Presence of a Transcriptionally Active Glucocorticoid Receptor α in Lens Epithelial Cells

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PURPOSE. The purpose of this study was to determine whether lens epithelial cells (LECs) contain a glucocorticoid receptor (GR) that is transcriptionally active and that is able to induce production of known glucocorticoid-inducible proteins.

METHODS. Protein and mRNA were obtained from human, rabbit, and bovine lens epithelia and from cultured human lens epithelial cells (B3, hLECs) and rabbit lens epithelial cells (N/N1003A, rLECs). Paraffin-embedded sections were prepared from human lenses for immunohistochemical localization of GR. RT-PCR was performed to amplify portions of GR, and the products were sequenced. Protein samples were analyzed by Western blot. hLECs and rLECs were transfected with pTAT3-luc and assayed for luciferase activity after treatment with dexamethasone (Dex) and/or RU486. Dextreated LECs were also analyzed by quantitative real-time PCR and by Western blot for expression of specific mRNA and proteins.

RESULTS. By PCR and sequencing, products consistent with GR sequences were obtained from human, rabbit, and bovine lenses and from hLECs and rLECs. The complete GRα sequence was obtained from rLECs and was found to be 89% identical with human GR. A 1757-bp 3′ fragment of bovine GRα cDNA was also amplified from bovine lens. By Western blot, bands of approximately 94 kDa, the expected size of GR, were identified from human, rabbit, and bovine lens samples and from hLECs and rLECs, using anti-GR antibodies. Anti-GR antisera localized GR to both the cytosol and anterior and bow region LECs and to the nuclei of epithelial and early-differentiating lens fiber cells. Luciferase expression was induced in pTAT3-luc–transfected rLECs and hLECs by Dex treatment and this expression was partially (rLECs) or completely (hLECs) blocked by pretreatment with RU486. mRNA levels for type-1 glucocorticoid-induced target genes and also mRNA and protein levels for type-2 genes were upregulated after Dex exposure.

CONCLUSIONS. The data confirm the existence of GR in hLECs, indicate that GR is present in rLECs, and resolve the controversy over the presence of GR in bovine lens. The GRs in hLECs and rLECs was shown to be transcriptionally active and the expression levels in hLECs of mRNAs and proteins known to be regulated by glucocorticoids were modified in these cells by glucocorticoid treatment. (Invest Ophthalmol Vis Sci. 2003; 44:5269–5276) DOI:10.1167/iovs.03-0401

As a consequence of the long-term application of steroids for treatments associated with conditions such as allergy, autoimmune disease, and transplantation, there is a high risk of development of posterior subcapsular cataract (PSC).1–3 Several mechanisms have been proposed for the induction of steroid-induced PSC, and studies have focused principally on two areas: the binding of steroids to lens crystallins and the oxidation of lens proteins after steroid administration.4–7 The evidence has not yet linked steroid treatment through either of these mechanisms to the generation of steroid-induced cataract.

Steroids have been reported to be capable of binding to lens proteins8; however, evidence against a steroid binding mechanism for PSC induction has been advanced by Dickerson et al.9 who reported findings more consistent with activation of a glucocorticoid receptor (GR) and with steroid binding to lens proteins playing an incidental role. Lenses treated with glucocorticoids in vitro become opacified. Some reports have indicated that this opacification can be prevented by administration of α-tocopherol,9,10 suggesting a role for free radicals. A reduction in the level of glutathione (GSH), in other studies of glucocorticoid-treated lenses7,9 may support a role for oxidants in this opacification. However, only steroids with glucocorticoid activity possessed this ability and the reduction could be substantially prevented by the glucocorticoid antagonist, RU38486 (RU486), further suggesting a possible role for GR. The recent study by Lyu et al.10 who found no GSH reduction in rat lenses coincident with the development of posterior opacities, concurs with a role for GR rather than oxidants in the development of PSC.

Although this circumstantial evidence may suggest GR involvement in changes to lens cells that could lead or contribute to steroid-induced cataract, the existence of a functional GR in lens epithelial cells (LECs) remained to be demonstrated. Immunohistochemical studies by Stokes et al.11 and Suzuki et al.12 indicated reactivity to anti-GR antibodies in lens epithelium, but anti-GR antibodies can be temperamental requiring additional confirmatory evidence. Early studies13,14 of glucocorticoid binding to lens proteins were consistent with the presence of GR in lens epithelium, but the Western blot analysis and binding assays of Jobling and Augustyn1 indicated that this presence of GR in bovine lens epithelium. Two recent studies of human and mouse epithelial cells15,16 and rat lenses10 have provided evidence for the existence of lens GR.

The purpose of this study was to determine the existence of a functional GR in LECs. Our results using polymerase chain reaction (PCR), Western blot analysis, and immunohistochemistry confirmed the presence of GR in human lens cells and indicate that GR is present in rabbit and bovine lens cells, thus resolving the controversy over the presence of GR in the bovine lens. Furthermore, sequencing data indicate that the lens cells for all three species contain GRα, the active isoform of GR. Treatment of cultured LECs with Dex induced expres-
sion from the pTAT3-luc expression vector indicating that the GRα in LECs can induce transcription from positive glucocorticoid-responsive elements (GREs). In addition, transcription of mRNAs for direct type-1 glucocorticoid-induced genes (that contain positive GREs) and mRNA and protein for indirect type-2 glucocorticoid-induced genes (reported in other studies to be regulated by glucocorticoids) were induced in LECs by Dexam treatment, indicating that the GRα in lens cells is transcriptionally active and functional.

**Materials and Methods**

**Cells and Tissues**

The rabbit lens epithelial cell line (rLECs), N/N1003A (kindly provided by John Reddan, Oakland University, Rochester, MI), was maintained by John Reddan, Oakland University, Rochester, MI), was maintained in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich Co., St. Louis, MO) containing 10% rabbit serum, 2.2 mg/mL sodium bicarbonate, and 50 μg/mL gentamicin (Sigma-Aldrich Co.). The human lens epithelial cell (hLEC) line, B-3, was obtained from the Department of Comparative Medicine, Medical University of South Carolina (MUSC). The use of animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Human lenses, designated for research after receipt of informed consent, were obtained through the services of the South Carolina Lions Eye Bank. With a dissecting microscope, the anterior chamber of each eye was opened, the iris removed, the zonule attachments severed, and the lenses removed without adherent tissues. From a central anterior

**PCR and Sequencing**

mRNA was isolated from rLECs and hLECs using extraction reagent (Trizol; Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol, and first-strand cDNA (fs cDNA) was prepared using avian myeloblastosis virus (AMV) reverse transcriptase; also according to the manufacturer’s instructions (Invitrogen). Oligonucleotide primers (Table 1) for PCR detection and amplification of rabbit GR were designed to regions of homology between published human GR (GenBank accession no. U01351) and mouse GR (accession no. X04335) sequences (http://www.ncbi.nlm.nih.gov.Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD). These primers (human GR nucleotide numbering) were also used to amplify bovine GR from lens fs cDNA. Reaction conditions were: dissociation at 95°C for 30 seconds, annealing at 52°C for 1 minute, and extension at 72°C for 2 minutes for 35 cycles. Reaction products were detected by ethidium bromide 2% agarose gel electrophoresis. DNA excised from the gel was extracted by centrifugation of the gel slice over a bed of acid-stripped Ottawa sand in an 0.8-mL microfuge tube with an apical 30-gauge hole, and into a nested 1.5-mL tube. DNA was precipitated in 75% ethanol, centrifuged, and reconstituted in deionized (d) H2O and submitted to the MUSC Sequencing Facility for sequencing.

**Western Blot**

Bovine, human, and rabbit lens capsules with adherent LECs were processed for protein in RIPA extraction buffer (150 mM NaCl, 50 mM Tris [pH 8.0], 2% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitor cocktail for mammalian cells (Sigma-Aldrich Co.) with sonication (50 W, three times 10 seconds). Cultured rLECs and hLECs were washed in cold PBS, scraped off the culture flask surface, and centrifuged at 800g for 5 minutes before addition of RIPA. After extraction for 30 minutes on ice, samples were centrifuged at 12,000g for 10 minutes and the supernatant saved. Protein concentrations were determined using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). Samples were electrophoresed on 10% to 20% Tris-glycine-polyacrylamide gels (BioWhittaker, Rockville MD) using a commercial apparatus (Mini-Protein 3; BioRad, Hercules, CA) and then blotted to nitrocellulose membrane using a transblot cell (Mini-Protean 3; BioRad, Hercules, CA) and then reconstituted with 2% agarose gel electrophoresis. DNA excised from the gel was extracted by centrifugation of the gel slice over a bed of acid-stripped Ottawa sand in an 0.8-mL microfuge tube with an apical 30-gauge hole, and into a nested 1.5-mL tube. DNA was precipitated in 75% ethanol, centrifuged, and reconstituted in deionized (d) H2O and submitted to the MUSC Sequencing Facility for sequencing.
FIGURE 2. Alignment of derived amino acid sequence of rabbit GRα (accession no. AY161275) with human GRα (accession no. U01351). The glucocorticoid binding domain (aa 564–748) and DNA binding domain (aa 414–486) are in boxes. The peptide regions recognized by antibodies PA1-511A (human aa 346–347) and BuGR2 (rabbit aa 383–399) are underscored.
FIGURE 3. Western blot of protein samples (100 μg/lane) prepared from the lens capsule and LEC layer of bovine (lane 1), human (lane 2), and rabbit (lane 3) lenses and from hLECs (B-3; lane 4) and rLECs (N/N1003A; lane 5). The blots were probed with the anti-GR monoclonal antibodies, NCL-GCR and BuGR-2, and the rabbit polyclonal antiserum, PA1-511A, H-300, and P-20. The approximate size of GR is 94 kDa.

using a gel documentation apparatus (Versadoc; BioRad) and digital camera. Antiserum used for detection of GR were BuGr2 (Alexis Biochemicals, San Diego, CA), H-300, and P-20 (Santa Cruz Biotechnology, Santa Cruz, CA), PA1-511A (Affinity Bioreagents, Golden, CO), and NCL-GCR (Novocastra Laboratories, Newcastle-upon-Tyne, UK).

Western blot analysis for Dex-induced protein expression were prepared from duplicate cell pellets derived from the same samples as used for quantitative (Q)-PCR (described later) and were also processed with RIPA extraction buffer. Media in all lanes were replaced with serum-free DMEM. Forty-eight hours before cells were harvested, 1 μM Dex was added to the 48-hour Dex flask, and DMSO was added to the other flasks. For the 24-, 6-, and 3-hour samples, the media were replaced with media containing Dex at 24, 42, and 45 hours, respectively. Cells in the control flask were exposed to DMSO for 48 hours. Cells from all flasks were harvested at the same time. Blots were probed using antibodies for cellular inhibitor of apoptosis protein (cIAP-2; Trevigen, Gaithersburg, MD) and mitochondrial superoxide dismutase (MnSOD; Stressgen, Victoria, British Columbia, Canada) at 1:100 dilution, followed by an anti-rabbit IgG secondary antisera (Sigma-Aldrich Co.). Blots were reprobed with anti-actin mAb with anti-rabbit IgG secondary antisera (Sigma-Aldrich Co.). Movies of the antigenic signal were obtained from hLECs (B-3; lane 4) and rLECs (N/N1003A; lane 5). The blots were probed with the anti-GR monoclonal antibodies, NCL-GCR and BuGR-2, and the rabbit polyclonal antiserum, PA1-511A, H-300, and P-20. The approximate size of GR is 94 kDa.

Immunohistochemistry

Lenses were placed in Bouin’s fixative for 5 days, bisected anterior to posterior, embedded in paraffin, and sectioned at 5 μm. Sections were deparaffinized and brought through ethanol to H2O. Sections were then immersed in 10 mM citrate buffer (pH 6.0) and heated at pressure in a pressure cooker for 1 minute to unmask antigens (Vector Laboratories, Burlingame, CA). Sections were blocked in 10% normal goat or rabbit serum, washed three times in PBS and exposed overnight to the primary antiserum: H-300 and P-20, NCL-GCR, and PA1-511A. Tetrazolium blue (Sigma-Aldrich Co.) was used to localize GR and the sections visualized by fluorescence microscopy (Axioplan-2 with Axioplan software; Carl Zeiss Meditec, Thornwood, NY).

Gene Expression

The plasmids, pTAT3-luc, kindly supplied by Keith Yamamoto, (University of California, San Francisco), and pRL-TK (Promega, Madison, WI) were purified from JM-101 cultures by standard miniprep techniques (Qiagen, Valencia, CA). Subconfluent LECs and hLECs were cotransfected with pTAT3-luc and pRL-TK (Renilla luciferase) according to the protocols provided with the transfection agents (Genejamer; Stratagene, La Jolla, CA for rLECs; SuperFect, Qiagen, for hLECs), then incubated overnight in serum-free medium. After treatment with Dex or antagonist, triplicate cell samples were harvested and luciferase expression analyzed using the dual-luciferase assay system, according to the manufacturer’s instructions (Promega), with a dual-injector plate luminometer (Berthold, Oak Ridge, TN). The pTAT3-luc luminescence was normalized to the Renilla signal for each sample and expressed relative to the transfected-untreated control as multiples of increase (or decrease) in luminescence.

Quantitative Real-Time PCR

mRNA reverse transcribed to ds cDNA, as described, was prepared for use in Q-PCR analyses. Oligonucleotide primers to human glucocorticoid-induced lucine zipper protein (GILZ), human serum/glucocorticoid-induced kinase (SGK), human and rabbit cellular inhibitor of apoptosis protein (cIAP-2) and human and rabbit mitochondrial superoxide dismutase (MnSOD) cDNAs were designed on computer (Primer Express software from Applied Biosystems, Foster City, CA). Primers were synthesized by Integrated DNA Technologies (Coralville, IA) (GILZ, SGK, cIAP-2, MnSOD) or GenBase Inc. (San Diego, CA) (actin). Q-PCR amplifications were performed in a 96-well plate (Applied Biosystems) in 25-μL volumes according to the manufacturer’s protocol using nucleic acid stain (SYBR Green; Applied Biosystems) as the indicator. Conditions were: dissociation at 95°C for 10 minutes followed by 45 cycles of 1 minute at 60°C and 50 seconds at 95°C (model 7000 Q-PCR Cycler; Applied Biosystems, with data analysis performed using the manufacturer’s software). Samples were run in triplicate alongside controls for actin, and the mean number of cycles to threshold (0.2) was compared with actin to determine multiples of increase or decrease in expression relative to untreated controls.

Results

Detection of GR by RT-PCR

PCR amplifications using primer combinations designed to amplify regions of the 5’ (GR001f with GR629r and GR460f with GR995r) and 3’ (GR1349f with GR2022r and GR2038f with GR2352r) portions of GR cDNA yielded products of approximately 630, 535, 670, and 310 bp, respectively, from cDNA prepared from human and rabbit (data not shown) lens samples and also from hLECs and rLECs (Fig. 1). These products were the anticipated size for cDNAs within exon 1 (5’ products) and spanning exons 3 to 7 (3’ products), respectively. Sequences obtained for the LECs-derived products subjected to BLAST analysis were similar to these same regions (www.ncbi.nlm.nih.gov/blast/ provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD).

The intervening rLECs GR sequences were obtained by PCR using primers designed to regions of sequence already obtained, and the full length sequence for rabbit GR from LECs was submitted to GenBank (accession no. AY161275). The rabbit GR nucleotide sequence is 89% identical with human GR and mouse GR. The derived protein sequence for rabbit GR is shown aligned to human GR in Figure 2 and shares 91% identity.

Primers GR1349f with GR2022r and GR2038f with GR2352r were also used in PCR amplifications of bovine fs cDNA prepared from bovine lens capsule and epithelial cells and generated two products of the expected sizes (Fig. 1). The intervening sequence was obtained using specific primers designed to regions of these products, and a reaction with rabGR548f and rabGR1473r was conducted to obtain additional 5’ sequence. A combined 1757-nucleotide (nt) bovine GR 3’ sequence was
obtained (accession no. AY238475). This bovine GR 3’ sequence is 89% and 93% identical with the comparable regions of human GR and the rabbit GR respectively and for the derived protein is 87% identical with both human and rabbit GRs.

Detection of GR by Western Blot

The anti-GR antibodies, NCL-GCR, PA1-511A, H-300, and P-20, gave positive reactions to GR in Western blots of bovine, human, and rabbit lenses (samples derived from lens capsules with adherent epithelial cells) and hLECs and rLECs, at the expected molecular size for GR of approximately 94 kDa (Fig. 3). BuGR2, which was raised to amino acids (aa) 407 to 423 of rat GR (identical with rabbit GR aa383 to 399: Fig. 2), as expected, reacted with the rabbit samples but did not react to those of human origin—the corresponding peptide region of human GR differs by 2 amino acids. BuGR2 also reacted to the bovine lens sample—the corresponding peptide region of bovine GR is also identical with that of rat GR. The most consistent response was observed with NCL-GCR, which was raised to the N-terminal of human GR, and this mAb reacted with samples from all three mammalian species and both cell lines. PA1-511A reacted strongly to hLECs and weakly to bovine, hu-
man, and rabbit lens, the epitope recognized by PA1-511A is identical in human and bovine GR but rabbit GR contains two conservative substitutions in this region. H-300 (maps to aa 121–420 of human GR) and P-20 (designed to the C-terminal of GR) both reacted with samples from all three species.

Detection of GR by Immunohistochemistry

Sections of human lenses were evaluated for the immunohistochemical localization of GR using four anti-GR antibodies, the mAb NCL-GCR and the rabbit polyclonals H-300, P20, and PA1-511A. The results are shown in Figure 4. PA1-511A reacted predominantly with the nuclei of epithelial cells in the anterolateral regions of the lens, the bow region and the differentiating lens fiber cells. NCL-GCR stained the epithelial cells poorly (data not shown).

Although reactivity of cell nuclei was only marginally increased over controls for H-300 and P-20, these antisera also indicated reactivity in the cytoplasm of the LECs that was absent from the controls. This cytoplasmic reactivity was seen in LECs from the anterior region to the bow region and appeared as a granular fluorescence.

Detection of GR Activity with pTAT3-luc

rLECs and hLECs preincubated in serum-free media overnight were exposed to 1 μM Dex for 0, 2, or 4 hours or to 100 μM RU/486 for 2 hours or to RU/486 for 2 hours followed by Dex for 2 or 4 hours. The data are shown in Figure 5. Addition of Dex induced luciferase expression, which increased between 2 and 4 hours, and RU/486 completely (hLECs) or partially (rLECs) blocked this expression.

Expression of GILZ, SGK, cIAP-2, and MnSOD mRNA in Response to Dex Treatment

Real-time Q-PCR analysis of Dex-treated hLECs and rLECs are shown in Figure 6. These data indicate that GILZ message increased by approximately 11-fold with 3 hours of Dex exposure and that mRNA levels remained elevated through the 48 hours of treatment. SGK mRNA levels were elevated by 3.8-fold at 3 hours of Dex treatment and declined to 2.4-fold by 48 hours. cIAP-2 message was increased by approximately two-fold in hLECs at 48 hours and threefold in rLEC. Levels of MnSOD mRNA were only marginally increased in hLECs but were approximately twofold increased by 3 hours in rLECs and declined subsequently.

cIAP-2 and MnSOD Protein in Response to Dex Treatment

Western blot analyses are shown in Figure 7 for hLECs and rLECs together with the densitometric analyses of these blots. The expression of cIAP-2 protein was elevated approximately 2.1-fold in hLECs and over 6-fold in rLEC after 48 hours of Dex exposure. MnSOD protein expression was elevated approximately 4.3-fold in hLECs and approximately 11-fold in rLECs.

**DISCUSSION**

Through PCR, sequencing, Western blot analysis, and immunohistochemistry, our data confirm the existence of GR in human lens cells, indicate the presence of GR in rabbit and bovine lens cells—resolving the controversy over the existence of GR in bovine lens—and indicate that lens cells of all three species contain the active GRα isoform.

![Figure 5](https://example.com/figure5.png)

**FIGURE 5.** Luciferase expression from hLECs and rLECs transfected with pTAT3-luc exposed to 1 μM Dex for 0, 2, or 4 hours; to 100 μM RU/486 for 2 hours; or to RU/486 for 2 hours followed by RU/486 + Dex for 2 or 4 hours; or to RU/486 for 6 hours and Dex for 4 hours.

![Figure 6](https://example.com/figure6.png)

**FIGURE 6.** Change in expression by hLECs of GILZ, SGK, cIAP-2, and MnSOD mRNA and by rLECs of cIAP-2 and MnSOD mRNA, analyzed by Q-PCR normalized to actin and expressed as multiples of increase or decrease relative to control (0) after Dex treatment for 0 to 48 hours.
Callicotization observed in the human lens sections in Figure 4 with regard to nuclear chromatin. The cytosolic versus nuclear immunolocalization of liganded GR translocates and binds to nuclear targets in many reports the predominant location appears to be cytosolic. From either location, liganded GR can translocate and bind to other proteins, particularly Hsp90, and is the transcriptionally active form. The partial bovine GR sequence was also compatible with the GR isoform. Its degree of phosphorylation was also confirmed to actin and expressed as multiples of increase or decrease relative to control (0) after Dex treatment for 0 to 48 hours.

Products of the expected sizes were obtained by PCR amplification of cDNA obtained from human, rabbit, and bovine lens and from hLECs and rLECs, with oligonucleotide primers designed to known GR sequences. Sequences from the amplified products of fs cDNA from these cells and also from bovine lens cells, obtained with the 3′ primers, matched with a region of cDNA spanning exons 3 to 7 from human GR, indicating the products represented mRNA and not genomic DNA. The complete sequence for rabbit GR was obtained from rLECs. This sequence was compatible with the predominant GR isoform and is the transcriptionally active form. The partial bovine GR sequence was also compatible with the GRα isoform.

Immunohistochemical staining with anti-GR antibodies localized the GR both to the nuclei and cytosol of LECs in the anterior and lateral capsule regions, bow region, and nuclei of cells in the early differentiating fiber cells. The four antisera used reacted at different intensities in the immunolocalization studies, and their reactivities in Western blot analysis were also different. In the case of BuGR2, this mAb was raised to the 407- to 423-aa region of rat GR and failed to react to samples of human origin, but did react in Western blot analysis with samples of cow and rabbit origin. The absence of antibody reactivity in Western blot analysis of bovine lens material in the earlier study of Jobling and Augusteyn may have resulted from their method of sample preparation or from their choice of antibody. PA1-511A (also referred to as clone 57 by the suppliers, and used by Jobling and Augusteyn) reacted only weakly with the bovine lens sample in Western blot analysis (Fig. 3), even though the epitope recognized by this antibody is conserved in bovine GR.

In the immunohistochemical study of human lens sections, PA1-511A reacted predominantly with the epithelial cell nuclei, whereas NCL-GCR, which responded most strongly by Western blot analysis of lens material, indicated that native GR in these lens cells is able to function as a transcription activator. These data substantiate similar results obtained recently in human and mouse LECs. In addition, competent direct glucocorticoid-induced type-1 gene transcription by GR was also suggested by the increase in transcription of GLIZ and SGK mRNAs from hLECs after exposure to Dex.

The GLIZ gene contains upstream GR recognition motifs and is directly induced by glucocorticoids. GLIZ also indirectly regulates the expression of other proteins through interaction with the transcription factors NFκB and AP-1. Among the upstream transcription factor recognition sites for both cIAP-2 and MnSOD are sites for GR, AP-1, and NFκB. Upregulation of both these proteins has been reported after glucocorticoid administration. In human and mouse LECs, indicate GR is present in rabbit lens and LECs, and resolve the presence of GR in the bovine lens. Furthermore, our data suggest that the GR present in LECs is transcriptionally active. The targets we selected for analysis of mRNA and protein expression levels clearly represent a very small sample of those proteins that are likely to have expression modified by glucocorticoid treatment. Reports link the four proteins included in this study to a range of different biochemical pathways, and thus glucocorticoid treatment of LECs is likely to perturb the dynamics of a number of cellular activities.

Conclusions

Our data indicate that the active GRα isoform is present in LECs and that the expression levels of proteins known to be regulated by glucocorticoids are modified in these cells by glucocorticoid treatment, indicating that this lens GR isoform is functional.

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References


