants of washed, surface-labeled T-cells (Fig. 2). At early time intervals ($\leq 3$ h for NWT and $\leq 4$ h for HPB-ALL), virtually all of the supernatant $^{125}$I was TCA precipitable (Fig. 2, A and B). Subsequently, the contribution of protein-bound, TCA-precipitable cpm to total supernatant radioactivity diminished, eventually plateauing at 10 to 15% of total culture cpm. In contrast, nonprecipitable radioactivity was virtually absent for the first 3 h of culture, but then increased rapidly and progressively to account for 50 to 60% of total culture cpm by 24 h for NWT, and 35 to 40% for HPB-ALL. The generation of TCA nonprecipitable cpm in culture supernatants in these experiments probably reflects intracellular degradation of $^{125}$I-64.1 followed by exocytosis of non-protein-bound $^{125}$I (7, 10). Intact $^{125}$I-64.1 shed from cells into the culture supernatants was presumably responsible for the TCA-precipitable component of supernatant radioactivity. Control cultures maintained at 0–4°C for 24 h to prevent endocytosis demonstrated comparable accumulation of TCA-precipitable supernatant cpm, but virtual absence of nonprecipitable cpm (0 to 2% of total cpm; Fig. 3A). Other control experiments performed by culturing plasma membrane fragments purified from disrupted Jurkat cells (see "Materials and Methods") with mAb 64.1 at 37°C for 24 h also generated negligible quantities of TCA-nonprecipitable radioactivity (<3% of total cpm).

Effects of Monensin and Ammonium Chloride on Degradation of $^{125}$I-labeled Anti-CD3 Antibodies by T-Cells. In cultures of both NWT and HPB-ALL, the generation of TCA-nonprecipitable supernatant radioactivity was markedly inhibited by lysosomotropic agents (Fig. 3, A and B). Non-protein-bound $^{125}$I represented >50% of total culture radioactivity after 24 h of culture of NWT cells with $^{125}$I-64.1 in the absence of lysosomotropic agents (Fig. 3A). In the presence of 50 μM monensin or 20 mM ammonium chloride, only 4% and 11% of total cpm, respectively, were TCA nonprecipitable after 24 h. Comparable results were observed with HPB-ALL (Fig. 3B) although the rate of antibody degradation was slower than with normal T-cells. When NWT or HPB-ALL was incubated with $^{125}$I-64.1 in the absence of monensin or ammonium chloride at 4°C, only 1 to 2% of radioactivity was non-TCA precipitable, demonstrating that spontaneous dehalogenation did not occur in the absence of endocytosis of antibody. The lysosomotropic agents appeared to induce a block in antibody processing which trapped radiolabeled 64.1 inside cells (34). This resulted in prolonged retention of radioactivity in intracellular, acid-resistant compartments (shown for HPB-
ALL in Fig. 4), and prevented exocytosis of free $^{125}$I to the culture medium (Fig. 5).

Immunelectron Microscopic Demonstration of Anti-CD3 Endocytosis. Direct morphological confirmation of the endocytosis of anti-CD3 antibodies was obtained by immunoperoxidase electron microscopy (Fig. 6). The general pattern of internalization was similar to that previously reported for other ligands (5, 38). Cells incubated with peroxidase-tagged anti-CD3 mAbs at 4°C initially displayed a circumferential surface distribution of label (Fig. 6A). Warming cells to 37°C induced rapid patching of the immunolabel ($<$5 min) followed by formation of distinct polar antibody caps within 15 to 30 min (Fig. 6B). Accumulation of peroxidase label in “coated pits” (Fig. 6C) rapidly led to endocytosis and appearance of peroxidase-containing endocytic vesicles or “receptosomes” (38) (Fig. 6D). Simultaneous appearance of label in prominent tubulovesicular organelles [reminiscent of the “compartment of uncoupling of receptor and ligand, “CURL” (39)] was very conspicuous with this antibody (Fig. 6, E and F). Gradual appearance of peroxidase in multivesicular bodies and dense bodies occurred from 30 min to 24 h after warming cells (Fig. 6, G and H).

Separate experiments with colloidal gold-adsorbed anti-CD3 antibodies confirmed findings demonstrated in the immunoperoxidase experiments described above. Once again, initial circumferential cell surface labeling with antibody was followed by rapid capping of bound antibody to one pole of the cell. The surface membrane in the region of the antibody cap was characterized by ruffling and by villous cytoplasmic projections which were absent from noncapped membrane domains (Fig. 7,a,d,e,f, and g). Anti-CD3 antibodies were internalized from the capped regions through “coated pits” bearing typical submembranous spikes and bristles (Fig. 7, a to e). Coated pits pinched off from the surface membrane to form coated vesicles, which subsequently fused with larger uncoated vesicles, with multivesicular bodies and ultimately with lysosomes (Fig. 7, d to f). Histocytochemical studies using the Gomori reaction for acid phosphatase (37) confirmed the existence of this lysosomal enzyme in organelles judged to be lysosomes on morphological grounds. Although the majority of gold-labeled 64.1 was internalized by receptor-mediated endocytosis through coated pits (Fig. 7, a to f), morphological evidence of nonspecific engulfment of some surface-bound 64.1 through noncoated membrane regions was also obtained (Fig. 7g).

Coinsertalional of mAb 64.1 and Transferrin-Gold Conjugates. Double-label experiments were performed with mAb 64.1 (labeled with peroxidase-conjugated Fab'-GAM1g) and transferrin (conjugated to 10-nm gold particles) to further define the pathway of endocytosis of mAb 64.1. HPB-ALL cells were initially densely labeled with both reagents in a circumferential surface pattern following incubation at 4°C (Fig. 8A). Following warming to 37°C to initiate endocytosis, both ligands were observed to enter cells through the same coated pits (Fig. 8B). After 10 to 30 min, multiple endocytic vesicles and multivesicular bodies densely labeled with both markers were visible in the cytoplasm of cells (Fig. 8C).

DISCUSSION

These studies demonstrate that anti-CD3 mAbs are cleared from the surface of normal and malignant human T-lymphocytes after binding by a combination of antibody shedding and endocytosis. Of the two processes, antibody internalization predominates as judged by the relative amounts of intact (presumably shed) $^{125}$I-64.1 and degraded, TCA-nonprecipitable $^{125}$I in supernatants of cells labeled with $^{125}$I-anti-CD3 mAbs. Intracellular lysosomes have been shown to be the sites of degradation of a variety of cell surface ligands (7, 10, 34) and are assumed to be responsible for the generation of free (TCA nonprecipitable) $^{125}$I. This assumption is supported by the marked inhibitory effects of ammonium chloride and monensin on the production of TCA-nonprecipitable cpm. These substances are known to block delivery of internalized ligands to lysosomes (12, 34, 40) and to inhibit lysosomal degradative hydrolases by neutralization of the acidic intralysosomal pH (41). The net result is an intracellular block in the processing of internalized $^{125}$I-64.1 with trapping of radioactivity in a compartment resistant to acid/papain elution. Total supernatant radioactivity is diminished in cultures containing lysosomotropic agents, since the TCA-nonprecipitable components are not released. Further studies will be required to discern the relative importance of lysosomal pH neutralization (41) versus intracellular redistribution of internalized ligand to nonlysoso-
Fig. 6. Immunoelectron microscopy of human T-lymphocytes labeled with peroxidase-conjugated antibody 64.1 (anti-CD3). A, circumferential labeling of T-cell at initiation of culture at 37°C. x 13,000. B, capping of surface label 30 min after warming T-cells to 37°C. x 12,000. C, endocytosis of surface label via "coated pit" after 15 min at 37°C. x 30,000. D, labeled receptosome (endocytic vesicle) after 15 min at 37°C. x 50,000. Note the characteristic internal architecture with microvesiculation. E and F, labeled "tubulovesicular apparatus" after 30 min at 37°C. x 86,000 and x 31,000, respectively. G and H, densely labeled lysosomes after 120 min at 37°C. x 10,500 and x 23,750, respectively.
Fig. 7. Endocytosis of gold-mAb 64.1 conjugates (20 nm) by normal human peripheral blood T-cells. a, b, and c, internalization of gold-labeled mAb 64.1 through multiple adjacent "coated pits" (small black arrows) of NWT cells after 10 min at 37°C. × 36,000, × 52,000, and × 38,000, respectively. Endocytosis was preceded by capping of antibody to one pole of a cell, and the cell membrane in this region of the cell usually contained numerous villous projections (arrowheads in a, d, and g). d, gold-labeled mAb 64.1 being internalized through coated pits (small black arrows) in an area of the cell where the membrane shows numerous villous projections (arrowheads). Numerous gold particles are located in endocytic vesicles with no apparent communication with the cell surface (open arrows). (Photo taken after 20 min at 37°C.) × 37,000. e, internalization of gold-64.1 through an early coated pit (small black arrow) and localization of numerous gold particles in a prominent tubulovesicular component of the endocytic compartment (open arrow). × 37,000. f, direct fusion of coated vesicles (small black arrows) with multivesicular bodies (open arrows). × 50,000. g, although most gold particles appeared to be internalized through coated pits, nonspecific engulfment of gold-64.1 by pinocytosis also appeared to occur in some cells through "uncoated" regions of the cell membrane. × 35,000.
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Fig. 8. Colocalization of peroxidase-labeled mAb 64.1 and gold-conjugated transferrin (10 nm) in HPB-ALL cells. A, double labeling of cell surface of HPB-ALL cell at 4°C with both ligands, x 55,000. B, internalization of both gold-labeled transferrin and peroxidase-labeled mAb 64.1 through the same cell surface microvaginations ("pits"), x 55,000. C, colocalization of gold-transferrin and peroxidase-mAb 64.1 in the same endocytic vesicle with 30-min incubation at 37°C. x 50,000.

When anti-CD3 mAbs are continuously incubated with target cells, the amount of antigen in culture supernatants is more rapidly depleted than that of mAbs internalized by the cell (13-16). The reason for this difference is not clear, but could result from the internalization of the antibody itself. It should be noted, however, that the pace of internalization of 125I-transferrin (t1/2 = 20 min) is slower (t1/2 = 120 min) than that of 125I-transferrin (t1/2 = 20 min) at 125I-transferrin (data not shown). The internalization of anti-CD3 antibodies is also receptor mediated. The process of modulation includes receptor and mAb unspecific recycling of the CD3 surface molecule back to the cell surface (42).

The current findings have implications for therapeutic trials using anti-CD3 mAbs either alone, or as components of immunotoxins or radioimmunoconjugates. Our work is consistent with previous reports (14) that T-cell clones and T-cell lines mediating the effects of amines and carboxylic ionophores. We found no evidence for exocytosis of intact 125I-64.1 by cells; conditions which inhibit endocytosis (e.g., incubation at 4°C) minimally affect the appearance of TCA-precipitable cpm in culture supernatants, but abolish the generation of TCA-non-precipitable cpm (data not shown).

Immunoelectron microscopic studies with peroxidase- and colloidal gold-labeled 64.1 have provided direct morphological confirmation of the internalization of anti-CD3 antibodies. The rate of endocytosis estimated from the cellular radioimmunoassay technique correlated well with the tempo observed ultrastructurally, with rapid internalization of bound antibody occurring within minutes of warming cells to 37°C. The pace of anti-CD3 mAb internalization subsequently decelerated, however, and small amounts of residual surface-bound antibody remained detectable by both methods even after 24 h of culture. Intralysosomal localization of antibody was first discernible with electron microscopy 30 min after warming cells to 37°C and increased over the next 3 h. These observations are in accord with the progressive appearance of TCA-non-precipitable cpm in culture supernatants beginning 2 to 4 h after cell warming.

We performed colocalization studies with peroxidase-labeled mAb 64.1 and gold-conjugated transferrin to elucidate the mechanism of endocytosis of 64.1. Both immunoconjugates were internalized through the same coated pits and localized in the same endocytic vesicles (receptosomes) and multivesicular bodies following internalization. Since transferrin is known to undergo receptor-mediated endocytosis (6, 42), it is assumed that the internalization of mAb 64.1 is also receptor mediated. It should be noted, however, that the pace of internalization of 125I-64.1 is slower (t1/2 = 120 min) than that of 125I-transferrin (t1/2 = 20 min, data not shown). Terminal targeting of the two ligands could not be contrasted in this system, since the terminal fate of transferrin is altered by gold adsorption (42). [Transferrin-gold conjugates are routed to lysosomes, whereas native transferrin and peroxidase-labeled transferrin are recycled to the cell surface (42).]

Several workers have studied the rate of CD3 antigenic modulation induced by continuous incubation with saturating concentrations of anti-CD3 mAbs by immunofluorescence or radioimmunoassay, and a wide variation in the rate and degree of modulation has been reported (from 100% modulation in 3 h to 60% in 48 h (13, 14, 16, 17, 24, 25, 43-45)). Parameters found to be of major importance in determining the maximal rates of modulation included antibody concentration (25, 43), facilitation by monocytes (17, 25, 44), antibody isotype (25), CD3 epitope recognized (24), and the type of cell being studied (14).

The present study assessed the rate of antibody endocytosis and degradation following pulse surface labeling rather than continuous incubation with antibody. This approach allowed us to quantify the small amount of mAb shedding which occurs, and to compare the magnitude of this process with that of internalization. Though antibody shedding clearly occurs in this system, experiments by others suggest that the CD3 antigen itself is not shed along with the antibody (24).

The slower kinetics of anti-CD3 internalization by HPB-ALL cells compared with normal blood T-cells is consistent with previous reports (14) that T-cell clones and T-cell lines modulate more slowly than freshly obtained blood lymphocytes, possibly reflecting the absence of facilitating monocytes (25, 44). Intralysosomal degradation of internalized 125I-64.1 also appeared to proceed more slowly in the malignant cell line than in normal T-cells as indicated by the higher peak levels of intracellular 125I-64.1 (despite slower endocytosis) in HPB-ALL, and the delayed appearance of non-TCA-precipitable cpm in culture supernatants of HPB-ALL.

A recent report has convincingly demonstrated the continuous recycling of the CD3 surface molecule back to the cell membrane following endocytosis, though the fate of anti-CD3 mAbs was not addressed in this study (46). Our report, in contrast, demonstrates terminal targeting of anti-CD3 mAbs to lysosomes. These two sets of observations suggest that either the CD3-anti-CD3 mAb complex dissociates during endocytosis or that the normal cellular pathway of CD3 recycling is disrupted by anti-CD3 mAb binding. Although this issue was not directly addressed by the experiments reported here, the latter possibility appears most likely, since anti-receptor mAbs in other systems usually result in delivery of receptor and mAb to lysosomes where both are degraded.

The current findings have implications for therapeutic trials using anti-CD3 mAbs either alone, or as components of immunotoxins or radioimmunoconjugates. Our work is consistent
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with the hypothesis (13, 21) that the immunosuppressive effects of anti-CD3 mAbs result from endocytosis of the CD3-T-cell receptor ("T3-T") complex, rendering T-cells incapable of antigen recognition. Such internalization is crucial for the effectiveness of anti-CD3 immunotoxins which rely on the delivery of toxic moieties (e.g., ricin A chain) to cytoplasmic targets (ribosomes) (4, 26). The potentiation of CD3-ricin A chain immunotoxin efficacy by ammonium chloride and monensin (4) likely results both from delayed delivery to the lysosomal compartment (12), and from inhibition of lysosomal degradation. The rapid intracellular degradation of 125I-64.1 and subse-quent exocytosis of free radioisotope may limit the efficiency of treatment with radiolabeled antibodies targeting this antigen. Since maximal cell kill with such therapy is most likely to result from prolonged retention of radiolabeled antibodies by target cells, it may be advantageous to select antibodies which are slowly and incompletely internalized, to inhibit lysosomal degradative activity (e.g., with chloroquine (47)), or to use radioisotopes that can be retained in an intracellular compartment after antibody internalization (48).

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REFERENCES


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