Development of Va14+ NK T cells in the early stages of embryogenesis

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Communicated by Kimishige Ishizaka, La Jolla Institute for Allergy and Immunology, La Jolla, CA, February 20, 1996 (received for review October 2, 1995)

ABSTRACT

The majority of T lymphocytes start to develop around day 15 of gestation (d15)–d17 in the thymus and comprise the peripheral repertoire characterized by the expression of polymorphic T-cell antigen receptors (TCRs). Contrary to these conventional T cells, a subset of T cells, called natural killer (NK) T cells (most of them expressing an invariant TCR encoded by the Va14Jα281 gene with a 1-nt N-region), preferentially differentiates extrathympically and dominates the peripheral T-cell population at a high frequency (5% in splenic T cells and 40% in bone marrow T cells). Here, we investigated the development of NK T cells and found that the invariant Va14+ TCR transcripts and the circular DNA created by Va14J and Ja281 gene rearrangements can be detected in the embryo body at d9.5 of gestation and in the yolk sac and the fetal liver at d11.5–d13.5 of gestation, but not in the thymus, whereas T cells with Vε1+ TCR expression, a major population in the thymus, were not observed at these early stages of gestation. Fluorescence-activated cell sorter analysis also demonstrated that there exist CD3+ αβ+ T cells, almost all of which are Va14/V ß8+ NK T cells, during early embryogenesis. To our knowledge, this demonstrates for the first time that a T lymphocyte subset develops in extrathymic tissues during the early stages of embryogenesis.

MATERIALS AND METHODS

Mice. Specific pathogen-free BALB/c and C57BL/6 mice were purchased from Shizuoka Animal Center (Hamamatsu, Japan). Recombination-activating gene (RAG)-1-deficient mice were provided by P. Mombaerts (12). Timed pregnancies were prepared as described (4). The day of the detection of a vaginal plug was designated d0.

Detection of TCR Transcripts. Total RNA was isolated from fetal tissues, including yolk sac, fetal liver, aorta-gonad-mesonephros (AGM), and thymus of d9.5–d13.5 embryos of (RAG-1−/− × BALB/c)F1 and (BALB/c × C57BL/6)F1 mice using Trizol Reagent (Life Technologies, Gaithersburg, MD). To amplify limited amounts of TCR transcripts in the early fetal tissues, we performed nested reverse transcriptase-PCR (RT-PCR) with 70–80 cycles of amplification according to the results on the single cell PCR reported by Brady et al. (13). First strand cDNA was synthesized by incubation of oligo(dT) primers with 0.1 μg of RNA as described (4). To detect TCR transcripts of (RAG-1−/− × BALB/c)F1 samples (see Fig. 1A), the cDNAs were amplified by nested PCR with primer pairs specific for Vaα/Cα sequences: VC1/VCS and VC2/VVC for the detection of Vaα mRNA, and VC4/VCS and VC3/VVC for Va1 mRNA. The first 20 cycles of amplification were carried out with denaturation at 94°C for 1 min, annealing at 62°C for Va1 or at 57°C for Va1 for 2 min, and extension at 72°C for 2 min, followed by the next 55 cycles under the same conditions except for annealing at 58°C for Va1. Oligonucleotide primers were as follows: VC1, 5′-CCGATTTCCAAAGTGGACAGTCTC-3′; VC2, 5′-GAATTCCTGAGTGGGAGACG-3′ for Va14; VC3, 5′-GGATTTCTCGTGAGTGGGAGACG-3′; VC4, 5′-GGATTTCTCGTGAGTGGGAGACG-3′ for Va1; VC5, 5′-GGAAGGAGGATTCGGGACTCCAT-3′; and VC6, 5′-GGAAGGAGGATTCGGGACTCCAT-3′ for Cα.

To detect CD3ε, RAG-1, and β-actin expression, RT-PCR was performed with specific primers under the following conditions: 40 cycles of denaturation at 94°C for 1 min, annealing at 54°C for CD3ε and β-actin or at 57°C for RAG-1 for 1 min, and extension at 72°C for 1 min. Oligonucleotide primers were as follows: for CD3ε, 5′-AACATTTTCCTGGG-GCATCTGTG-3′ and 5′-TGATGATTATGGCTACTGCTG-3′. Those for RAG-1 and β-actin have been published (11).

Abbreviations: d, day of gestation; AGM, aorta-gonad-mesonephros; NK, natural killer; RAG, recombination-activating gene; RT-PCR, reverse transcriptase-PCR; TCR, T-cell antigen receptor; TdT, terminal deoxynucleotidyl transferase; UT, untranslated.

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were PCR embryogenesis. From analysis (RAG-1-'x (BALB/c primers the 57°C primers designed sequences and VC1/CU1 at A sequences assayed. Detection U, . . .

\[ \text{Fig. 1. Detection of TCR transcripts and signal sequences in early embryogenesis. PCR was performed on nuclear DNA and RNA isolated from fetal tissues of d9.5–d13.5 embryos of (RAG-1-1/2 x BALB/c)F1 and (BALB/c x C57BL/6)F1 mice. (A) DNA blot analysis of products amplified by RT-PCR using RNA from d9.5–d13.5 (RAG-1-1/2 x BALB/c)F1 embryos. CD3e and RAG-1 expressions were also assayed. (B) DNA blot analysis of PCR products of TCR transcripts and signal sequences in the circular DNA from a d11.5 (BALB/c x C57BL/6)F1 embryo.} \]

To detect terminal deoxynucleotidyl transferase (TdT) expression (see Fig. 4), nested RT-PCR was carried out with primers: TDT1/TDT2 and TDT1/TDT3 under conditions of the first 40 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and extension under the same conditions. Oligonucleotide primers were: TDT1, 5’-AAAGAAGATGGAGAAACAATCG-3’; TDT2, 5’-AATCCCTCTGTTGCTTCTCAT-3’; and TDT3, 5’-GGGCATCCGGATAGTTGGT-3’.

To detect TCR transcripts of (BALB/c x C57BL/6)F1 samples (see Fig. 1B), RT-PCR was performed with primers specific for Vα14 (VC1)/Co (CU1), Vα1 (VC3)/Ca (CU1), and Vβ8/Cβ. To avoid detecting the transcripts of the maternal origin, as illustrated in Fig. 24, the primer CU1 was designed to include the 3’-half of the 18-nt insertion sequences specific for the 3’-untranslated (UT) region in the Ca gene of C57BL/6 origin (14). The primer sets of VCI/CU1 and VC3/CU1 exclusively amplify the TCR transcripts of paternal origin. The PCR conditions consisted of 40 cycles of denaturation at 94°C for 1 min, annealing at 63°C for Vα14Ca or 55°C for Vα1Ca and Vβ8/Cβ for 1 min, and extension at 72°C for 1 min. Oligonucleotide primers were as follows: VCI, VC3, CU1, 5’-TCGAATTCACAGACCCGCGTCTTAGAAGAC3’; Vβ8, 5’-CATATGCCTGCTGCAAGCCTT3’; and Ca (CB3), 5’-CCGATTCGGAAGCTTTTGGT-3’. The EcoR1 linker sequences are underlined.

PCR products were fractionated by electrophoresis on 2% agarose gel and were analyzed by Southern blot hybridization with 32P-labeled probes. Probes used for detection of RT-PCR products were as follows: Vα14, 5’-CCACACAGATGTTAGGTGAC-3’; Vα1, 5’-TCGACAGAGATAGAGAGCAG-3’; Cβ, 5’-TCCAAACAGAGACCGTGTGGT-3’; CD3e, 5’-CCATCTCAGGAACCAGTGTAG-3’; and 520-bp cloned PCR products of TdT cDNA. The signals were detected by the Bio Imaging Analyzer (Fujix BAS2000; Fuji, Tokyo, Japan).

**Sequencing.** The PCR products of (RAG-1-1/2 x BALB/c)F1 and (BALB/c x C57BL/6)F1 were cloned into pGEM-T Vector (Promega) and were sequenced by the dideoxy chain termination method with a Taq dye primer cycle sequencing kit (Applied Biosystems/Perkin–Elmer) in an automated DNA sequencer (model 370A, Applied Biosystems; ref. 11).

**Detection of A Signal Sequence in Circular DNA.** Nuclear DNA was isolated from fetal tissues of d11.5 (BALB/c x C57BL/6)F1 embryos and was investigated for the detection of signal sequences as described (11). In brief, the amounts of nuclear DNA were first measured by a quantitative PCR using RAG-2 primers and normalized. Nuclear DNA was then amplified by nested PCR with PCR primer sets of VA1/1A1 and VA2/2A2 for Vα14-Jo281, VA3/1A1 and VA4/2A2 for Vα1-Ja281, and VB4/DB3 and VB4/DB4 for Vβ8-DJβ2 rearrangements. Oligonucleotides for PCR primers were as follows: RAG-2, 5’-CACAGTCTTGGCAGAGATG-3’; Vα1, 5’-CTCTTTTCTGTGGATTTT3’; and Vβ8, 5’-CTCTTTTCTGTGGATTTT3’.

**Fig. 2. Nucleotide sequences of Vα14+ TCR transcripts detected in the early stages of embryogenesis. (A) A schematic illustration of RT-PCR primers designed for detection of Vα14 mRNA and its strain polymorphism. VC2/VC6 for detection of Vα14 mRNA (RAG-1-1/2 x BALB/c)F1 and VC1/CU1 for amplification of Vα14.1 of C57BL/6 type bearing the 18-nt insertion in the 3’-UT region in (BALB/c x C57BL/6)F1. (B) Nucleotide sequences with polymorphism in the Vα region and the VJ junction. Vα14.1 is C57BL/6 type, whereas Vα14.2 is BALB/c type. The frequencies are illustrated in the right margin. DNA blots shown in Fig. 1A were confirmed by the sequences from (RAG-1-1/2 x BALB/c)F1 mice. (C) Nucleotide sequences with genetic polymorphism in the 3’-UT region of Ca.Vα14.1 mRNA of C57BL/6 type possesses the 18-nt insertion at nucleotide position 837, whereas the insertion is missing in BALB/c type Vα14.2* mRNA.**
AGTGCCTCCCTGAGATG-3'; VA4, 5'-GCCACAGAGTAGAAACAGAAT-3'; VB4, 5'-GCCACCTCTTCTCCTTTATTT-3'; DB3, 5'-GACITTTCCAGCCCCTCTCATA-3'; and DB4, 5'-AACCTCTCTCCAGCTCTCTC-3'. PCR was carried out for 20 cycles with denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min in the first step, and subsequently for 40 cycles under the same conditions as the first step. PCR products were hybridized with 32P-labeled probes. Probes for the detection of signal sequences were as follows: 288-bp PCR product of Va14-Ja281 signal sequence for the Va14-Ja281 and Va1-Ja281 signal sequences; oligonucleotides of the 5' flanking region of DB2 (5'-CCCTGGCAGGCTTG-GGTTAGCCACTGTGGGGAAGAA-3') for Vβ8-Dβ2 signal sequences, and 852-bp PstI fragments of RAG-2 cDNA.

The signals were detected by the Bio Imaging Analyzer.

Antibodies and Flow Cytometry. Mononuclear cells in the d13.5 fetal liver of C57BL/6 mice were separated on a Ficoll/Hypaque density gradient, incubated with anti-FcR antibody (2.4G2) to block FcR-mediated binding, and stained with monoclonal antibodies. Anti-Va14 was raised in our laboratory (15). Other antibodies were obtained by PharMingen. Phycoerythrin-conjugated antibodies were as follows: anti-Va4, anti-Va3 (RR3-16), and anti-TCRβ GL3. Fluorescein isothiocyanate-labeled antibodies were as follows: anti-TCRβ (H57-597), anti-Vβ8 (MR5-2), anti-Vβ2 (B20.6), anti-Vβ11 (RR3-13), anti-Vβ13 (MR12-4), anti-CD3 (145-2C11), anti-CD4 (RM-4-5), and anti-CD8 (53-6.7). Biotinylated anti-NK1.1 (PK136) was used with Cy-chrome-streptavidin. Dead cells were gated out with propidium iodide staining, and 1-2 \times 10^6 cells were then analyzed by EPICS-XL (Coultet) with a logarithmic amplifier.

RESULTS AND DISCUSSION

Detection of Va14+ TCR Transcripts and Signal Sequences Generated by Va14 and Ja281 Gene Segments. Because Va14+ T cells can develop extrathympically, we investigated Va14+ transcripts and Va14 gene-mediated circular DNA in the early fetus at d9.5 (somites 21-25)-d13.5 of gestation before the thymus formation. For this purpose, we prepared nuclear DNA and cDNA from fetal liver, yolk sac, AGM, and thymus at different gestation times and carried out PCR to amplify Va14 gene products and the signal sequences composed of inverted heptamer repeats and nonamer sequences in the circular DNA (11), both of which are created only by rearrangements of the Va14 and Ja281 gene segments. Furthermore, to exclude the possibility of maternal contamination in the fetal tissues, we used two systems: one to elucidate the paternal type of Va14+ TCR in (RAG-1-deficient female \times conventional male)F1 mice, where no lymphoid cells of maternal origin are expected (12), and a second to identify the polymorphism (16) of the paternal type Va14+ TCR in (BALB/c \times C57BL/6)F1 fetuses amplified by paternal-specific primers. The results in Fig. 1A show the detection of Va14 transcripts in (RAG-1-/- \times BALB/c)F1 mice. At d9.5, Va14 transcripts were positive only in the embryo body but not in the yolk sac, whereas at d11.5-d13.5 Va14 mRNA expression increased significantly, and Va14 transcripts were present in the yolk sac and the fetal liver but not in the thymus, confirming the fetal development of Va14+ T cells in extrathympic tissues.

As shown in Fig. 1B, the signal sequence of the circular DNA generated by Va14 and Ja281 gene segments was successfully demonstrated at d11.5 in the yolk sac and the embryo body, including the fetal liver and AGM. These are known sites for the generation of hematopoietic precursors (2, 17). In contrast to Va14 gene expression, neither Va1+ transcripts nor the Va1 gene-mediated circular DNA were found in the same sample materials. Since Va1-bearing T cells are known to be a major repertoire in the adult and differentiated only in the thymus (11), it is clear that Va1+ TCR of the thymic type cannot be functional at this early stage of gestation or that Va1+ T cells cannot proliferate, resulting in being undetectable of Va1 TCR transcripts. The results suggest that Va14+ TCR is expressed in the extrathympic sites at an early stage of gestation before conventional αβ TCR appear in the thymus.

Molecular Evidence for Va14+ TCR Expression in the Early Stage of Gestation. We tried to detect paternal polymorphism in the V and 3'UT regions of Va14+ mRNA in (BALB/c \times C57BL/6)F1 samples with VCA/UC1 PCR primer set. The UC1 primer is specific for the TCRα mRNA of C57BL/6 but not BALB/c origin (see Fig. 2A). Therefore, only paternal Va14+ TCR transcripts are supposed to be amplified by the system. Indeed, we defined Va14.1 TCR transcripts of C57BL/6 type with 154GACAAAAAA163G sequences in the V region whose 3'UT region possessed the 18-nt insertion beginning at nucleotide position 837 only found in C57BL/6 (ref. 14; Fig. 2 B and C). These results suggest that the Va14+ TCR mRNA detected are of embryonic origin, not a result of maternal contamination. In addition, we confirmed the Va14+ TCR expression in (RAG-1-/- \times BALB/c)F1 mice shown in Fig. 1 at a sequence level which should be of paternal origin, because RAG-1-/- mice demonstrated no Va14+ TCR transcripts under the same PCR conditions (data not shown).

Interestingly, the Va14 sequences in the embryos were encoded by Va14 and Joa281 with a 1-nr N-region, in which all VJ junctions were substituted by guanine (G) or thymine (T) (Fig. 2B). The Va14 sequences are identical to the one found in the adult (18). Although TdT is known to be essential for the N-region addition (19, 20), recent studies by Giffillan et al. (21) using TdT-- mice clearly show that a short N-region addition occurs even in the absence of TdT. As we have successfully detected TdT mRNA in the early fetal tissues (see Fig. 4), the N-region of Va14Joa281 is likely to be generated by TdT. It is also quite surprising thatVa14+ TCR sequences detected were exclusively invariant. Therefore, it seems that invariant Va14+ TCR is due to the selection in the fetal tissues.

Detection of Va14+ NK T cells in the Fetal Liver at the Early Stage of Gestation. Fluorescence-activated cell sorter analysis on the d13.5 fetal liver revealed that TCRαβ+ T cells were in fact present (Fig. 3). Interestingly, the majority of αβ T cells belong to CD4-CD8- population and expressed NK1.1 and Va14+ TCR, which is apparently associated with the CD3 complex. Because no anti-Va3 staining was observed in this population and Va14 staining was completely blocked by excess amounts of cold anti-Va4, anti-Va14 staining seems to be specific. Moreover, as almost all Va14 T cells were stained by anti-Vβ8 but not by other anti-Vβ antibodies, Vβ8 was preferentially used as TCRβ by Va14+ T cells at this stage. TCRβ expression in the fetal Va14+ T cells was also confirmed at a molecular level. We investigated rearrangements and expression of Vβ8 gene in fetal tissues. As shown in Fig. 1B, Vβ8-Dβ2 circular DNA as well as Vβ8 transcripts were successfully demonstrated in the fetal liver, AGM, and yolk sac at d11.5 of gestation. Because peripheral NK T cells expressing the invariant Va14 on the cell surface in the adults preferentially use Vβ8 (6), Va14+ NK T cell repertoire in the fetus is similar to those in the adult.

NK T cells are originally found as a minor subset in the thymus (22-26). Recently, it is reported that NK T cells in the periphery comprise a relatively large fraction in spleen (5% of splenic T cells) and in bone marrow (40% of bone marrow T cells) (7). However, some recent reports on peripheral NK T cells, such as cell surface phenotypes including Va14 TCR expression, are demonstrated; most thymic NK T cells are negative for invariant Va14 TCR expression on the cell surface, whereas the majority of peripheral NK T cells are positive (7). In addition, the invariant Va14+ NK T cells are detected in athymic nude mice, indicating their extrathympic origin (11). Taken together with the present data, peripheral Va14+ NK T cells in the adult may be the same as those in the
with Va14+ liver. Clear cells CD3, NK1.1, conventional TCR as and at antibody lineage in intraepithelial from the tissues demonstrated in the body, particularly results to tissues is likely because Va14 only the experiments.

However, experiments demonstrated that Va14 only NK T cells, but not conventional T cells of thymic type such as Va14+ TCR, are of great advantage of the selection and proliferation in the early fetal tissues. Thus, NK T cells were only detected. However, after thymus formation, T cells bearing conventional TCR are rapidly increased in number so that NK T cells bearing invariant Va14+ TCR become a minor population until 2 weeks after birth (18). This assumption is likely because the invariant Va14+ TCR sequence was indeed detected in the neonatal thymus at a relatively low frequency as shown (18). The second possibility is that selective rearrangement of Va14/Ja281 TCR in the NK T cells is the mechanism. In fact, B1a cells, unlike conventional B cells, develop earlier and preferentially use a rather unique receptor composed of VH11/JH1 gene segments (31). It is also true that TCRγ genes show ordered rearrangements whose expression patterns are dependent on the cell types (2, 32). Thirdly, TCR rearrangements and transcripts were mainly investigated so far in the thymus but not in the non-thymic fetal tissues under the usual conditions with low cycles of PCR amplifications, resulting in the failure to detect TCR transcripts with limited amounts. In fact, Brady et al. (13) used 80 cycles of PCR amplifications for detection of transcripts from the single cell.

The function of NK+ αβ T cells has not yet been well defined. However, several investigators have shown that NK T cells play a role in bone marrow allograft rejection (33), the inhibition of metastasis of lymphoid cell tumor (34), and the control of abnormal T cell development in lpr mice (35). In fact, a decrease in Va14+ T cells is tightly correlated with the development of autoimmune diseases in lpr or gld mice (36). Because the target cells of Va14+ T cells seem to be bone marrow-derived cells (8–10), Va14+ NK T cells in the early embryogenesis may control hematopoietic systems. In any event, Va14 gene-deficient mice will provide a model in which to identify the functional role of Va14+ NK T cells in the early embryogenesis.

We would like to thank N. Yumoto, K. Masuda, and M. A. Mieza for technical assistance, and Ms. Hiroko Tanabe and Margaret D. Oto for preparation of the manuscript. This work was partly supported by a Grant-in-Aid for Scientific Research (B) 04452298 from the Ministry of Education, Culture, and Science, Japan, by the special coordination of funds for promoting science and technology of the Science and Technology Agency, Japan and by the Sugimoto Memorial Fund.


**Fig. 3.** Fluorescence-activated cell sorter profiles of d13.5 fetal liver. Mononuclear cells in the d13.5 fetal liver of C57BL/6 mice were separated and stained with antibodies. They (1–2×10⁶) were analyzed with EPICS-XL. The majority of αβ T cells (0.5% of total mononuclear cells separated) expressed Va14+ TCR which was associated with CD3, NK1.1, and Vβ8. Specificity of staining was confirmed by cold antibody blocking. All monoclonal antibodies used stained 0.1% Va3, 0.9% Vβ2, 0.5% Vβ11, 0.4% Vβ13, and 0.4% NK1.1 in adult (8-week-old) total thymocytes, 26% Vβ8, >95% CD3e, and >95% TCRβ in TCRβ+ fraction of thymocytes, and 37% TCRβ in intestinal intraepithelial lymphocytes.

**Fig. 4.** DNA blot analysis on RT-PCR products with TdT primers in the early fetal tissues. PCR was performed with RNA from (RAG-1−/− × BALB/c)F1 embryos as well as 8-week-old adult thymus of C57BL/6 as a control.

**Origin of Va14 T Cells.** RAG gene expression is known to be essential for the rearrangement of TCR genes (27) and is demonstrated at d9 of gestation (28). Fig. 1A illustrates that by d9.5 RAG-1 and CD3ε mRNAs are readily seen in all fetal tissues in which Va14/Vβ8 expression is detectable except for the d9.5 yolk sac and is rather diminished from the yolk sac at d13.5 of gestation. Thus, the results suggest that lymphoid precursor cells might be present in the yolk sac at d9.5, but migrate from the yolk sac at d13.5 of gestation. Palacios and Imhof (29) have reported that the yolk sac contains lymphoid precursors at d8.0–d8.5 of mouse embryogenesis; the above result on RAG-1 expression in the d9.5 yolk sac in part support their results. However, the d9.5 yolk sac does not seem to contain Va14+ T-cell precursors, because Va14+ TCR mRNA is detected only in the embryo body but not in the yolk sac at d9.5 (Fig. 1A). Because B1a cell progenitors are demonstrated to originate from AGM at d8.5–d9.0 of embryogenesis (30), it is likely that Va14 precursor cells are derived from the embryo body, particularly from AGM, although the possibility that the precursor cells are originated from yolk sac cannot be ruled out in the experiments.

The findings of the successful detection of Va14+ T cells as well as the machinery necessary for TCR expression in non-thymic tissues at an early embryogenesis are intriguing and interesting, because rearrangements and transcription of conventional TCR are known to start at around d15 of gestation in the thymus (5). These findings might be explained by the several possibilities. First, as the machinery for the TCR rearrangement and expression, such as RAG, TdT, and CD3 (see Fig. 1 and 4), is already prepared in the early fetal tissues, it is possible that any of TCR can be transcribed. However, only NK T cells, but not conventional T cells of thymic type such as Va14+ TCR, are of great advantage of the selection and proliferation in the early fetal tissues. Thus, NK T cells were only detected. However, after thymus formation, T cells bearing conventional TCR are rapidly increased in number so that NK T cells bearing invariant Va14+ TCR become a minor population until 2 weeks after birth (18). This assumption is likely because the invariant Va14+ TCR sequence was indeed detected in the neonatal thymus at a relatively low frequency as shown (18). The second possibility is that selective rearrangement of Va14/Ja281 TCR in the NK T cells is the mechanism. In fact, B1a cells, unlike conventional B cells, develop earlier and preferentially use a rather unique receptor composed of VH11/JH1 gene segments (31). It is also true that TCRγ genes show ordered rearrangements whose expression patterns are dependent on the cell types (2, 32). Thirdly, TCR rearrangements and transcripts were mainly investigated so far in the thymus but not in the non-thymic fetal tissues under the usual conditions with low cycles of PCR amplifications, resulting in the failure to detect TCR transcripts with limited amounts. In fact, Brady et al. (13) used 80 cycles of PCR amplifications for detection of transcripts from the single cell.

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