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Thymocytes Express the golli Products of the Myelin Basic Protein Gene and Levels of Expression Are Stage Dependent

Ji-Ming Feng,* Irene M. Givogri,* Ernesto R. Bongarzone,* Celia Campagnoni,* Erin Jacobs,* Vance W. Handley,* Vilma Schonmann,* and Anthony T. Campagnoni2*†

The golli products of the myelin basic protein gene have been shown to be expressed in mouse thymus and brain. The full repertoire of thymic cell types expressing golli products has not yet been determined, although immunoreactivity has been found in some macrophages. We have analyzed the cellular expression of golli mRNAs and proteins in the thymus. The results showed that MTSS1+ cortical/MTS10+ medullary epithelial cells and NLDC145+ dendritic cells did not express golli, while some macrophages did exhibit strong immunoreactivity. Golli mRNAs were not detected in macrophages by in situ hybridization. Thymocytes expressed significant levels of golli mRNAs and proteins by in situ hybridization and immunohistochemistry. Interestingly, golli immunoreactivity varied with thymocyte stage of differentiation. For example, CD4+CD8+ expressed significant levels of golli protein, while CD4+CD8+ thymocytes did exhibit strong immunoreactivity.

When thymocytes developed into CD8+ (double-negative) thymocytes expressed relatively high levels of golli. Upon further differentiation into CD4+CD8+ (double-positive) thymocytes, golli protein expression declined dramatically. When thymocytes developed into CD8− or CD4+ (single-positive) thymocytes, golli protein expression increased again, but it never achieved the levels found in double-negative thymocytes. Thus, the altered levels of expression of golli proteins in developing thymocytes correlated with the transitions from double-negative to double-positive and double-positive to single-positive stages. The lack of significant golli expression in thymic stromal cells may offer an alternative explanation for the mechanism of inefficient negative selection of those autoreactive thymocytes with specificity for myelin basic proteins.


E xperimental allergic encephalomyelitis (EAE)3 has served as a model of autoimmune disease (1); and the myelin basic proteins (MBPs) were the first proteins identified in the CNS to induce this disease in laboratory animals (2–5). For many years, it was assumed that the reason MBPs induced the disease was because the immune system was protected from exposure to these proteins by the blood-brain barrier.

In recent years, it has been found that the MBP gene encodes two families of products containing classic MBP epitopes. In addition to the classic MBPs found in the myelin sheath and expressed by myelin-forming cells, a second set of products, called golli proteins and mRNAs, is produced by the gene in the immune and nervous systems of mice, rats, and humans (6, 7). The MBP gene, the two families of products it expresses, and the common MBP sequences in the golli and classic MBPs are shown in Fig. 1. The golli products differ from the classic MBP proteins through the presence of a 133-aa sequence unique to the golli proteins, encoded by several exons upstream of the classic MBP transcription start site. These unique regions of the golli proteins and mRNA gene products have permitted us to generate specific Abs and specific probes (8) for the analysis of cellular expression of golli products at the protein and mRNA level. The presence of golli products in the early developing thymus in mouse and human (9, 10) suggests that lack of exposure of the immune system to MBP-related products cannot completely explain the autoimmune activity of the MBPs. Studies have shown that MBP epitopes found within the context of golli proteins can be recognized by T cell lines specific for encephalitogenic epitopes of classic MBP (11), which can induce severe EAE in mice. It has also been reported that golli-specific peptides alone can induce a mild autoimmune disease in the rat (12). Previous work has shown that golli mRNA expression increases during the relapsing phases of EAE, which has led to the proposition of an alternative model for epitope spreading during the course of EAE based upon endogenous expression of golli in lymphoid tissue (13).

Several lines of evidence indicate that golli proteins (or epitopes) can be found within some macrophages in the thymus and lymph nodes of mice and humans and that they are capable of presenting MBP epitopes derived from golli on their surface (13, 14). However, the expression of golli products in other cells in the thymus could not be excluded because of the high autofluorescence in the tissues (9). Thus, we undertook a more detailed examination of golli protein and mRNA expression in the thymus to identify all the cell types expressing these products of the gene. Such a study is an important component of determining the mechanisms underlying the induction of tolerance and the autoimmune response to MBPs in EAE. While the results confirmed the presence of immunoreactivity in some thymic macrophages, we were surprised to find that the majority of golli expression occurs in thymocytes, that this expression changes with the state of thymocyte maturation, and that there is little expression of golli in epithelial or dendritic cells.

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Materials and Methods

Antibodies

The rabbit polyclonal anti-golli Ab was generated against a recombinant peptide corresponding to the 133 aa specific to the golli proteins; the antisera were affinity purified with the golli peptide and characterized, as described previously (8). The other primary Abs used were: CD4 (L3T4) FITC, CD8a (Ly-2) PE, MTS5 (rat IgM), and MTS10 (rat IgM) mAbs (PharMingen, San Diego, CA); NLDC-145 (rat IgG2a; Serotec, Oxford, U.K.); and F4/80 (rat IgG2b; Caltag, South San Francisco, CA).

Tissue preparation for immunohistochemistry and in situ hybridization

BALB/cByJ mice bred in our animal colony from stock obtained from The Jackson Laboratory (Bar Harbor, ME) were used for analysis. Thymuses were removed from the mouse at postnatal day 6, embedded in OCT medium (Tissue-Tek, Sakura Finetek, Torrance, CA), and stored at −20°C until use. Tissue was sectioned at 2–5 μm, mounted on Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA), dried briefly at room temperature (RT), fixed in pure acetone at RT for 20 min, dried again in air, and stored at 4°C.

Ag detection by immunohistochemistry

The slides were hydrated in PBS for 15 min, and incubated in 1% BSA in PBS for 30 min. Sections were then incubated with primary Ab mixtures in 0.2% BSA/0.05% sodium azide (Ab dilution buffer) in PBS for 45 min at room temperature in a humid chamber. Following three washes (10 min each) with 0.05% Tween 20 in PBS, the sections were incubated for 45 min at RT with fluorescence-labeled secondary cocktails. The slides were then washed three times (10 min each) and mounted in 75% glycerol in PBS (pH 9.5). Fluorescence images were obtained using an excitation wavelength of 495 nm. Excitation wavelengths of 488 and 550 nm were used for PE and tetramethylrhodamine isothiocyanate red fluorescence, respectively.

For double labeling, the primary Ab cocktails consisted of the following combinations: golli Ab (1:500) + MTS5 (1:200) MTS5 (1:100), F4/80(1:50), or NLDC-145 (1:50), respectively. The secondary Ab cocktails consisted of the anti-rabbit IgG tetramethylrhodamine isothiocyanate (Sigma, St. Louis, MO; 1:200) + anti-rat Ig light chain FITC (Sigma; 1:200).

Isolation of thymocytes

Cells were dispersed from the thymus tissue by extrusion through a stainless steel 200 mesh into PBS. After 2-h culture in the plastic dishes to remove the possible contaminated adherent stromal cells, the nonadherent thymocytes were collected by centrifugation. Following three washes in PBS, the thymocytes were subjected to FACS analysis or RNA extraction. The thymocyte preparations were routinely 99% pure, as determined by FACS analysis.
Triple labeling of thymocytes for FACS analysis and confocal microscopy

Approximately 5 × 10^6 to 1 × 10^7 freshly isolated thymocytes were incubated for 45 min at 4°C with CD4 FITC (0.5 μl) and CD8 PE (0.5 μl) in 200 μl Ab dilution buffer, washed with PBS twice, suspended, and fixed in 2% paraformaldehyde in PBS. After overnight fixation, the stained thymocytes were incubated with golli polyclonal Ab (45 min at 4°C). Following three washes, cells were incubated for 45 min at 4°C with Cy-5-conjugated secondary Ab (anti-rabbit IgG, 1:400; Jackson ImmunoResearch, West Grove, PA). After three washes, the cells were subjected to FACS analysis on FACScalibur (Becton Dickinson, San Jose, CA) or were examined by confocal microscopy. All results displayed in FACS analysis are representatives of at least three independent experiments. An emission wavelength of 650 nm was used to examine Cy-5 fluorescence in the confocal microscope, but the fluorescence image was converted into a blue pseudo color.

Extraction of RNA and RNase protection assays (RPA)

**RNA extraction.** Total RNA was extracted from isolated thymocytes, normal thymus, and 20-h dexamethasone (DEX)- injected thymus (i.p.) using the Trizol procedure (Life Technologies, Gaithersburg, MD).

**Riboprobe.** The golli antisense [32P]UTP riboprobes specific to exons 2, 3, and 5A (called G19) were synthesized from linearized plasmids with T7 polymerase.

**RPA assays.** RPA were performed with a commercially available kit from Ambion (Austin, TX). For RNase protection experiments, 10 μg total RNA was hybridized to the probe at 42°C. Samples were electrophoresed in a 6% polyacrylamide/8 M urea gel, and then subjected to autoradiography.

**Tyramide signal amplification for fluorescence in situ hybridization (TSA-FISH)**

After treatment with 0.2 M HCl for 10 min, sections were prehybridized for 30 min at 37°C in a solution containing 50% formamide, 750 mM NaCl, 0.1% SDS, 50 mM PIPES, pH 6.9, 0.2% Ficol, 0.2% BSA, 0.2% polyvinylpyrrolidone, and 0.5 mg/ml ssDNA. Sections were then hybridized overnight at 45°C in prehybridization solution containing 15 ng/μl digoxigenin-labeled antisense golli cRNA probe (G19). After hybridization, sections were washed in RNase buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA, 0.5 M NaCl) for 30 min at 37°C, and then digested for 30 min at 37°C in RNase buffer containing 20 μg/ml RNase. Finally, sections were washed in RNase buffer without RNase (30 min, 37°C), 0.5× SSC (1 h, 60°C). The remaining steps were the same as described for immunohistochemistry. Briefly, sections were treated with 3% H2O2 in methanol for 15 min to quench the endogenous peroxidase, and then incubated with the antidigoxigenin Ab conjugated with peroxidase (RT, 1 h). After a series of washes in TNT buffer (0.1 M Tris-HCl, pH 7.5, 0.15% NaCl, and 0.05% Tween-20), sections were incubated in fluorescent tyramide (Green FISH Kit from NEN Life Science Products, Boston, MA) for 5 min at RT. Sections were mounted in PBS-glycerol (1:3, pH 9.5) after complete washes in TNT buffer, and then analyzed by microscopy.

**Results**

**Distribution of golli immunoreactivity in thymic stromal cells**

Because of the role that stromal cells play in thymic selection, we were interested in determining the extent of golli expression in these cells by double immunofluorescence with anti-golli and known markers of stromal cells. Because autofluorescence increases markedly in the thymus with age, we performed the study on BALB/cByJ mouse thymuses at postnatal day 6 (P6).

Fig. 2 shows thymic sections stained for golli immunoreactivity in red and several markers of stromal cells in green. F4/80 was used to mark thymic macrophages (15, 16). Occasionally, some F4/80+ macrophages within the medulla showed strong golli immunoreactivity, an example of which is shown in the inset, upper right of Fig. 2A, although most of the F4/80+ macrophages were golli negative (Fig. 2A). MTS10 and MTS5 were used to identify medullary (Fig. 2B) and cortical (Fig. 2C) epithelial cells, respectively (17); and NLDC-145 was used to mark dendritic cells and some cortical epithelial cells (Fig. 2D) (18–20). None of these dendritic or epithelial cells exhibited detectable levels of golli immunoreactivity.

**Thymocytes express golli proteins**

As Fig. 2, A–D indicates, many small round cells with prominent nuclei showed relatively low golli immunoreactivity. The location and morphology of these cells suggested they were thymocytes. To

**FIGURE 2.** Double-label immunofluorescence studies with golli (red fluorescence) and several stromal cell markers (green fluorescence). A, F4/80 was used to identify macrophages. Most F4/80+ macrophages did not exhibit detectable golli immunofluorescence. However, a subset of macrophages exhibited intense expression of golli, an example of which is shown in the inset in A. B, MTS10 Ab was used to visualize medullary epithelial cells. A section was chosen illustrating the border between the medulla and cortex, as shown. No detectable golli staining was seen in MTS10+ cells. C, Cortical epithelial cells, identified by MTS5 staining, did not express detectable golli proteins. D, No detectable golli immunoreactivity was associated with NLDC-145+ dendritic cells. Magnification in A and B, ×400; magnification in C and D, ×630.
verify this, thymocytes were isolated and immunostained for golli proteins. Interestingly, only cells fixed and permeabilized were stained by the golli Ab (Fig. 3, A and B). Live staining of thymocytes showed no immunoreactivity for golli proteins (Fig. 3, C and D). This suggested that unlike surface markers such as CD4, CD8a, or CD3ε, golli proteins were localized inside the thymocytes, and not on their surface, consistent with their localization in neural cells. Also, the level of intensity of golli staining varied widely among individual, isolated thymocytes. Larger thymocytes with blastocytic features (arrows, Fig. 3A) tended to exhibit more robust golli staining than smaller, apparently more mature, cells.

**Changes in golli expression in developing thymocytes**

Developing thymocytes can be divided into four populations, according to their expression of CD4 and CD8 surface markers. The least differentiated thymocytes are CD4\(^{-}\)CD8\(^{-}\) double-negative (DN) cells. They then transition to a CD4\(^{+}\)CD8\(^{-}\) double-positive (DP) stage and mature into either CD4\(^{+}\) or CD8\(^{+}\) single-positive (SP) populations (21, 22). Colocalization of golli in thymocytes at different developmental stages was performed by triple-label immunofluorescence and confocal microscopy (Fig. 4). The majority of isolated cells were DP thymocytes, staining for both CD4 and CD8 surface markers with varying intensity. Fewer cells in the population were SP or DN thymocytes. Almost all the thymocytes exhibited golli staining, but they seemed to distribute into two populations based upon staining levels, i.e., high expressers (golli\(^{\text{high}}\)) and low expressers (golli\(^{\text{low}}\)). It seemed that the golli\(^{\text{low}}\) cells colocalized principally with DP thymocytes, but golli\(^{\text{high}}\) cells colocalized primarily with SP (not shown in Fig. 4) and DN thymocytes. In DP thymocytes, there also seemed to be a correlation
In the nervous system, golli localization of the nucleus and the cytoplasm, and in certain populations of neurons there occurs a shift in subcellular localization during development and migration. For this reason, we wished to determine the localization of golli proteins in thymocytes. Conventional microcopy clearly showed cytoplasmic labeling and suggested punctate labeling of golli in thymocyte nuclei (e.g., see Fig. 2, C and D), but to confirm this, sections were examined by confocal microscopy. Since ∼98% of thymocytes (including CD4⁺CD8⁻ DP and CD4⁺ CD8⁺ SP) express CD4 molecules on the surface, we used CD4 to mark the location of thymocytes in tissue sections. The optical sectioning images through the tissue clearly demonstrated the presence of golli immunoreactivity in the nuclei of thymocytes (Fig. 6A). In agreement with the cell-sorting data, the thymocyte population in the subcapsular zone (i.e., DN cells) exhibited stronger staining than the DP thymocytes in deeper regions of the cortex. The subcapsular region contains primarily immature DN cells. Immaturity and cell division are indicated by the presence of many mitotic figures and increased cell size of thymocytes in this zone revealed by staining with cresyl violet (arrows, Fig. 6B).

**Thymocytes express golli mRNAs**

Further proof of the expression of the golli-MBP gene in thymocytes was obtained by in situ hybridization of tissue sections using the TSA-FISH technique. A digoxigenin-labeled cRNA probe specific for golli exons 2, 3, and 5a was prepared and used to detect golli mRNAs in situ. Hybridization signal was clearly evident throughout thymocyte populations in the tissue. Again, high intensity of expression was noted in the subcapsular region, presumably arising from DN thymocytes (Fig. 7A–C).

Additional proof of golli expression in thymocytes and the large contribution of thymocyte expression to golli expression in the thymus was demonstrated by RPA experiments. As shown in Fig. 8A, high levels of golli mRNAs could be seen in both thymus and brain at both P6 and P24. Surprisingly, the levels of golli mRNAs were very similar in the two tissues at these ages, indicating proportionately high expression of this myelin protein gene product in the thymus. Low levels of golli mRNA expression were evident in P6 liver, which disappeared by P24, consistent with the decline in hematopoietic activity in this tissue during early postnatal development in the mouse. Another experiment is shown in Fig. 8B. Isolated, pure thymocytes expressed high levels of golli mRNAs, comparable with levels in the intact P6 thymus, and consistent with thymocytes as a major source of golli expression in this tissue. Further evidence of this was obtained by injecting P24 mice with DEX, known to severely deplete the thymus of thymocytes. Twenty hours after injection with DEX, RNA was isolated and a RPA analysis performed and compared with RNA from a PBS-injected control. The results showed a substantial decrease in the levels of golli mRNAs in the thymus, consistent with the loss of thymocytes brought about through the DEX injection (Fig. 8B).

**Discussion**

**Golli expression occurs primarily in mouse thymocytes**

Thymic cells can be divided into two populations, one of which is T lymphoid derived and represents ∼95% of the total cells in the thymus. This population includes T lineage cells at various stages of differentiation, located throughout the entire thymic parenchyma. The other population consists of non-T lymphoid cells, also called stromal cells, which include many epithelial cells that form a network extending from the cortex to the medulla, bone marrow-derived hemopoetic cells including macrophages and dendritic cells, and mesenchymal cells associated with connective tissue coursing throughout the thymus (23).

The results of this study indicate that thymocytes are the primary cell type to express golli in the thymus. Expression of golli mRNAs and proteins in thymocytes was established by several
means, including direct double- and triple-label immunofluorescent analysis on tissue sections and isolated thymocytes using cell-specific markers, FACS analysis, and RNase protection analyses of thymuses from normal and DEX-treated animals. In mice injected with DEX, there is a massive death of thymocytes and an increased proliferation of thymic macrophages. Our results showed a substantial reduction in \textit{golli} mRNA in the DEX-treated thymus, consistent with a primary localization of these mRNAs in thymocytes. There was no increase in \textit{golli} immunoreactivity in thymic macrophages as a consequence of DEX treatment (data not shown).

\textit{Golli} expression does not occur in stromal cells, except for a few macrophages

In contrast with the immunofluorescent results indicating pervasive expression of \textit{golli} in thymocytes, there was essentially no detectable immunofluorescence in stromal cells (i.e., MTS5\textsuperscript{+}, NLDC-145\textsuperscript{+}, and most F4/80\textsuperscript{+} cells), with the sole exception of some F4/80\textsuperscript{+} macrophages. Using TSA-FISH analysis, we were unable to detect significant expression of \textit{golli} mRNAs in thymic macrophages. The presence of \textit{golli} immunoreactivity in these cells could be due to the phagocytosis of apoptotic thymocytes or to instability of \textit{golli} mRNAs in thymic macrophages compared with thymocytes.

\textit{The lack of \textit{golli} expression in thymic stromal cells may explain inefficient central tolerance to MBP autoreactive \textit{T} cells}

It has been established that CD4\textsuperscript{+} Th cells reactive to MBP mediate EAE, an animal model for some aspects of multiple sclerosis (24). The presence of autoreactive T cells specific to MBPs in healthy individuals makes it possible that these autoreactive T cells are normal components of the T cell repertoire, and suggests that in the thymus there must be inefficient negative selection for T cells autoreactive to MBPs (25).

There is substantial evidence, including this study, to indicate that products of MBP gene are expressed in the thymus, but it is not clear why the escape of autoreactive T cells specific for MBP occurs in the thymus. An affinity hypothesis has been put forward based on the fact that the dominance of encephalitogenic epitopes varies among strains depending upon the MHC background of the strains (26). For example, in the SJL(H-2\textsuperscript{s}) strain, the 83–102 sequence is the immunodominant region, while 1–11 is the subdominant region; but in the PL and B10.PL (H-2\textsuperscript{u}) strains, the immunodominant region is aa 1–11. In these instances, inefficient

\textbf{FIGURE 6.} CD4\textsuperscript{+}CD8\textsuperscript{−} DN cells exhibit strong expression of \textit{golli} proteins, particularly within thymocyte nuclei in vivo. \textit{A}. A confocal image through a section of a P6 thymus illustrates the localization of \textit{golli} immunoreactivity (red) in thymocyte nuclei. The section, which is costained for CD4, also illustrates that the DN thymocytes situated in the subcapsular region exhibit stronger staining than CD4\textsuperscript{+} thymocytes located deeper in the cortex. \textit{B}. A neighboring section, stained with cresyl violet, shows many mitotic cells (arrows) of larger size and with visible chromosomes characteristic of dividing, immature cells in the subcapsular region. The double-headed arrows delineate the subcapsular region. Magnification, ×630.

\textbf{FIGURE 7.} TSA-FISH detects expression of the \textit{golli} mRNAs in thymocytes in thymic tissue sections. \textit{A}. In situ hybridization histochemistry with a \textit{golli}-specific cRNA probe (green fluorescence) showing pervasive expression of \textit{golli} mRNAs in thymocytes throughout the cortex and subcapsular region (arrows) of the P6 thymus. \textit{B}, Nomarski image of tissue section shown in \textit{C}, in which the TSA-FISH procedure was conducted without the cRNA probe. Magnification, ×630.
negative selection was attributed to a lower and unstable binding capacity for a certain epitope to a particular MHC II molecule (27).

Previous work on thymic selection indicates that for any self Ag, expression and presentation by different thymic cells will significantly affect the shaping of the autoimmune T cell repertoire (28). In this regard, our findings of the lack of golli immunoreactivity in stromal cells and the presence of this immunoreactivity in thymocytes are significant. MHC II⁺ thymic stromal cells (macrophages, dendritic cells, and cortical epithelial cells) are responsible for the negative selection of CD4⁺ T cells (28–31), whereas MHC I⁻ thymic cells, including thymocytes themselves, are responsible for the negative selection of CD8⁺ T cells (32). Given the lack of significant golli expression in thymic MHC II⁺ stromal cells, it might be expected that CD4⁺ autoreactive T cells specific for MBP gene products are generated as a consequence of inefficient negative selection in the thymus. In contrast, the significant expression of golli proteins in thymocytes can generate efficient negative selection for the CD8⁺ autoreactive T cells specific for MBP gene products. This might explain why CD4⁺ T cells, but not CD8⁺ T cells, are the predominant effector cells in EAE and multiple sclerosis lesions. Our data also imply that central tolerance is caused by the autoreactive T cells against the MBP family of proteins.

Golli expression changes during thymocyte development

Although recent RT-PCR analysis has suggested the presence of RNA products of the MBP gene in purified rat thymocytes, there was no evidence presented for the expression of the gene at the protein level (33, 34). Our findings indicate that the golli products of the MBP gene are expressed in thymocytes at both the mRNA and protein levels, and that the levels of golli expression change depending upon the differentiation state of the thymocyte.

Thymocyte development can be followed using the CD4 and CD8 T cell surface markers through their least mature CD4⁺CD8⁻ (DN) stage to their most mature, CD4⁺ or CD8⁺ (SP) stage. Associated with this maturation is a change in the localization of these cells within the thymus. For example, DN cells are located in high concentrations in the proliferative, subcapsular zone of the thymus, where they appear to be a precursor population with the capacity of dividing. After proliferation, DN cells differentiate into DP cells and move deeper into the cortex, where they are subjected to thymic selection. DP cells are relatively inactive and are pre-programmed to die if they do not undergo positive selection. The majority of DP cells (95%) undergo apoptotic cell death, and only about 5% are rescued from apoptosis by positive selection. These cells differentiate further by selectively down-regulating one of the two coreceptors, CD4 or CD8, and migrate to the medulla, where SP cells are ready to emigrate to the periphery as mature T lymphocytes (21, 22). There are many transitional cells between the DN and DP stages, and the DP and SP stages; CD4lowCD8⁺ cells lie between the DN and DP stages and CD4⁺CD8low cells lie between the CD4⁺CD8⁻ and fully CD4⁺ or CD8⁺ SP stages (35–37).

Higher levels of golli were found in DN cells and some transitional DP cells (i.e., CD4⁺CD8⁻ and CD4⁺CD8low cells), but were 2-fold less in the DP cells. Interestingly, when thymocytes survived thymic selection and developed into SP cells, golli protein expression increased again. This pattern of golli expression that golli products may play a role in events that occur during the transition of thymocytes from DN to DP, and from DP to SP stages of development. The subcellular localization of golli in both cytoplasm and nuclei of thymocytes and other unpublished work from our laboratory suggest that golli products may be associated with a signal transduction pathway in T cells and neural cells.

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References


