Cholesterol-producing transgenic *Caenorhabditis elegans* lives longer due to newly acquired enhanced stress resistance

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Abstract

Because *Caenorhabditis elegans* lacks several components of the de novo sterol biosynthetic pathway, it requires sterol as an essential nutrient. Supplemented cholesterol undergoes extensive enzymatic modification in *C. elegans* to form other sterols of unknown function. 7-Dehydrocholesterol reductase (DHCR) catalyzes the reduction of the \( \Delta^7 \) double bond of sterols and is suspected to be defective in *C. elegans*, in which the major endogenous sterol is 7-dehydrocholesterol (7DHC). We microinjected a human DHCR expression vector into *C. elegans*, which was then incorporated into chromosome by \( \gamma \)-radiation. This transgenic *C. elegans* was named *cholegans*, i.e., cholesterol-producing *C. elegans*, because it was able to convert 7DHC into cholesterol. We investigated the effects of changes in sterol composition on longevity and stress resistance by examining brood size, mean life span, UV resistance, and thermotolerance. *Cholegans* contained 80% more cholesterol than the wild-type control. The brood size of *cholegans* was reduced by 40% compared to the wild-type control, although the growth rate was not significantly changed. The mean life span of *cholegans* was increased up to 131% in sterol-deficient medium as compared to wild-type. The biochemical basis for life span extension of *cholegans* appears to partly result from its acquired resistance against both UV irradiation and thermal stress.

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Sterols are important constituents of the plasma membranes of eukaryotic cells. Cholesterol, which is found predominantly in animal cells, influences membrane permeability and the fluidity of the lipid bilayers. Data accumulated in recent years demonstrate that cholesterol is not only a membrane structural component but is also actively involved in modulation of cell signaling [1]. Fungi, plants, and mammals have complex sterol biosynthetic pathways, with more than 39 enzymatically catalyzed reactions. In contrast, nematodes such as *Caenorhabditis elegans* cannot synthesize sterols de novo [2]. *C. elegans* does express homologues of the enzymes up to the point of farnesyl pyrophosphate biosynthesis, a major branching point for the biosynthesis of other major isoprenoids [3]. Therefore, in order to culture *C. elegans* in the laboratory, agar plates must be supplemented with cholesterol or other sterols, as nematodes can modify many externally added sterols by transforming them into sterols permissible for nematode growth.

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Only the final products of the plant and mammalian biosynthetic pathways such as ergosterol, sitosterol, cholesterol or closely related sterols or their precursors can fully support worm growth. However, when one of the steps converting these sterols (e.g., sitosterol) to cholesterol is blocked by an inhibitor (e.g., azacaprostone), there are serious defects in egg laying, development, and growth [4]. A mammalian counterpart to this phenomenon occurs in the case of the enzyme 7-dehydrocholesterol reductase (DHCR), which biosynthesizes cholesterol from its immediate precursor, 7-dehydrocholesterol (7DHC). The accumulation of 7DHC in human fetuses deficient in DHCR leads to a morphological disorder termed the Smith–Lemli–Opitz syndrome. Thus, 7DHC does not seem to fulfill the cholesterol requirement of the developing embryos in humans and exerts an additional embryotoxic effect, probably via oxidized byproducts [5]. In contrast to mammals where cholesterol is the major sterol, *C. elegans* contains 7DHC as its major sterol [2].

*Caenorhabditis elegans* has proven to be an excellent experimental system for dissection of the aging process, which is modulated by complex genetic regulation (e.g., *age-1, daf-2* genes). Aging is associated with an accumulation of molecular and cellular damage that correlates with a decline in normal physiological function. Where tested, all known *C. elegans* longevity mutants exhibit increased resistance to environmental stress such as oxidative stress (e.g., hydrogen peroxide, paraquat), heat shock, UV irradiation, and heavy metal stress, which result in the decline of cellular homeostasis [6,7]. It is not known whether enhanced resistance to stress is required for extended life span in *C. elegans*, but various interventions that enhance stress tolerance result in longevity.

Despite the possible importance of sterols in stress tolerance in *C. elegans*, either as biophysical modulators of membrane fluidity or as precursors to bioregulatory steroids, physiological studies on the role of cholesterol in the aging of *C. elegans* have not been reported. Therefore, we attempted to create a transgenic cholesterol-producing *C. elegans*, termed *cholegans*, and examined changes in behavior, reproductive activity, and longevity. Our results with this transgenic strain indicate that cholesterol plays an important role in *C. elegans* longevity apparently by imparting stress resistance.

**Materials and methods**

*Nematode strains and culture*. Wild-type *C. elegans* (Bristol strain, N2) was provided by the *Caenorhabditis* Genetics Center. Worms were routinely grown at 20 or 25 °C on 50-mm-diameter nematode growth medium (NGM) agar plates containing *Escherichia coli* (OP50) as a food source [8]. For aging experiments, worms were grown in three different media: cholesterol (5 μg/ml) supplemented medium (CSM), 7DHC supplemented medium (DSM), and sterol depletion medium (SDM), prepared as previously described [9].

*DNA transformation in C. elegans*. DNA transformation in wild-type was achieved by inserting plasmid DNA into the distal arm of the hermaphrodite gonad as described [10]. The 1.5 kb *myc*-tagged human DHCR (hDHCR) cDNA fragment [11] was directionally cloned into expression vector pPD49.78, which has the hsp 16.41 small heat shock promoter and no enhancer (Fig. 1A). The sequence of the resulting cloned plasmid was confirmed by automated dye terminator, dideoxy sequencing (ABI Prism 377 Sequencer, Applied Biosystems, Foster City, CA). The hDHCR expression construct and the plasmid pRF4 containing a dominant genetic marker were microinjected into the gonads of wild-type at a final concentration of 100 ng/μl. Integration of the extrachromosomal arrays was achieved by γ-irradiation of transformants with γ-rays of 4000 Rad (15 R/s) from a 60Co source. Two strains had stably integrated chromosomal transgenes: these worms were backcrossed with wild-type males to exclude the rare possibility of random mutation.

*Northern blot analysis*. Total RNA extracted from *cholegans* was separated on a 1.0% (w/v) agarose gel in 30% (v/v) formaldehyde and transferred to nylon membranes followed by immobilization with UV-cross-linking. hDHCR cDNA probe was labeled with [α-32P]dCTP by random-priming. After hybridization (42 °C, 50% (v/v) formamide), the membranes were washed with 0.1 x SSC containing 0.1% SDS at 65 °C. Hybridization signals were visualized by autoradiography using an X-ray film.

Fig. 1. (A) Expression vector of the recombinant *myc*-tagged hDHCR. The 1.5 kb *myc*-tagged human DHCR cDNA fragment was directionally cloned into the expression vector pPD49.78 (3.8 kb), which has hsp 16.41 small heat shock promoter and no enhancer. (B) The radiolabeled hDHCR cDNA was hybridized to total RNA extracted form *cholegans*. After incubation at 20 or 25 °C daily, or 30 °C for 2–6 h, total RNA was prepared from the worms, and 20 μg of RNA was subjected to Northern blot analysis. hDHCR mRNA levels in this figure were normalized to the quantity of 28S rRNA expression.
Ergosterol or lathosterol (dissolved in Tween 80 at a fraction, we used the Mitochondria Isolation Kit (Sigma). A weight ratio of 70:1, detergent:sterol) was added at a final concentration of 40 μM after worms had reached adulthood [13]. To measure survival and mean life span (MLS), worms were counted every day by eliminating dead ones. Death was recognized as a loss of spontaneous movement and lack of response to touch. The mean survival rate was then spun at 105,000 x g for 1 h. The supernatant (cytosol) at 4 °C was used for enzymatic activity assays and sterol analysis by gas–liquid chromatography (GLC).

Preparation of membrane fractions. Worms were harvested from small prewarmed (35 °C) NGM plates and incubated at 35 ± 0.5 °C. At the end of incubation times ranging from 1 to 9 h, plates were removed and worms were examined for mobility, response to provoked movement, and pharyngeal pumping. Worms failing to display any of these traits were scored as dead. The mean survival rate (MSR, time of 50% survival) was determined [17]. As a control, adult hermaphrodites were incubated at 35 °C until all worms were dead.

Preparation of membrane fractions. Worms were harvested from liquid culture and washed repeatedly in S medium to remove bacteria. Washed worms were collected by centrifugation at 1500g for 10 min. The pellets were resuspended in two volumes of reaction buffer (0.1 M potassium phosphate buffer, pH 7.4, 1 mM reduced glutathione, 0.5 mM EDTA, and 20% glycerol v/v) containing a proteinase inhibitor cocktail (Boehringer Mannheim GmbH, Penzberg, Germany) and were disrupted with a Teflon homogenizer. The homogenate was centrifuged at 1500g for 10 min at 4 °C to remove cuticles and large debris. The supernatant (whole homogenate: WH) was then spun at 105,000g for 1 h. The supernatant (cytosol) at 105,000g, the pellets (microsomes) containing the membrane fraction and mitochondrial fraction, resuspended in reaction buffer, were used as the DHCR enzyme source [19]. To obtain an intact mitochondrial fraction, we used the Mitochondria Isolation Kit (Sigma).

Results

Creation and biochemical validation of transgenic cholerealgs

To create a transgenic C. elegans that can produce cholesterol (choleragalgs), first we obtained a transient line containing the transgene hDHCR on extrachromosomal arrays, termed choleragalgs-transient, which was identified by observation of the roller phenotype. To prepare a stable line containing hDHCR integrated into the host chromosome, the choleragalgs-transient line was irradiated with γ-rays, and a choleragalgs-stable (thereafter referred to simply as choleragalgs) line was selected after the 6th generation of progeny and used for our experiments. The stable line exhibited 100% transmission of both roller markers and the presence of the chromosomal-localized hDHCR gene [12], as confirmed by Southern blotting, suggesting that the myc-hDHCR transgene was successfully integrated into choleragalgs (data not shown). Because hDHCR construct had an hsp 16.41 promoter, we were concerned about whether hDHCR was expressed well at 20 °C. Our Northern blot data (Fig. 2B) demonstrated that hDHCR mRNA is expressed in a temperature-dependent manner, confirming the function of hsp 16.41 promoter in the expression vector. Transgenic worms expressing hDHCR (e.g., choleragalgs, age-1 (l545d)) showed similar properties as those of wild-type in many aspects: body size, dauer formation rate, and time required for larval development at each stage (data not shown).

To validate the expression of recombinant myc-tagged hDHCR in choleragalgs, whole cell lysates of the transgenic lines carrying hDHCR were analyzed for expression of the transgene by Western blotting, enzymic assays, and GLC analysis. First, to determine the steady state level of exogenously introduced hDHCR expressed in choleragalgs, Western blot analysis was carried out using cell homogenates obtained from choleragalgs that contain integrated myc-tagged hDHCR cDNA in
the host chromosome. As anticipated, a 55 kDa recombinant protein was detected by an antibody against the myc protein in Western blotting, confirming the correct expression of the myc-tagged hDHCR protein (Fig. 2A).

Second, having successfully established the DHCR expression system in *cholegans*, we examined the subcellular distribution of DHCR by analyzing the enzyme activity of whole homogenates (WH), mitochondria, microsomes, and cytosol (Fig. 2B). As in mammals [19], DHCR activity was mainly present in the microsomal fractions, although low levels of activity were also found in WH, indicating that exogenous hDHCR is localized in membranous organelles. There is a difference in DHCR specific activities between rat liver and *cholegans* microsomes; the former containing usually 450–500 pmol/mg/min while the latter contained 100–300 pmol/mg/min. Third, to examine whether transgenic worms can convert endogenous 7DHC to cholesterol, we compared the presence of endogenous cholesterol in wild-type and *cholegans* (Fig. 2C). The GLC chromatograms clearly demonstrated that *cholegans* converted 7DHC (rt. = 5.247 min, rrt. to cholesterol = 1.08, m/z = 384) to cholesterol (rt. = 4.858 min, m/z = 386) efficiently. Note that there was a huge difference between *cholegans*-transient (>40%) and *cholegans* (>80%) in terms of cholesterol conversion (Fig. 2C). The major sterol of *cholegans* detected by GLC was found to be cholesterol whether the worms were grown in the presence of 7DHC or cholesterol in the agar plate. Taken together, these results indicate that hDHCR cDNA was successfully integrated in *cholegans* and active enough to convert most of the detectable endogenous 7DHC to cholesterol.

Biological properties and characteristics of *cholegans*

The first observation we made from *cholegans* was its extended life span that was dependent on the sterol-supplemented condition. When we calculated mean life span (MLS) values from survival curves of nematodes grown in sterol-supplemented vs. sterol-depleted medium, the MLS of wild-type, *cholegans*, and age-1 in cholesterol supplemented medium (CSM) were 21, 24, and 34 days, respectively (Table 1). However, when worms were grown on sterol depletion medium (SDM), *cholegans* lived at least 31% longer than the control wild-type animals (*n* = 20) (21 days vs. 16 days), and even 10.5% longer than the age-1 mutant (21 days vs. 19 days). When grown in the 7DHC plate (5 μg/ml) (*n* = 30), MLS of F2 *cholegans* was 19.6 ± 2.8 days, 24% longer than that of wild-type (19.6 days vs. 15.8 days) (data not shown), indicating that extended life span of *cholegans* in 7DHC supplemented medium (DSM) is essentially similar to that in SDM.

Second, we found differences in average brood size of progeny among the three strains (*n* = 20): 252 ± 22
Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>NGM (+)cholesterol</th>
<th>Mean life spana</th>
<th>Brood sizeb</th>
<th>Adult number</th>
<th>Percent developmentc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S µg/ml</td>
<td>(±SD, days) n = 20</td>
<td>(mean ± SD) n = 20</td>
<td>(mean ± SD) n = 20</td>
<td>(egg → adult)</td>
</tr>
<tr>
<td>N2</td>
<td>+</td>
<td>21 ± 5.1</td>
<td>252 ± 22</td>
<td>229 ± 14</td>
<td>90.8 ± 5.5</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>16 ± 1.2</td>
<td>119 ± 10</td>
<td>76 ± 6</td>
<td>63.9 ± 5.0</td>
</tr>
<tr>
<td>Cholegans</td>
<td>+</td>
<td>24 ± 3.6</td>
<td>187 ± 19</td>
<td>172 ± 13</td>
<td>92.1 ± 6.9</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>21 ± 4.1</td>
<td>108 ± 7</td>
<td>93 ± 9</td>
<td>86.4 ± 8.3</td>
</tr>
<tr>
<td>Age-l (hx546)</td>
<td>+</td>
<td>34 ± 2.5</td>
<td>153 ± 17</td>
<td>140 ± 13</td>
<td>91.5 ± 8.5</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>19 ± 2.0</td>
<td>82 ± 9</td>
<td>73 ± 5</td>
<td>89.1 ± 6.1</td>
</tr>
</tbody>
</table>

Each mean ± SD was derived from three separate determinations.

a To measure survival and mean life span (MLS) of F1 progeny in cholesterol supplemented medium (CSM) or sterol depletion medium (SDM), worms were counted every day by eliminating dead ones.

b Brood sizes of F3 progeny in CSM or SDM plates are the total number of non-hatched and hatched eggs of each hermaphrodite, expressed as mean ± SD (n = 20).

c Percent development is the number of adults divided by the total number of eggs.

Changes in physiological characteristics of transgenic *C. elegans* in response to sterols

(wild-type), 187 ± 19 (cholegans), and 153 ± 17 (age-l) (Table 1). The reduction of brood size (~40%) was also seen in the case of age-l, a long-lived mutant. Interestingly, percentage reductions in brood size were very similar (43–53%) among these strains when the growth conditions were shifted from CSM to SDM. However, in SDM there was also a clear difference in the percent development from eggs to adult among the three strains (wild-type: 90.8% [CSM] to 63.9% [SDM], cholegans: 92.1–86.4%, and age-l: 92–89.1%). The long-lived mutant age-l and cholegans showed similar percent development to adult (89.1% vs. 86.4%) in SDM, suggesting that other factors might exist besides sterol presence for development of eggs to adults. When worms were grown in SDM, cholegans had a substantially higher rate (>35.2%) of adult development from eggs (86.4 ± 8.3%) as compared to wild-type (63.9 ± 5.0%). This result suggests that both cholegans and age-l exhibit a clear advantage over wild-type in growth rate and longevity when faced with a harsh nutritional environment (e.g., SDM).

To examine whether extended life span in cholegans might correlate with stress resistance, we first determined mean survival rate (MSR) after the initial exposure of worms to increasing intensity of UV light with worms grown in CSM plates. We measured MSR of worms grown only in CSM, to exclude the possibility of change in MSR due to sterol composition in the media, which was already well established as described above. The worms of all three strains, wild-type N2, cholegans, and age-l, showed the same pumping rate per min during the measurement of MSR. The MSR of cholegans was very similar to that of age-l (4.17 days). The results from the UV-resistance assay at 20 °C showed that the MSR of both cholegans and age-l was almost twice that of wild-type (4.17 days [cholegans, age-l] vs. 2.38 days [wild-type]) (Fig. 3A). The age-l observation is consistent with that previously published [16]. Further examination of stress resistance was performed by measuring the thermotolerance of wild-type, cholegans, and age-l against heat (35 °C) in CSM plates. Cholegans showed about an 18% higher MSR compared to wild-type [497 h vs. 420 h]. The longevity mutant age-l displayed a higher thermal tolerance that could not be calculated from the survival curve (Fig. 3B) [16,17]. Therefore, we tentatively conclude that production of cholesterol by DHCR might account for the observed decrease in lethality upon exposure of these transgenic worms to UV and elevated temperature.

Subsequently, we determined the relative activities of two representative antioxidant defense enzymes, catalase and SOD. The relative activities of each enzyme in cholegans were similar to those of wild-type regardless of developmental stages and nutritional conditions (e.g., for SOD in young adults, 4.7 ± 0.5 U [wild-type] vs. 6.5 ± 0.3 U [cholegans]), suggesting negligible influence of cholesterol production on these defense enzymes (data not shown).

Discussion

In this report, we demonstrate the first example of transgenic *C. elegans* that produces cholesterol, a line that we have named cholegans. It is important to note that cholegans and wild-type are both incapable of de novo sterol biosynthesis; cholegans, however, possesses the enzyme that converts 7DHC (typically the major sterol of *C. elegans*) to cholesterol. Interestingly, cholegans acquired an ability to resist stress, perhaps caused by the change of endogenous sterol composition from 7DHC to cholesterol. Despite the lack of difference between wild-type and cholegans in general behaviors and morphology, the change in sterol composition appeared to affect growth, development, reproduction, and longevity of *C. elegans*. Although the phenotypic differences are about 30% between the wild type and cholegans, it is a statistically meaningful and reproducible difference in longevity extension. Among the transgenic lines made
by γ-radiation, we found that some of the lines were not able to convert 7-dehydocholesterol to cholesterol, suggesting that γ-radiation may create random integration of DHCR gene into the host chromosomes. The transgenic lines we selected were clearly stable strains which consistently express DHCR activity when tested.

In particular, the increase in MLS of *cholegans* may be due to enhanced stress resistance when the worms are faced with adverse environments such as UV and heat. Resistance to thermal stress has previously been shown to be related to longevity in *C. elegans* [23]. We speculated that production of cholesterol may lead to protect *cholegans* against damage by UV or heat, perhaps by promoting the formation of oxidation products of cholesterol less toxic than the primary free radicals formed by oxidative stresses, as seen in mammals [24].

As a step toward understanding the specific biological roles of cholesterol in *C. elegans*, we investigated a mechanistic connection between stress resistance and longevity. Our survival experiments demonstrate that DHCR protects transgenic worms from damage generated by UV and heat. Apparently, SOD and catalase are not much involved in the DHCR-mediated stress resistance in *cholegans*. In the process of stress resistance, the presence or absence of sterol in the media affects significantly the changes in the MLS of worms. For example, F1 *cholegans* in sterol depletion medium (SDM) showed a 20–30% longer MLS than wild-type, but this difference was only 14% when worms were grown in cholesterol plates. This increased MLS of *cholegans* in SDM was even more prominent as the generations passed. That is, in the F4 generation, MLS of *cholegans* was almost double of wild-type 2 (10 days [wild-type] vs. 18 days [*cholegans*]). Interestingly, the MLS of wild-type grown in cholesterol was longer than wild-type grown in 7DHC in subsequent generations (e.g., F4). For UV and thermotolerance studies, it was very difficult to measure MSR because wild-type did not tolerate these kinds of stresses well when grown in SDM. We also wanted to eliminate the possible cause of changes in MSR by switching the media because differential effects of sterol composition on nematode biological processes have already been demonstrated.

The specific mechanism through which *age-1* enhances UV resistance is unknown. In the case of *cholegans*, we can only note that cholesterol may protect against UV-derived mitochondrial DNA damage, as previously reported in mammalian brains [24]. The brood sizes of all strains tested were reduced in SDM, which may result from the fact that embryogenesis did not progress well under sterol deficiency, regardless of the ability to produce cholesterol from 7DHC. One possible explanation for the thermotolerance of *cholegans* is that when worms were exposed to heat, the hsp promoter certainly directed the expression of DHCR cDNA, mostly in the L1 and L2 larval stages (data not shown), which then caused an increase in cholesterol production from 7DHC. As a result, membrane rigidity in localized microdomains may have also increased, which may be advantageous to survive under heat stress. Alternatively, a cholesterol-derived steroid of unknown identity could be involved in other responses to heat.

When sterol metabolism-related gene sequences were searched in the *C. elegans* genomic database, only two mRNA clones, GenBank Accession Nos. NM063363 and NM063362, were found as members of the sterol desaturase family; they are expressed at low levels. The sterol 5-desaturase family also contains sterol 4-methyl...
oxidases. Although we expected that sterol 7-desaturase activity would be very prominent in wild-type in order to convert exogenous or endogenous cholesterol to 7DHC, we were not able to detect any activity by enzyme assays. In mammals, 7DHC and its oxidized products are embryotoxic [5] and perhaps adversely interact with proteins or enzymes containing a sterol-sensing domain [25]. In *cholegans*, we believe that the transgenic DHCR produces cholesterol from 7DHC, consequently overriding the activity of the existing sterol 7-desaturase (Fig. 4), but conclusive evidence remains to be established.

There seems to be a correlation between sterol structure and the MLS of *cholegans*. For example, when worms were grown in sitosterol or stigmasterol, both having an ethyl group at C-24, *cholegans* showed a 10% longer MLS in the F2 generation than lanosterol-supplemented worms. A similar MLS was observed in the worms grown in either 7DHC or ergosterol, as compared to wild-type grown in cholesterol (data not shown).

 CHOLEGANS and wild-type worms grown in lanosterol showed the same MLS pattern as worms grown under SDM conditions, suggesting that *C. elegans* does not uptake or metabolize lanosterol for further use. The three bulky methyl groups at C-4α, 4β, and 14α might result in poor uptake or subsequent metabolism. For example, when worms were grown in the presence of miconazole (0.1, 1.0, and 10 mM) plus lanosterol or azacoprostane plus sitosterol [4], the MLS of wild-type and *cholegans* was similar to that of wild-type and *cholegans* grown in SDM (data not shown), suggesting that *C. elegans* lacks sterol 4- or 14-demethylase.

In summary, the biochemical basis for the extended life span of *cholegans* may be due to two factors: first, the readily available cholesterol possesses a protective function against extreme stresses, either by a biophysical interaction in membranes or by the production of cholesterol metabolites that interact with other molecules. Second, DHCR overexpression in *cholegans* may stimulate uptake of sterol that is present in extremely low amounts in SDM and *E. coli*, or promote translocation of sterol within the nematode. Both aspects of the cholesterol-associated enhancement of longevity in *C. elegans* warrant further investigation.

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Fig. 4. Proposed pathway of sterol transformation in *cholegans* with acquired DHCR activity. Despite the lack of enzymes for de novo sterol synthesis, nematodes can modify externally added yeast, plant or mammalian sterols by introducing double bonds, reducing or isomerizing them, and dealkylating the side chain [15]. Arrows long and bold shape indicate that endogenous sterols in *cholegans* are converted from 7DHC to cholesterol.
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References


