Mechanisms of Immunosuppression: Effects of Cyclophosphamide on Cellular Immunity

By Alan Winkelstein

A course of cyclophosphamide administered to guinea pigs after immunization with mycobacteria led to a transient inhibition of cutaneous hypersensitivity to tuberculin protein. This treatment resulted in generalized depletion of lymphoid cells, including lymphopenia, and a substantial reduction in the number of macrophages found in induced peritoneal exudates. Results of in vivo cell transfer studies indicated that cyclophosphamide-treated recipients were rendered transiently unresponsive to purified tuberculin protein (PPD). However, sensitized lymph node cells from drug-treated donors were capable of transferring immunity to normal recipients. In vitro tests of cellular immunity with lymph node cells from drug-treated animals yielded discordant results. The proliferative response to both PHA and PPD was significantly impaired. However, these lymphoid cells functioned normally in assays for migration inhibition factor (MIF). Thus, these data suggest that cyclophosphamide can limit proliferation without impairing intermitotic functions of sensitized lymphocytes, such as the release of MIF. Results of this study suggest that cyclophosphamide does not impair development of a population of specifically sensitized T-type lymphocytes. However, several components of the expression phase are affected; the resultant anergy is probably due to a summation of effects on lymphocytes and macrophages.

Cyclophosphamide is a potent immunosuppressive agent, capable of inhibiting both humoral and cell-mediated immune responses. In animal models, this cytotoxic agent can suppress graft-vs.-host disease, prevent development of certain autoallergic conditions, and promote tolerance to several antigens. Because of this activity, cyclophosphamide is being evaluated in the treatment of certain human disorders believed to result from aberrant immunity and as an agent useful in preparing recipients for marrow transplants.

The present investigations were designed to further delineate cellular events associated with cyclophosphamide-induced immunosuppression. The model chosen was the inhibition of delayed hypersensitivity reactions in guinea pigs sensitized to tuberculin protein. To evaluate the drug’s activity, both in vivo and in vitro immune parameters were assessed. Results suggest that the major cause of immunosuppression accrues from toxicities directed at those cellular events concerned with effecting a cell-mediated immune response. However, the acquisition of antigen sensitivity by T-type lymphocytes is
not impaired. Furthermore, these data demonstrate paradoxical results in in vitro tests of cellular immunity. Although lymph node cells showed limited proliferative response to both phytohemagglutinin (PHA) and purified tuberculin protein (PPD), they function normally in assays for migration inhibition factor (MIF).

MATERIALS AND METHODS

Male Hartley strain guinea pigs, weighing between 300–450 g, were sensitized to H37 Ra mycobacteria in Freund’s adjuvant (Difco) by multiple subcutaneous injections using a total of 1 ml of the immunogen. Except as noted, drug-treated animals received 20 mg/kg of cyclophosphamide intramuscularly daily for 7 out of 8 days starting on the day of immunization (day 0). Controls received a similar course of sterile saline injections.

Animals were skin tested with 25 μg purified tuberculin protein (PPD) 7 days after immunization (day + 7); cutaneous reactivity was assessed 24 hr later. Reactions were judged positive if there was an area of palpable induration greater than 5 mm in diameter. In all sensitized controls, the zone of induration exceeded 15 mm.

Multiple parameters that relate to cellular immunity were evaluated in each animal. White blood counts were performed with the aid of a Model B Coulter counter, and the number of circulating lymphocytes was determined by a 200 cell differential count. In addition, recoverable peritoneal exudate macrophages, appearing 4 days after the intraperitoneal injection of 30 cc sterile paraffin oil, were measured. The peritoneal cavity was washed with 120 ml Hanks’ balanced salt solution containing 1 mg/100 ml heparin. Cells were enumerated, and a 500 cell differential count was performed on Wright-Giemsa-stained smears.

The histology of the lymph nodes and spleen was assessed by standard techniques. Tissues were fixed in formalin; paraffin embedded sections were cut at 5 μ and were stained with hematoxylin and eosin.

The response of lymph node cells to the nonspecific mitogen, phytohemagglutinin (PHA), was measured by previously described techniques. For each animal, five cultures, each containing 10 × 10⁶ lymph node cells, were established in 5 ml media TC 199 supplemented with 20% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Three cultures contained 0.01 ml PHA (Burrough-Wellcome); the other two served as controls. All cultures were incubated at 37°C for 2 days. One hour prior to completion of the incubation period, 2 μCi of tritiated thymidine (3HTdr, New England Nuclear, specific activity, 6.7 Ci/mM) were added to each culture. Isotope incorporation was measured by precipitating DNA with cold 5% trichloroacetic acid, dissolving the residue in Soluene, and diluting with 10 ml Liquiflor-toluene scintillation fluid. Each sample was counted in a Packard Tri-carb liquid scintillation counter for five minutes; results were corrected for background and quenching and were expressed as cpm/10⁶ cells.

In experiments designed to assess the duration of cyclophosphamide-induced suppression of PHA responsiveness, a single dose (150 mg/kg) was administered to guinea pigs. Animals were evaluated serially; the response was determined as described above, except that 3HTdr was added to the cultures 4 hr prior to harvesting.

To evaluate the in vitro proliferative responses to PPD, lymph node cultures were prepared from nine cyclophosphamide-treated and 15 sensitized control animals. In these assays, individual cultures contained 10 × 10⁶ lymph node cells in media RPMI 1640 supplemented with 20% fetal calf serum, glutamine (200 μg/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml). Three replicate cultures containing 25 μg PPD and three unstimulated cultures were established for each animal. All cultures were incubated at 37°C in a 5% CO₂ atmosphere for 5 days; 3HTdr was added 4 hr prior to harvest. As the degree of stimulation with the specific antigens is known to be considerably less than with PHA, results from these experiments were expressed both as the stimulation ratio and the counts per minute per culture. The former value was determined in each experiment by dividing the 3HTdr incorporation in PPD-stimulated cultures by that measured in controls.
Suspensions of lymph node cells from each animal were also tested in assays for migration inhibition factor (MIF) using previously reported techniques. Results of these experiments were based on assays of 27 drug-treated and 21 sensitized controls. Individual capillary tubes were filled with $10 \times 10^6$ cells, centrifuged at 800 rpm, and cut at the cell-supernate interface. The cellular portion was incubated in a Sykes-Moore culture chamber containing 1 ml Eagle's minimal essential media supplemented with 20% guinea pig serum, penicillin (100 U/ml), streptomycin (100 $\mu$g/ml) amphotericin B (0.25 $\mu$g/ml), and 25 $\mu$g PPD.

After 24-hr incubation, the area of migration was measured by planimetry. For each lymphocyte sample, results were calculated on the basis of four replicate capillary tubes. The percent migration was determined by comparison with the area measured using the same peritoneal exudate cell suspension (PEC) mixed with lymph node cells from nonsensitized donors. A positive response required at least a 20% reduction in area.

Further evaluation of cyclophosphamide's effects were obtained in a series of in vivo cell transfer experiments. Initial studies indicated that the intraperitoneal injection of $50 \times 10^6$ lymph node cells from sensitized animals consistently transferred cutaneous reactivity to normal recipients. In the first group of animals, a course of cyclophosphamide was initiated on the day of immunization. On day +8, treated animals were killed, and aliquots of $50 \times 10^6$ lymph node cells were administered intraperitoneally to nonimmunized donors. Cutaneous reactivity to tuberculin protein was assessed at 2 and 10 days following the cell transfer using the same criteria described above. A reaction was considered positive if there was an area of induration greater than 5 mm in diameter. In the second group, the recipients were pretreated with a 7-day course of cyclophosphamide. One day after the last injection, these animals received lymph node cells from normal animals sensitized to tuberculin protein. Skin reactivity to intradermal PPD was assessed on day 2 and day 10.

RESULTS

In Vivo Effects of Cyclophosphamide on Cutaneous Reactivity

An appropriately timed course of cyclophosphamide proved highly effective in inhibiting cutaneous reactivity to PPD. As shown on Table 1, the response to this antigen was markedly inhibited by drug therapy begun on the day of immunization. No animal showed a response that exceeded 5 mm; the majority had no palpable induration. Furthermore, a course of this alkylating agent was effective when initiated 3 days after immunization; none of the seven animals developed a significant cutaneous reaction (greater than 5 mm). In this system, the suppression of immune reactivity was transient; following

| Table 1. Effects of Cyclophosphamide on Cutaneous Reactivity to PPD |
|--------------------|-----------------|-----------------|------------------|
| Course of Cyclophosphamide Treatment* | No. of Animals | Day Tested | No. Responding, (%)† |
| 0--7 | 27 | +7‡ | 0 (0%) |
| +3--10 | 7 | +10 | 0 (0%) |
| +10--17 | 6 | +17 | 2 (33%) |
| −5--0 | 6 | +7 | 6 (100%)* |
| Controls§ | 21 | +7 | 21 (100%)* |

*Animals received 20 mg/kg intramuscularly daily for 7/8 days.
†Reactions judged positive if induration greater than 5 mm. Intradermal reactivity assessed 24 hr after injection of 25 $\mu$g PPD.
‡Day 0 indicates day of immunization.
§Received sterile saline from day 0 to +7.
Fig. 1. Photomicrograph of cervical lymph nodes from cyclophosphamide-treated (A) and control (B) guinea pigs. A marked reduction in lymphocytes, involving both thymic-dependent and independent areas, is observed following drug treatment. × 32.

cessation of drug therapy, animals developed a positive test (15–20 mm) when rechallenged on day +15.

Cyclophosphamide proved partially effective in inhibiting cutaneous reactivity when a course of therapy was initiated 10 days after immunization. Prior to drug treatment, all animals showed a positive skin test to PPD; the area of induration ranged from 15 to 25 mm. When retested on day +18, following a 7-day course of cyclophosphamide, all six animals showed either minimal or no reactions to this antigen. Four animals showed no palpable

<table>
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<th>Table 2. In Vivo Cell Transfer Studies</th>
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<tr>
<td>Normal</td>
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<td>Normal</td>
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<td>Cycl Rx‡</td>
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*Received 50 × 10^6 lymph node cells intraperitoneally on day 0.
†Treated with 20 mg/kg cyclophosphamide intramuscularly from day −7 to 0.
‡Treated with 20 mg/kg cyclophosphamide intramuscularly from day of immunization to sacrifice (day +8).
induration; the other two developed a minimal zone of induration (6 and 8 mm, respectively). Thus, cyclophosphamide was capable of inhibiting intradermal reactivity even in animals with preexisting positive reactions.

In contrast to the effectiveness of this agent when employed following initial antigen challenge, no inhibition of the intradermal reaction was observed when treatment was administered prior to immunization. All six animals, treated with a course of cyclophosphamide starting 5 days prior to immunization, showed strongly positive reactions.

In cell transfer experiments, treatment of the recipient with cyclophosphamide, immediately prior to receiving sensitized cells, temporarily inhibited the cutaneous response to PPD (Table 2). In these experiments, \(50 \times 10^6\) lymph node cells from sensitized animals consistently promoted a delayed hypersensitivity response in normal recipients. However, treatment of the recipients with a 7-day course of cyclophosphamide, prior to the intraperitoneal administration of sensitized cells, led to a transient inhibition of intradermal reactivity. None of the eight recipients showed a positive response (greater than 5 mm induration) when challenged with PPD 2 days after transfer; however, when retested 10 days after the transfer, four of five animals had positive responses.

Although a course of cyclophosphamide was capable of inhibiting cutaneous reactivity in donors of cell transfer experiments, lymph node cells from these animals promoted delayed hypersensitivity responses in normal recipi-
Fig. 3. Effect of cyclophosphamide on total recoverable cells and number of macrophages in induced inflammatory exudates. Animals received 30 cc sterile paraffin oil intraperitoneally 4 days prior to sacrifice. As noted, a 7-day course of this alkylating agent produced marked reduction in number of recoverable macrophages.

Fig. 4. Comparative activity of lymph node cells in assays for MIF. Addition of 2.5% lymph node cells from normal donors did not result in any reduction in the area of macrophage migration. However, lymphocytes from both sensitized controls and cyclophosphamide-treated animals produced marked inhibition in migration. There was no significant difference in resulting inhibition between the two groups, indicating that drug therapy did not impair ability of persisting lymphocytes to release MIF. Area of migration is shown in planimetry units on the left and per cent on the right (mean ± 1 SE).

Effects of Cyclophosphamide on Lymphocytes and Macrophages

Cyclophosphamide was effective in reducing body pools of lymphocytes. Following a 7-day course of treatment, peripheral blood lymphocytes were reduced from an average of 3600 ± 490 cells/cu mm to 1200 ± 130 cells/cu mm. Histologic sections of the spleen and lymph nodes (Figs. 1 and 2) confirmed the generalized depletion of these cells. The loss of lymphocytes in the peripheral tissues appeared to involve both the thymic-dependent and independent areas.14
Fig. 5. In vitro proliferative response of lymph node cells to PHA. Stimulation is measured by incorporation of \(^{3}H\)-Tdr, added to cultures 1 hr prior to harvesting; results expressed as cpm/10^6 lymphocytes. It is apparent that cells from drug-treated animals show a markedly impaired response (mean ± 1 SE).

In addition to depleting both blood and tissue lymphocytes, a course of cyclophosphamide led to a marked reduction in the number of recoverable cells in inflammatory exudates (Fig. 3). Four days after intraperitoneal injection of paraffin oil, the total cellularity of these exudates was reduced from a mean of 44.7 ± 5.6 \times 10^6 cells to 5.5 ± 0.6 \times 10^6 cells. Differential cell counts showed that this reduction was primarily due to a decrease in macrophages; the number of these phagocytic cells was reduced by 84%.

**In Vivo Assays of Cellular Immunity**

Two types of assays that evaluate cellular immunity were employed in these studies: the ability of sensitized cells to elaborate MIF, and the lymphoproliferative responses to both the nonspecific mitogen PHA and the specific antigen PPD. In assays for MIF, lymph node cells from unsensitized donors did not inhibit the migration of normal peritoneal exudate cells (PEC). The area of migration was virtually identical to that measured using PEC alone. In contrast, lymph node cells from saline-treated animals immunized with mycobacteria produced a 61% reduction in the area of migration (Fig. 4). An equal number of lymphocytes, obtained from immunized animals treated with cyclophosphamide, proved as effective as those from the saline-treated group. The reduction in migration averaged 53%, a value not significantly different from that observed with cells from the sensitized controls. Thus, lymphocytes surviving alkylating agent treatment appeared active in elaborating this mediator of cellular immunity.

In contrast to the ineffectiveness of cyclophosphamide in reducing the MIF response of lymph node cells, treatment resulted in a marked diminution in the proliferative response to PHA (Fig. 5). Following mitogenic stimulation, lymph node cells from drug-treated animals showed a mean \(^{3}H\)-Tdr uptake of 68 ± 16 cpm/10^6 cells; comparable values for the saline-treated controls were 231 ± 37 cpm (71% reduction).

In addition to inhibiting the proliferative response to the nonspecific mitogen
PHA, the ability of lymph node cells to replicate following stimulation with a specific antigen, PPD, was also impaired (Table 3). Using cells from control animals, antigenic stimulation resulted in increased isotope uptake in 13/15 determinations; the average stimulation ratio was $5.1 \pm 1.8$. In contrast, the ratio determined in the nine animals treated with cyclophosphamide was $1.2 \pm 0.1$; in no instance did the isotope uptake in stimulated cultures exceed twice the control value. The difference between the stimulation ratios in the controls and drug-treated animals was significant ($p < 0.02$), indicating that the in vivo administration of cyclophosphamide inhibits the in vitro lymphoproliferative response to PPD.

The inhibitory effect of cyclophosphamide on PHA responsiveness was transient (Table 4). In experiments employing a single dose of cyclophosphamide (150 mg/kg), maximum inhibition of PHA response occurred 2 hr after drug administration. At this point, the activity was reduced to 11% of the controls. Proliferative responses rapidly returned to the control levels; within 3 days after drug administration, the average isotope uptake had returned to normal. An “overshoot” in proliferative activity was apparent 5–7 days following administration of this alkylating agent.

**DISCUSSION**

Data presented in this study indicate that a course of cyclophosphamide is effective in inhibiting cell-mediated immune responses. The suppressive activity appears to result from the summation of effects on those cellular elements involved in the reaction. Histologically, treated animals show a decrease in both blood and tissue lymphocytes and a pronounced reduction in the number of macrophages localized to inflammatory exudates. In vitro, the lymphoproliferative responses to both the nonspecific mitogen PHA and the specific antigen PPD are greatly reduced. However, by both in vivo and in vitro criteria, drug treatment does not inhibit acquisition of tuberculin hypersensitivity by T-type lymphocytes. Furthermore, this agent does not block at least one activity attributable to intermitotic lymphocytes, the elaboration of migration inhibition factor (MIF).

In vivo, a course of cyclophosphamide initiated coincident with or 3 days after immunization induced a transient state of anergy. In addition, this agent suppressed cutaneous reactivity in animals with previously established delayed hypersensitivity responses. However, responsiveness to this antigen was not

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<th>Table 3. Proliferative Response to PPD</th>
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<tr>
<td>No. of Animals</td>
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<td>----------------</td>
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<tr>
<td>PPD-sensitized controls</td>
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<tr>
<td>Cyclophosphamide-treated animals</td>
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*Ratio of $^3$H-Tdr incorporation in PPD-stimulated cultures to controls $\pm 1$ SE (values determined separately for each animal).

†Different from cultures containing PPD ($p < 0.02$).

‡Different from sensitized controls ($p < 0.01$).
impaired if drug therapy was administered before sensitization. These observations suggest that cyclophosphamide does not eliminate potentially responsive populations of uncommitted lymphocytes, rather its major activity relates to an inhibition of the effector phases of this reaction.

A prominent histologic effect of cyclophosphamide was to deplete lymphocytes in both the peripheral blood and lymphatic tissues. Reduced cellularity was apparent throughout peripheral lymphatic tissues, affecting both thymic-dependent and independent areas. As the former contains T-type lymphocytes, this depletion may contribute to the drug-induced anergy. However, this reduction may be a minor component; only a few specifically sensitized lymphocytes are required to initiate cutaneous reactions. Furthermore, Cole and Miller showed that those cells that have acquired reactivity to certain antigens become highly resistant to cyclophosphamide.

As macrophages are believed to be important in expressing delayed hypersensitivity responses, the observed anergic state may, in significant measure, result from the effects of cyclophosphamide on precursors of these phagocytic elements. The present data indicate that repetitive administration of this drug markedly reduces the macrophage response in induced peritoneal exudates. As the majority of these cells are known to arise from rapidly dividing monocytic precursors in the marrow, this suggests that therapy severely limits monocyte formation. Similarly, the effector cells found in delayed hypersensitivity reactions are thought to be derived from rapidly proliferating marrow monocytic elements. From this, it may be inferred that the reduced macrophage response reflects an overall diminution in the monocytic or expression phase of cellular immune reactions. Thus, a marked deficit in this component may constitute a major cause for tuberculin nonreactivity.

The importance of bone marrow-derived cells in the cutaneous response is further emphasized by in vivo cell transfer experiments. Cyclophosphamide-treated recipients proved transiently anergic to PPD following cell transfer. A likely cause for nonreactivity is a quantitative deficiency in the nonspecific effector component of the response. This finding is in accord with recent

<table>
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<th>Days After Cyclophosphamide*</th>
<th>No. of Animals</th>
<th>PHA Response (cpm/10⁶ cells)†</th>
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<tr>
<td>2 hr</td>
<td>9</td>
<td>163 ± 46</td>
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<tr>
<td>1 day</td>
<td>9</td>
<td>249 ± 73</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>266 ± 80</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>2359 ± 529</td>
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<td>5</td>
<td>6</td>
<td>2207 ± 592</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>5830 ± 1613</td>
</tr>
<tr>
<td>11</td>
<td>6</td>
<td>1402 ± 355</td>
</tr>
<tr>
<td>Controls</td>
<td>11</td>
<td>1542 ± 285</td>
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</table>

*Received 150 mg/kg cyclophosphamide intramuscularly.
†Net ³H-Tdr incorporation (PHA stimulated less unstimulated). Isotope added 4 hr prior to harvesting.
‡Mean ± 1 SE.
studies by Tubergen and Feldman; their data also suggest the importance of nonimmunologic precursors located in the marrow in developing delayed hypersensitivity responses.21

The activity of cyclophosphamide was further evaluated by two types of in vitro tests that correlate with cellular immunity.22–24 The ability of lymph node cells to inhibit macrophage migration was not impaired by drug therapy. Cells from sensitized controls and cyclophosphamide recipients proved equally capable of inhibiting the migration of unsensitized peritoneal exudate cells. Thus, it may be concluded that lymph nodes from drug-treated animals contain a population of sensitized cells. This conclusion was further substantiated by in vivo cell transfer experiments. As noted, lymph node cells from cyclophosphamide-treated donors were capable of transferring reactivity to normal recipients.

Although treatment with cyclophosphamide did not limit the ability of lymphocytes to elaborate MIF, a definite reduction in the lymphoproliferative response to PHA was observed. This test also is believed to measure the integrity of cellular immunity.24 A similar reduction has also been shown to occur in cultures of lymphocytes obtained from patients with rheumatoid arthritis treated with this drug.25 These results suggest that cyclophosphamide may limit the capacity of small lymphocytes to undergo in vitro proliferative expansion. A similar activity may exist in vivo; a proportion of persisting T-type lymphocytes in treated animals may be incapable of completing repetitive mitotic divisions following antigen stimulation. Such an inhibition is consistent with the pharmacologic activity of this drug,26 suggesting that intermitotic cells are "sterilized" but not destroyed until stimulated to divide.

The in vitro proliferative response to PPD was also impaired by the in vivo administration of cyclophosphamide. In contrast to the increment in 3HTdr uptake observed in lymph node cultures from sensitized controls stimulated with PPD, virtually no response was noted in tests using cells from drug-treated animals. These results are in accord with the limitation of proliferative expansion noted with PHA and further suggest that this alkylating agent can limit the replicate activity of those lymphocytes specifically sensitized to tuberculin protein. This may imply a specific immunosuppressive activity directed against a committed population of T-type lymphocytes.

The variant results in the two types of in vitro assays for cellular immunity are believed to reflect different components of the response of stimulated lymphocytes. Although cyclophosphamide may induce cellular injuries sufficient to prevent replication, it does not appear to impair the metabolic functions of stimulated cells prior to their entry into active cell cycle. In both PHA- and antigen-stimulated cultures, increased DNA synthesis is not observed during the initial 24 hr;27 however, this incubation period is sufficient for these cells to elaborate MIF.28 Thus, it may be concluded that the elaboration of this mediator occurs as an event independent of DNA synthesis. As such, immunologically competent cells may be capable of responding in those tests of cellular immunity not dependent on replication, while simultaneously showing impaired ability to undergo proliferative expansion. These results indicate that different in vitro studies of cellular immunity may provide
important information related to the different functions of lymphocytes and further demonstrate the need to evaluate multiple parameters in order to completely assess the status of cell-mediated immunity.

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