Increased Cardiomyocyte Apoptosis and Changes in Proapoptotic and Antiapoptotic Genes \textit{bax} and \textit{bcl-2} During Left Ventricular Adaptations to Chronic Pressure Overload in the Rat

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\textbf{Background}—Left ventricular hypertrophy (LVH) represents both an adaptive response to increased cardiac work load and a precursor state of heart failure. Recent evidence linked cardiac myocyte death by apoptosis with LVH and heart failure. It remained unclear, however, whether apoptosis participated in the transition from LVH to left ventricular dysfunction (LVD).

\textbf{Methods and Results}—Cardiac myocyte apoptotic events and changes in apoptosis-specific genes were studied in a rat model of chronic pressure overload induced by transverse aortic constriction. The changes in left ventricular geometry and function were assessed by echocardiography. Transverse aortic constriction rats progressively developed “concentric” LVH and subsequently, LVD. A similar distribution of LVH and LVD was found 18 weeks after surgery. At this time point, we determined the occurrence of myocyte apoptosis by DNA laddering, in situ DNA TUNEL labeling, and light and electron microscopy. The monitoring of proapoptotic and antiapoptotic genes was determined by Western blot and immunohistochemistry. Our data demonstrated that cardiomyocyte apoptotic events increased from virtually undetectable (in sham-operated controls, SH) to 0.8/10\textsuperscript{3} and 1.5/10\textsuperscript{3} positive nuclei in LVH and LVD, respectively. Fibrosis also increased in the subendocardial and midwall regions of LVH and LVD rats compared with SH. Expression of the proapoptotic gene \textit{bax} increased, whereas that of antiapoptotic gene \textit{bcl-2} decreased in LVH and LVD compared with SH.

\textbf{Conclusions}—These data suggest that in response to chronic pressure overload, cardiomyocyte-specific apoptosis contributed to the transition from LVH to LVD. LVH and LVD were accompanied by a dramatic cardiomyocyte upregulation of the proapoptotic gene \textit{bax} and reduced \textit{bcl-2/bax} ratio, predisposing cardiomyocytes to apoptosis. (\textit{Circulation}. 1999;99:3071-3078.)

\textbf{Key Words:} cells ■ heart failure ■ genes ■ apoptosis ■ hypertrophy

During heart failure (HF), the left ventricle (LV) is unable to pump blood at a rate commensurate with the metabolic requirements of peripheral tissues. An increase in imposed cardiac work load promotes an adaptive response consisting of cardiac hypertrophy. Although this response is initially compensatory, prolonged cardiac overload leads to a decreased global cardiac function and subsequently to HF. The mechanisms underlying the transition from compensatory hypertrophy to cardiac muscle dysfunction have not been completely elucidated yet. Among the several mechanisms involved, a critical role is played by loss of myocardial cells.\textsuperscript{1} Myocardial cell death may be accounted for by 2 independent mechanisms, necrosis and apoptosis (programmed cell death), which differ in several morphological and biochemical features.\textsuperscript{2} Necrosis is characterized by rapid cell swelling, plasma membrane breakdown, and a concomitant inflammatory response. It typically involves contiguous cells and is associated with tissue architectural disruption. In contrast, apoptosis generally affects scattered single cells and is identified by an enzymatic internucleosomal degradation of chromatin in the absence of membrane rupture and...
inflammatory response. Unlike necrosis, apoptosis is a cellular suicide, controlled by specific genetic programs, which can be activated by a wide range of physiological and pathophysiological events. Recent studies report that apoptosis is induced in cardiac myocytes in vivo and in vitro. Apoptosis may be a significant factor in the myocardial hypertrophy induced by pressure overload in rats, in spontaneously hypertensive rats, in the aging rat myocardium, in myocardial ischemia-reperfusion injury, and in acute myocardial ischemia in mice. Apoptosis is described to occur both in the early stage of LV hypertrophy and in overt human HF. However, little is known about the contribution of apoptosis to cardiac myocyte loss during the transition from LV hypertrophy to LV dysfunction after pressure overload.

Methods
The experimental procedures performed in this study followed the guidelines of the American Physiological Society and were approved by the National Animal Care Committee.

Surgical, Echocardiographic, and Hemodynamic Procedures
Male Wistar rats weighing 175 to 200 g (Charles River) were anesthetized with a mixture of 120 mg/kg ketamine and 10 mg/kg xylazine, given by intraperitoneal injection. Transverse aortic constriction (TAC) between the innominate artery and the left carotid artery was performed. Echocardiograms were performed on anesthetized animals (Sonos 100, equipped with a 7.5-MHz transducer, Hewlett-Packard). Two-dimensional short-axis views of the LV were obtained. Whole studies were recorded on half-inch S-VHS videotape (Panasonic AG7350). Freeze-frames were printed on a color printer (UP 5000, Sony) and analyzed with the use of the NIH image program. Anterior and posterior end-diastolic wall thickness (AWT, PWT) and LV diastolic and systolic internal dimensions (EDD, ESD) were measured according to standard procedures as well as calculations of LV mass (LVM), with the standard cube formula

\[
LVM = 1.04((EDD + PWT + AWT) - EDD)^3
\]

where 1.04 is the specific gravity of muscle. LV percent fractional shortening (LVFS) was calculated as LVFS = ([EDD - ESD]/EDD) * 100, where ESD is LV internal systolic dimension. Relative wall thickness (RWT), was calculated as 2 * PWT/EDD.

In our hands, the calculated LVM by echocardiography correlated significantly with postmortem LV weight (\(r = 0.989 \), \(SE = 0.73 \), range = 0.37 to 1.86 g, \(n = 11; P < 0.01 \)). In addition, the intraobserver and interobserver coefficients of variation were 7% and 8%, respectively.

Two days after their final echocardiogram, rats were anesthetized, both right and left carotid arteries were then cannulated with a fluid-filled catheter pericardial effusion 50, catheters were connected to pressure transducers (Statham P23db), and hemodynamic data were recorded on a Gould recorder and then acquired in a computerized system by Gould’s DASA. Analysis of the pressure curves was performed with the use of View II software (Gould Instruments Systems). Heart rate was obtained from the arterial pressure pulse. After arterial blood pressures were recorded, the catheter positioned in the right carotid artery was advanced into the LV under constant pressure monitoring to evaluate the LV systolic and end-diastolic pressures.

LV meridional peak systolic and end-diastolic wall stresses were estimated by use of the formula validated by Litwin et al. Additional information is available on request.

Tissue Histology Sections
Five animals per group were analyzed for histological changes.

TUNEL Assay
LV were embedded in OCT (Miles Laboratories), frozen in liquid nitrogen, and kept at ~80°C until used. The LV was divided into 3 regions: base, middle and apex. Ten serial cryostat sections (4 \( \mu m \)) per region were then analyzed. Labeling of 3'-OH fragmented DNA ends (TdT-mediated dUTP nick end labeling) was performed by an in situ apoptosis detection kit, following manufacturer instructions (In Situ Cell Death Detection, AP; Boehringer Mannheim). Eosin was used as a counterstain. Photography was performed with a Zeiss microscope.

Immunofluorescence
The TUNEL staining was performed with the use of fluorescein-dUTP, omitting the AP-converting step. Sections were then exposed to 1 \( \mu g/mL \) of phallolidin-TRITC label (Sigma) for 20 minutes at room temperature and washed in PBS twice for 3 minutes. Sections were incubated with 10 \( \mumol/L \) of a chromatin dye (Hoechst 33342, Molecular Probes Inc) for 8 minutes to stain nuclei. The slides were analyzed with the use of confocal laser scanning microscopy (Fluoview+IX70, Olympus) under appropriate wavelength.

Tissue Histology and Electron Microscopy
Ultrastructural analysis of morphological changes for evidence of apoptosis and replacement and interstitial fibrosis was performed on fixed specimens. The embedding was performed in epoxy resin (Epon 812) and polymerized at 60°C overnight. Semithin sections (0.5 \( \mu m \)) cut in an Ultracut-E (Reichert-Young) were mounted onto slides and stained with warm methylene blue. Screening and photography were performed with the use of Zeiss microscope.

Ultrathin sections were mounted on nickel grids and counterstained with uranyl acetate for 10 minutes and lead acetate for 1 to 2 minutes. The specimens were analyzed with the use of a JEOL 1220 electron microscope. Necrosis was calculated as area of fibrosis, according to the point-counting method. The staining for the connective tissue was performed with the use of a modified basic trichrome technique on myocardial semithin sections embedded in epoxy resin.

Immunohistochemistry
The expressions of bcl-2, bax, and bcl-xl were determined following manufacturer instructions (Vectastain Quick Kit, Vector). Serial frozen heart sections were used for this staining; 3-amino-ethylcarbazole was used as a colorimetric substrate.

In Vitro DNA Extraction and Labeling
Extraction of DNA from 30 \( \mum \) LV sections of 30 \( \mum \) was performed as described. Five microliters of each DNA and 1 \( \mug \) of 1 kb DNA marker were incubated with 10 \( \muCi \) of (\( \alpha^{32} \))dCTP (Amersham), a mix of cold dNTPs (–dCTP), and 10 U of Klenow polymerase (Gibco) for 15 minutes at 30°C. The reactions were stopped with 10 mmol/L of EDTA. Samples were then run on 1.5% agarose gel, blotted onto a Magna nylon membrane (Micron Separations), and exposed to Kodak X-OMAR x-ray film for 1 to 2 hours at room temperature.

Western Blots
Antibodies against rat bcl-2, bax, and bcl-xl were purchased from Santa Cruz Biotechnology. Anti-rat ANF was from Penylnula Inc and was used at the following dilutions 1:500 ANF; 500 ng/mL for bcl-2, bcl-xl, and bax.

LV was lysed and 30 \( \mug/lane \) of proteins were loaded on gel. Western blots, done in triplicate, were developed with the ECL kit (Amersham). Additional information is available on request.

Statistical Analysis
Results are shown as mean±SEM. We identified 2 main adaptive LV phenotypes on the basis of morphological and functional function and in acute myocardial ischemia in mice.

Apoptosis is induced in cardiac myocytes in vivo and in vitro. Apoptosis may be a significant factor in the myocardial hypertrophy induced by pressure overload in rats, in spontaneously hypertensive rats, in the aging rat myocardium, in myocardial ischemia-reperfusion injury, and in acute myocardial ischemia in mice. Apoptosis is described to occur both in the early stage of LV hypertrophy and in overt human HF. However, little is known about the contribution of apoptosis to cardiac myocyte loss during the transition from LV hypertrophy to LV dysfunction after pressure overload.
parameters. LV hypertrophy (LVH) was defined by an LVM increase of at least 2 SD of that recorded in sham-operated controls (SH), with a preserved LV function (LVFS comparable with that observed in SH). In contrast, LV dysfunction (LVD) was characterized by a deterioration of LV function (LVFS decrease of at least 2 SD of that observed in SH) independently by LVM value. All tissue samples were analyzed in a blind fashion, and the LV condition was unknown before statistical analysis was performed. One-way ANOVA and 2-way ANOVA were performed to estimate the changes of hemodynamic and histological data.

Post hoc simultaneous multiple comparisons were done with Bonferroni analysis.

**Results**

**Hemodynamics**

Two main adaptive phases are detectable during chronic pressure overload in the LV: The first is characterized by concentric LVH; the second is distinguished by LVD. To estimate the percentage of each of these 2 LV phenotypes throughout the study, we assessed the distribution of both LVH and LVD at each time point. There was a net prevalence of LVH (83%) at 12 weeks compared with LVD phenotype, whereas at 18 weeks a similar distribution of both phenotypes was observed (LVH 55% vs LVD 45%). Mostly LVD phenotype was detectable at 24 weeks (>90%).

Eighteen weeks after surgery, arterial blood pressure recorded in the right carotid of LVH and LVD rats was comparable and, as expected, markedly higher than that measured in SH rats (Table). Even LV end-diastolic pressure was markedly increased in LVH and LVD as compared with SH, and a significant difference was detectable between the 2 groups of TAC rats. The analysis of the LV wall stress revealed that LV diastolic wall stress was higher in both groups of TAC rats compared with SH. In contrast, LV systolic wall stress was significantly elevated in LVD compared with both SH and LVH rats.

**Evaluation of Interstitial and Replacement Fibrosis**

The extent of fibrosis was determined in the subendocardium, midwall, and subepicardium of SH, LVH, and LVD animals. Whereas interstitial and replacement fibrosis were virtually absent in SH animals, interstitial fibrosis was present in the subendocardial region in LVH and represented 0.125 of a 0.33 mm² observational area. Replacement fibrosis represented 0.030 of a 0.33 mm² observed area in the same region. In the midwall region of LVH, interstitial fibrosis represented an area of 0.05 of a 0.33 mm², whereas replacement fibrosis was absent. The epicardial region showed no fibrotic process. In LVD, although the epicardial region was spared, the endocardial region was affected by the fibrotic process to 0.033 of 0.33 mm², whereas interstitial fibrosis represented ~20% of the fibrotic process. Interstitial edema also was significant in the subendocardial region in LVD. In LVD as opposed to LVH, areas of recent necrosis, recognized as pale with methylene blue staining, also were present (Figure 1).

In both LVH and LVD, the LV free wall was more affected than the septal region, which was almost completely spared from the fibrotic process.

### In Vivo LV Morphological and Hemodynamic Parameters

Detected 18 Weeks After Surgery in Sham-Operated Rats and in Aortic-Banded Rats With LVH or LVD

<table>
<thead>
<tr>
<th></th>
<th>SH (n=10)</th>
<th>LVH (n=9)</th>
<th>LVD (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, g</td>
<td>530±22</td>
<td>518±18</td>
<td>499±11</td>
</tr>
<tr>
<td>PWT, mm</td>
<td>1.5±0.02</td>
<td>1.9±0.3*</td>
<td>1.4±0.05†</td>
</tr>
<tr>
<td>AWT, mm</td>
<td>5.5±0.1</td>
<td>2.1±0.07*</td>
<td>1.4±0.05†</td>
</tr>
<tr>
<td>EDD, mm</td>
<td>8.1±0.1</td>
<td>7.7±0.2</td>
<td>9.7±0.05†</td>
</tr>
<tr>
<td>ESD, mm</td>
<td>4.3±0.2</td>
<td>3.6±0.2*</td>
<td>6.7±0.2†</td>
</tr>
<tr>
<td>LVM, mg</td>
<td>917±35</td>
<td>1263±65*</td>
<td>1147±56*</td>
</tr>
<tr>
<td>RWT</td>
<td>0.37±0.01</td>
<td>0.61±0.01*</td>
<td>0.27±0.01†</td>
</tr>
<tr>
<td>LVFS, %</td>
<td>47±1</td>
<td>53±1*</td>
<td>31±1†</td>
</tr>
<tr>
<td>E/A</td>
<td>1.3±0.05</td>
<td>1.7±0.2*</td>
<td>2.1±0.1†</td>
</tr>
<tr>
<td>PAaort. carot., mm Hg</td>
<td>134±5</td>
<td>219±13*</td>
<td>207±5*</td>
</tr>
<tr>
<td>PAext. carot., mm Hg</td>
<td>132±2</td>
<td>136±6</td>
<td>131±5</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>102±2</td>
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<td>LVEDP, mm Hg</td>
<td>7±2</td>
<td>11±2*</td>
<td>24±2†</td>
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<td>LVWS, kdyne/cm²</td>
<td>107±7</td>
<td>140±15*</td>
<td>327±5†</td>
</tr>
<tr>
<td>LVDDS, kdyne/cm²</td>
<td>19±6</td>
<td>30±6</td>
<td>70±7†</td>
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<tr>
<td>HR, bpm</td>
<td>285±18</td>
<td>295±20</td>
<td>321±18</td>
</tr>
</tbody>
</table>

BW indicates body weight; PWT, posterior wall thickness (diastole); AWT, anterior wall thickness (diastole); EDD, left ventricular diastolic diameter; ESD, left ventricular systolic diameter; LVM, left ventricular mass; RWT, relative wall thickness; LVFS, left ventricular fractional shortening; E/A, ratio of early to late diastolic filling velocities; PA, systolic arterial blood pressure; LVEDP, left ventricular end-diastolic pressure; LVWS, left ventricular systolic wall stress; LVDDS, left ventricular diastolic wall stress; and HR, heart rate.

All values are mean±SEM.

*P<0.05 vs SH; †P<0.05 vs LVH.

### DNA Laddering

Analysis of genomic DNA obtained by agarose gel demonstrated DNA degradation in both LVH and LVD compared with the control SH group. In addition, there was a marked increase of low-molecular-weight DNA fragments in LVH and LVD compared with SH (Figure 2). The density of the DNA ladder from LVD hearts was higher than that measured in LVH. In addition, the pattern of DNA laddering supports both an apoptotic (internucleosomal) and nonspecific, necrotic-type DNA degradation.

### Evaluation of Cardiac Myocyte Apoptosis

Analysis of the number of cardiomyocyte nuclei with DNA fragmentation detected by TUNEL increased progressively from SH to LVD. In particular, the number of TUNEL-positive nuclei increased in LVH and LVD compared with SH. Cardiac myocyte nuclei can be recognized because they have an elliptical shape on longitudinal sections and are included within myofibers (Figure 3, white arrowheads). We calculated that the number of myocytes undergoing apoptosis increased from approximately undetectable to 0.06±0.01 to 0.11±0.03 nuclei/0.33 mm², respectively (P>0.05). Because there are 72±11 cardiomyocyte/0.33 mm², we calculated that 0.8/10⁶ and 1.5/10⁶ cells scored positive for TUNEL. Nuclear damage in cardiac myocytes undergoing apoptosis was confirmed by electron microscopy analysis, which showed nuclear col-
lapse and the presence of apoptotic bodies within the nucleus in LVH and LVD but not in SH (Figure 4). The number of nuclei of nonmyocytes stained for TUNEL were $0.15 \pm 0.02/0.33 \text{ mm}^2$ in LVH and $0.15 \pm 0.02/0.33 \text{ mm}^2$ in LVD, whereas in SH animals the number of nuclei of nonmyocyte was almost undetectable.

A triple fluorescence staining was also performed in which nuclei were identified by Hoechst and immunofluorescence; the cytoplasm of cardiomyocytes was recognized by typical staining with phalloidin, which reacts with actin fibers (Figure 5). These results confirmed the number of cardiomyocytes considered positive on TUNEL.

Changes in Expression of Proapoptotic Gene \textit{bax} and Antiapoptotic Gene \textit{bcl}-2 in LVH and LVD

\textit{bcl}-2 was the first gene to be identified of a family that includes molecules sharing sequence homology and with proapoptotic and antiapoptotic activity.\textsuperscript{14} \textit{bcl}-2 gene expression in cardiomyocytes has been shown to decrease in physiopathological states associated with increased stretching of myocardial cells in vitro\textsuperscript{15} or in ischemic myocardium in vivo.\textsuperscript{16} Under similar conditions, the expression of \textit{bax} was upregulated.\textsuperscript{15,16} Therefore the ratio between \textit{bcl}-2 and \textit{bax} expression has been proposed as an important marker of myocardial cell survival probability. To determine whether the increase in cardiomyocyte apoptosis rate in LVH and LVD was accompanied by a change of the ratio between \textit{bcl}-2 and \textit{bax}, Western blot and immunohistochemical analyses were conducted. Results show a relative decrease in \textit{bcl}-2 and an increase in \textit{bax} expression in LVH and LVD compared with SH. In contrast, \textit{bcl}-\textit{xl} levels did not change significantly. ANF protein levels, induced in ventriculocytes subject to pressure overload, were upregulated in LVH and LVD, as expected (Figure 6).

Because changes in cardiac expression of the apoptotic genes determined by Western blot from whole tissue cardiac
extracts might not reflect changes in cardiomyocyte specific genes, expression of bcl-2, bax, and bcl-xl was also determined by immunohistochemistry. The results confirmed the data obtained by Western blot, because the number of bax-positive cells was markedly increased in LVH and LVD and peaked in LVD, whereas bcl-2/bax ratio was reduced in LVH and LVD compared with SH (Figure 7). Also, the number of bcl-xl–positive cells was equal in the 3 conditions (not shown). These experiments demonstrated that the bcl-2/bax ratio was decreased in LVH and LVD compared with SH, and bax expression was upregulated in almost all cardiocytes under these conditions. LVD hearts showed maximal levels of bax expression.

Discussion

This model of cardiac pressure overload allows determination of an initial compensatory phase characterized by concentric LVH followed by an enlargement of cardiac chambers associated with a further deterioration of LV function. Twelve weeks after TAC, we detected LVH almost exclusively, whereas at 24 weeks there was a net prevalence of LVD. Similar distribution of the 2 types of LV response to pressure overload was observed 18 weeks after TAC, the time interval selected for histopathological analysis. TUNEL analysis showed a moderate increase of cardiomyocyte apoptosis in LVH. In LVD, the rate of apoptotic events was further increased compared with either LVH or SH. Triple fluo-
cent staining confirmed TUNEL data. In this model of HF, apoptosis is localized in the subendocardial-midwall region in LVH and LVD. In addition, cardiomyocyte apoptotic figures are mainly present in areas in which interstitial and replacement fibrosis are more abundant. In both LVH and LVD, apoptosis was confined within the free wall of the LV, whereas the septal region was almost spared. Our model cannot discriminate between the single contributions of apoptosis and necrosis in determining HF. It does suggest, however, that these 2 phenomena are tightly linked. Moreover, we did not determine the rate of apoptosis throughout the course of the study but at 1 point only (18 weeks). Therefore the contribution of cardiomyocyte apoptosis to the pathogenesis of HF might be underestimated.

Our data share similarities with the pathological analysis conducted on spontaneously hypertensive rats during the LVD phase. LVD was accompanied by increased cardiac apoptosis, which was more frequent in the free wall than in the septal region of the LV and was associated with subendocardial fibrosis. The number of apoptotic cells reported by these investigators is within the range described in the current study. A different myocardial wall stress in our rats could account for the small difference in the number of apoptotic cells between the 2 studies.

Quantitation of the expression of genes involved in the apoptotic pathway might represent a good index of the probability for a cell to undergo apoptosis. The proapoptotic bax gene shares structural similarities with bcl-2 and is thought to inactivate bcl-2 by binding to it. The number of bax-expressing cells in our study dramatically increases in LVH and LVD, whereas a tendency toward a decrease in bcl-2 expression is also revealed. Interestingly, bcl-xl (antiapoptotic) levels did not change. Although the
threshold of bcl-2/bax ratio at which apoptosis is triggered in the cardiac cell is not known, our data, together with others,\textsuperscript{15,16} suggest that a decreased bcl-2/bax ratio increases the probability for a myocardial cell to undergo apoptosis in LVH and LVD. The role of other proapoptotic and antiapoptotic genes needs further investigation.

Recent studies indicate that trophic factors or cytokines might influence the rate of cardiomyocyte survival and could be an important determinant in cardiomyocyte apoptosis. In fact, transgenic mice overexpressing insulin-like growth factor-1 (IGF-1) show a better survival rate after coronary occlusion trials.\textsuperscript{17} The beneficial effects of IGF-1 in enhancing cardiac function during the onset of cardiac failure also was demonstrated in rats.\textsuperscript{18} Other reports demonstrated the hypertrophic\textsuperscript{19} or proapoptotic\textsuperscript{20,21} effect of tumor necrosis factor-\textalpha on cardiomyocytes in rodents.

Possibly, pressure overload activates specific signal transduction pathways in an autocrine-paracrine way, which triggers the apoptotic cascade. In fact, it has been shown that Gq\alpha, a key postreceptor signal transduction molecule activated by AT1, ET1, and \textalpha1-adrenergic receptors in cardiomyocytes, is able to trigger the apoptotic program in cardiomyocytes.\textsuperscript{22} Moreover, cardiac-selective overexpression of Gq\alpha in transgenic mice induces hypertrophy\textsuperscript{23} and HF.\textsuperscript{22} Results of these studies suggest a scenario in which the hypertrophic cell is more prone to apoptosis and that similar signal transduction pathways are involved in cardiomyocyte hypertrophy and apoptosis.

In conclusion, our results support the hypothesis that apoptosis is involved in the transition from compensated LVH to LVD. Whether or not apoptosis is the primary event leading to LVD during chronic pressure overload cannot be answered by our data. Whatever the initial event, our data point out the possibility that apoptosis in LVH may further contribute to the progression toward HF.

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References


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