Characterization of soluble FAS, FAS ligand and tumour necrosis factor-alpha in patients with chronic HCV infection

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Abstract

Background: There are limited reports on the role of the cell surface receptor Fas and its ligand molecule in mediating apoptosis during infection with the hepatitis C virus (HCV).

Objectives: The aims of this study were (1) to assess the susceptibility of the Fas antigen expressed on peripheral blood mononuclear cells to Fas ligand-induced-death in patients with chronic HCV infection and (2) to investigate the correlation between the plasma levels of soluble Fas (sFas), soluble Fas ligand (sFasL), tumour necrosis factor-alpha (TNF-alpha), alanine amino transferase (ALT), and HCV viral load.

Study design: The susceptibility of peripheral blood mononuclear cells from 17 subjects with chronic HCV infection to Fas ligand induced cell death was assessed using a water soluble tetrazolium assay. The plasma levels of associated markers such as sFas, sFasL, and TNF-alpha were quantified using immunoassays. ALT values were obtained from hospital records. Viral loads were quantified using a commercially available quantitative assay—the Amplicor Monitor (version 2.0). Controls for comparison included a group of healthy individuals and individuals infected with the human immunodeficiency virus 1.

Results: The percentage of cell death induced in hepatitis C virus infected individuals was lower than that seen in the healthy control group. Patients infected with HCV had higher average values of sFas and TNF-alpha as compared to both control groups. Plasma levels of sFas in patients with chronic HCV infection showed significant positive correlations to ALT and TNF-alpha levels. TNF-alpha levels also showed a significant positive correlation with ALT levels.

Conclusions: PBMC in HCV infection exhibit decreased susceptibility to Fas ligand induced cell death. This may signify a means by which HCV escapes immune surveillance. This phenomenon merits further investigation. The strong correlations observed between plasma sFas, ALT and TNF-alpha suggest a potential role for these markers as an alternative to an invasive liver biopsy.

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1. Introduction

The immune response to the hepatitis C virus (HCV) has two facets. While an effective immune response is critical for viral clearance (Thimme et al., 2001), the other facet of this immune response may be the major mediator of liver injury. HCV viral persistence or clearance is determined by a complex interplay of host and viral factors. The host immune system responds to HCV infection with HCV specific cytotoxic T lymphocytes (CTLs) that kill hepatocytes by employing Fas ligand and/or perforin based mechanisms (Rehermann, 2000). In mice transgenic for HCV, the expression of HCV proteins has been observed to be associated with an inhibition of Fas-mediated apoptosis (Machida et al., 2001) or by increasing the production of soluble molecules of Fas that can act as decoy receptors for CTL clones bearing Fas ligand
The soluble form of the Fas antigen (sFas) is produced as a result of alternate splicing of the gene coding for the Fas antigen. The amount of sFas is thought to correlate with the level of Fas expression in hepatocytes and the severity of liver inflammation (Hayashi and Mita, 1997). High levels of sFas production may be associated with poor CTL-mediated immune response to the viral antigens by preventing the Fas/Fas ligand interaction (Ohkawa et al., 2001). Soluble isoforms of the Fas ligand (sFasl) are also produced as a result of alternative splicing mechanisms resulting in a loss of the transmembrane domain of the molecule (Cheng et al., 1994) and are implicated in the downregulation of potentially harmful apoptotic activity (Schneider et al., 1998).

A liver biopsy to demonstrate or quantify Fas ligand mediated apoptosis is not routinely feasible due to the risk and discomfort that this procedure entails. Additionally, liver biopsies cannot be performed in subjects with mild or acute disease in whom a liver biopsy is not clinically indicated. Peripheral blood mononuclear cells (PBMC), on the other hand, are easier to obtain. Support for the use of PBMC as a model to study Fas ligand mediated apoptosis comes from the following facts: (1) HCV replication has been demonstrated in PBMC (Azzari et al., 2000) suggesting that PBMC serve as an extra hepatic site for virus persistence; (2) the similarity between the cytotoxic T lymphocyte (CTL) response seen in the liver and that seen in the PBMC (Wong et al., 2001) suggest that the immunological correlates observed with PBMC in HCV infection may well be applicable to that seen in hepatic tissue.

The role of the Fas/Fas ligand and the TNF-alpha system in those with chronic HCV infection has not been studied comprehensively to date especially in Indian patients. Therefore, the study was carried out with the aims of (1) ascertaining if there is any difference in the rates of cell death mediated by Fas ligand between those infected with HCV, or HIV and in healthy volunteers (2) to compare plasma levels of sFas, sFas ligand, and TNF-alpha in patients infected with HCV and to correlate this with ALT and viral load in patients with chronic HCV (CHC) infection.

2. Methods

2.1. Patients and controls

Seventeen patients with CH-C infection were recruited. All seventeen were positive for HCV RNA by the nested PCR reaction. Of these, 16 patients had chronic HCV infection and liver damage demonstrated by either an ultrasound or liver biopsy and a positive HCV antibody (HCV Ab) status. The seventeenth patient had been HCV Ab positive for 6 months prior to recruitment. HCV viral loads were estimated using the HCV Amplicor Monitor (version 2.0) in accordance with the manufacturer’s instructions. The average viral titer of the CH-C group was $9 \times 10^5$ International Units/ml (IU/ml) (range: $1 \times 10^4$ to $3.5 \times 10^6$ IU/ml). HCV antibody (HCV Ab) status was determined by a third generation ELISA (UBI HCV EIA 4.0, United Biochemicals, NY, USA) or the Assym HCV version 3.0 Abbott Diagnostics, IL, USA), and the presence of HCV RNA was determined by an in-house nested PCR using primers for the 5' non-coding region as described previously (Radhakrishnan et al., 2000).

The patients were recruited ensuring (1) that they were not on any antiviral or immunosuppressive therapy and (2) that they did not have any other immunological disorders, as determined by the treating physician.

These 17 patients were negative for hepatitis B surface antigen (HBsAg), and antibody to the human immunodeficiency virus 1 and 2. The clinical profile of these patients is shown in Table 1. All patients were questioned to elicit possible routes of HCV acquisition. Risk factors were known for 10 of the 17 CH-C patients (primarily blood transfusion) whereas for the remaining no particular risk factor could be elicited. The average time between the probable acquisition of infection and time of testing was 11 years (range 1–32 years). Of the 17 patients, 10 (59%) were cirrhotic as diagnosed by a liver biopsy or an ultrasound. Since patients arriving at this tertiary hospital seek treatment only at an advanced stage of disease, there was an overrepresentation of the cirrhotic disease stage in the study group.

2.2. Controls

2.2.1. Disease controls

Nineteen patients infected with human immunodeficiency virus-1 (HIV-1) were recruited. The HIV infected individuals comprised 9 asymptomatic (average CD4 count: 428 ± 189, range 230–770 cells/mm$^3$) and 10 symptomatic (average CD4 count: 221 ± 203, range 30–576 cells/mm$^3$), treatment naive patients.

2.2.2. Healthy controls

Ten healthy laboratory personnel, free of major illness for 3 months prior to testing and negative for HBsAg, HIV-1 and HBV Ab positivity for 6 months were recruited. The average viral load was $10^4$ to $3.5 \times 10^6$ IU/ml. HCV antibody (HCV Ab) status was determined by a third generation ELISA (UBI HCV EIA 4.0, United Biochemicals, NY, USA) or the Assym HCV version 3.0 Abbott Diagnostics, IL, USA), and the presence of HCV RNA was determined by an in-house nested PCR using primers for the 5' non-coding region as described previously (Radhakrishnan et al., 2000).

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Table 1

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age ± S.D. (in years)</td>
<td>47.52 ± 12.46</td>
</tr>
<tr>
<td>Male:female</td>
<td>10:7</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
</tr>
<tr>
<td>Biopsy proven cirrhosis$^a$</td>
<td>10 (58.8%)</td>
</tr>
<tr>
<td>Biopsy proven chronic hepatitis$^a$</td>
<td>2 (11.7%)</td>
</tr>
<tr>
<td>Chronic liver disease on ultrasound$^a$</td>
<td>4 (23.5%)</td>
</tr>
<tr>
<td>HCV Ab positivity for 6 months</td>
<td>1 (5.80%)</td>
</tr>
</tbody>
</table>

$^a$ All patients were positive for HCV RNA in plasma.

Available for 16 individuals only.
2 Ab and HCV Ab were recruited to generate baseline values for the parameters. Blood was drawn from patients and controls after informed verbal consent. In addition, a general consent is also obtained from all patients in this hospital for all investigations as part of routine patient management. Plasma from all subjects was collected and frozen at –60 °C for testing of humoral parameters.

2.3. Methods

2.3.1. Cell death assays

Peripheral blood mononuclear cells (PBMC) from CH-C individuals were used as a model to study the Fas–Fas ligand system in HCV infection. PBMC were obtained from heparinized whole blood by density gradient centrifugation over Ficoll–Hypaque (Amersham BioSciences, Uppsala, Sweden) as per the recommendations of the manufacturer. The required number of PBMC were suspended in RPMI 1640 (Sigma–Aldrich, Missouri, USA) supplemented with 10% fetal calf serum (Gibco, Auckland, NZ). PBMC were set up for the cell death assays in triplicate at a concentration of 1 × 10^5 cells per well. The PBMC were incubated with 1 g/ml of anti-Fas antibody (clone 2R2, IgG3, mouse monoclonal antibody, catalog number: 1 922 432, Roche Biochemicals, Germany). To serve as the control, 1 × 10^5 PBMC were incubated with culture medium. Both test and control PBMC were incubated for 72 h at 37 °C with a 5% CO2 atmosphere in an incubator (NuAireTM IR Autoflo, MN, USA). At the end of the incubation, 50 μl of a XTT labelling mixture (consisting of sodium 3′-(1-phenylaminocarbonyl)-1, 4-tetrazolium)-bis-(4-methoxy-6-nitro) benzene sulfonic acid hydrate) and the electron coupling reagent–PMS (N-methyl dihydrazine methyl sulfate) (Cell Proliferation Kit II (XTT), Roche Molecular Biochemicals, Germany) was added and incubated for a further 4 h under the same assay conditions. The formazan formed at the end of the incubation was spectrophotometrically quantified by reading the absorbance at 492 nm (reference wavelength of 690 nm) using an ELISA plate reader. Tetrazolium is reduced to formazan by mitochondrial dehydrogenases, enzymes that are present only in metabolically active cells. Hence, measurement of the amount of formazan formed by cells is an indirect method for quantifying the degree of cell death. The percentage of cell death induced by anti-Fas was calculated using the formula given below (Taya et al., 2000):

\[
\text{percentage cell death} = \frac{\text{absorbance without anti-Fas antibody}}{\text{absorbance with anti-Fas antibody}} \times 100
\]

The mean of absorbance in each set of triplicate wells was used for calculating the average absorbance. Absorbance values that were greater than or less than the mean ± S.D. value were excluded and the means were recalculated. Not more than one value was excluded per determination.

The Fas sensitive Jurkat cell line (E6-1 clone, human lymphoblastoid T cell line) was used as the positive control for estimating the degree of cell death with the anti Fas antibody under the experimental conditions used in the study. The Jurkat E6-1 cells are known to express Fas antigens on the surface and are susceptible to Fas ligand mediated apoptosis. The Jurkat cell line was procured from the National Centre for Cell Science (Pune, India). It was maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS), at a concentration of 1 × 10^5 cells per flask, as recommended. The viability was measured by the trypan blue exclusion test. The assays for measurement of cell death were carried out only when the viability of the cells was greater than 90%.

The cell death assays were carried out in triplicate, as described above and the percentages were averaged to obtain the mean ± S.D. values.

2.3.2. Quantitation of soluble Fas, sFas ligand and TNF-alpha levels in plasma

Soluble Fas (sFas) levels were quantified using the Human APO-1/Fas kit (Catalog Number: KHS 9501, BioSource International Inc., CA, USA). The quantitation of the sFas levels is achieved by the concomitant assaying of standards ranging in concentration from 0 to 15 ng/ml. Plasma TNF-alpha levels were quantified using the TNF-alpha EASIA/K2 assay (Catalog number: KAC 1752, BioSource International, Belgium). The standards ranged in concentration from 15 to 1500 pg/ml. The levels of sFas Ligand (sFasL) were quantified using the immunoassay kit for sFas ligand (Catalog Number: KHS 9521, BioSource International Inc., CA, USA) by concomitant assaying of seven standards of known sFasL concentrations (0.16, 0.31, 0.63, 1.25, 2.5, 5, and 10 ng/ml) with the samples. All assays were carried out in accordance with the manufacturer’s instructions.

2.3.3. Viral load quantitation

Viral loads in infected patients were estimated using the HCV Amplicor Monitor version 2.0 (Roche Diagnostics, NJ, USA).

2.3.4. Alanine aminotransferase (ALT) levels

The alanine amino transferase levels for the HCV infected patients were obtained from the hospital records.

2.3.5. Statistical analysis

All data (i.e., raw data) was used for calculations. No kind of criteria was employed for data inclusion at any stage. The differences in the percentages of cell death and the average sFas values were compared using the non-parametric Mann–Whitney U or Wilcoxon Rank–Sum test for difference in medians using the NCSS/PASS 2000 Data Edition statistical package. These non-parametric tests were used whenever the data showed non-normal distributions. Differences in the average TNF-alpha values among
the three study groups was analyzed using the analysis of variance (ANOVA) test (Epi-Info version 6.04c statistical package). The correlation coefficients were calculated using the Microsoft Excel 2000 program. The scatter plot with two regression lines was generated using the ‘Statistical Calculator’ program hosted by the Department of Statistics at the University of California at Los Angeles website (http://calculators.stat.ucla.edu/correlation.php).

3. Results

3.1. Cell death assays

The mean ± S.D. percent cell death induced by Fas ligand in the control Jurkat E6-1 cells using the same experimental conditions as that used for the patient and control samples was 50.9 ± 6.5%.

The average cell death induced by the anti-Fas antibody in the PBMC from different groups of patients is shown in Table 2. CH-C patients showed significantly lower cell death as compared to normal healthy controls (3.91% versus 8.37%, P = 0.03, Mann–Whitney U or Wilcoxon Rank–Sum Test). CH-C patients showed a lower percentage of cell death as compared to HIV infected individuals but this difference was not statistically significant (3.91% versus 6.98%, P = 0.819, Mann–Whitney U or Wilcoxon Rank–Sum Test).

3.2. Quantitation of soluble Fas (sFas), soluble Fas ligand (sFas ligand) and tumour necrosis factor-alpha (TNF-alpha)

The mean (±S.D.) sFas titers was 0.0035 ± 0.011 ng/ml in the healthy controls, 2.69 ± 2.86 ng/ml in the CH-C patients and 0.809 ± 1.806 ng/ml in HIV infected patients. The mean cell death induced by the Fas ligand and ALT values (r = 0.480, P = 0.05).

Among the CH-C patients, a correlation at the limit of significance was observed between the percentage cell-death induced by the Fas ligand and ALT values (r = 0.480, P = 0.05). A highly significant correlation was observed between the levels of soluble Fas and ALT values (r = 0.78795, P < 0.000) (Fig. 3) and between TNF-alpha and ALT levels (r = 0.803, P < 0.000) (Fig. 4).
Table 3

<table>
<thead>
<tr>
<th>Study parameter</th>
<th>Normal healthy controls (n = 10)</th>
<th>CH-C group (n = 17)</th>
<th>HIV infected group (n = 19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean plasma TNF-alpha value ± S.D. (in pg/ml)</td>
<td>23.97 ± 4.86</td>
<td>73.11 ± 63.41*</td>
<td>62.76 ± 27.47</td>
</tr>
<tr>
<td>Percentage of values greater than cut off of 35.58 pg/ml</td>
<td>-</td>
<td>78.9</td>
<td>76.4</td>
</tr>
</tbody>
</table>

* Cut off = mean ± 3 S.D. of levels seen in healthy controls.

* The average TNF-alpha value was significantly higher as compared to both control groups (P = 0.01, ANOVA).

4. Discussion

In this study, HIV infected patients were recruited as ‘disease’ controls. HIV infection shows several similarities to HCV infection. Several studies have demonstrated Fas/Fas ligand system mediated apoptosis in the PBMC of patients with HIV (Katsikis et al., 1995; Kobayashi et al., 1990; Ogasawara et al., 1993; Sloand et al., 1997). Additionally, (1) both viruses have the ability to induce chronic persistent infection, (2) both viruses have a remarkable similarity in terms of viral kinetics (Eyster, 1998) and (3) in both viral infections the cell mediated immune response is more effective than the humoral response.

In this study, percentage of cell death in PBMC from patients with CH-C infection was significantly lower than that observed with PBMC from healthy controls but was similar to that seen with the PBMC from the HIV infected group. This would suggest that either (i) the Fas antigen expressed on the surface of PBMC in HCV infected patients is not as susceptible to Fas ligand induced cell death as the Fas antigen on the PBMC from healthy subjects or that (ii) the Fas expression is down-regulated on the surface of peripheral...
the cytoplasmic tail of the lymphotoxin demonstrated that the core protein of HCV interacts with persistent infections. A study by Matsumoto et al., 1997 of modifying the mechanisms of apoptosis to establish site. Earlier studies have indicated that HCV is capable T lymphocytes (CTL) viral response in this extra hepatic evasion Fas ligand mediated apoptosis by activated cytotoxic blood mononuclear cells as a form of survival mechanism to evade Fas ligand mediated apoptosis by activated cytotoxic T lymphocytes (CTL) viral response in this extra hepatic site. Earlier studies have indicated that HCV is capable of modifying the mechanisms of apoptosis to establish persistent infections. A study by Matsumoto et al., 1997 demonstrated that the core protein of HCV interacts with the cytoplasmic tail of the lymphotoxin β receptor (LTβR) (a member of the TNF receptor family). Since the LTβR is known to be a key player in the transduction of apoptotic signals, it may be hypothesized that the HCV core protein may downregulate apoptotic signals through this pathway.

Support for our findings and a possible explanation of the molecular basis for Fas-associated suppression of cell death come from another study conducted in HCV transgenic mice. The study by Machida et al. (2001) in transgenic mice showed that HCV proteins may directly or indirectly inhibit Fas-mediated apoptosis and death by limiting the release of cytochrome c from mitochondria, leading to a suppression of activity of the proapoptotic enzymes-caspase-9 and -3/7. Other explanations proposed for the attenuation of Fas-mediated apoptosis in transgenic mice include the reduction in the amount of Bid (a cytosolic proapoptotic protein belonging to the Bcl-2 family of apoptosis regulators) (Disson et al., 2004). While an investigation into the molecular mechanism responsible for the suppression of Fas associated cell death is beyond the scope of the study, we can speculate that similar mechanisms operate in human subjects also.

Many other viruses escape elimination from the host by producing proteins that change or circumvent the host’s defense system. Viral proteins frequently target members of the tumour necrosis factor superfamily of ligands and receptors as evidenced by the fact that diverse viruses such as the adenovirus, poxvirus and the herpesvirus produce proteins that target ligands and receptors of this superfamily (Tedoro and Branton, 1997). It is interesting to speculate on the therapeutic potential of a drug that would cause Fas up-regulation and hence make the infected cell “more visible” to the immune system.

Lymphoproliferative disorders such as B cell non-Hodgkin’s lymphoma are associated with HCV infection (Zuckerman and Zuckerman, 2000). A possible avenue for further research would be to investigate whether the decreased cell death seen in PBMC from HCV infected patients leads to the expansion, proliferation and immortalization of lymphoid cells. The finding of decreased cell death in PBMC from chronically infected HCV patients is contradicted in the study by Taya et al. (2000). Possible explanations for discrepancy are as follows: (1) firstly, the clinical stage of disease in the recruited patients. In this study all patients were diagnosed with chronic infection and 59% of patients had a histological diagnosis of cirrhosis. The study by Taya et al. (2000) recruited patients with chronic HCV infection but did not indicate how many were cirrhotic. It is possible that the wide variety of cellular changes associated with the development of cirrhosis may have caused decreased surface expression of Fas or altered the pathway by which Fas transduces apoptotic signals to the interior of the cell and (2) secondly, the difference in immune response between the patient groups investigated by Taya et al. (2000) from those reported on in our study.

A HCV specific CTL response has previously been shown to be associated with a lower viral load (Nelson et al., 1997a). In a majority of chronic carriers HCV persists in spite of a detectable CTL response. The CTL response keeps viral replication in check, but is unable to completely eliminate the virus. In this study, viral load showed negative correlations with plasma levels of sFas, tumour necrosis factor and serum levels of ALT though these correlations were not significant. It is possible that liver injury is more a reflection of the host immune response to the virus. The host immune response in an attempt to control viral replication causes concomitant liver injury by the production of proapoptotic molecules and proinflammatory cytokines. However, these findings need to be substantiated with larger sample sizes.

A recent study has shown that CTL lines derived from chronically infected HCV patients lyse a large number of bystander cells expressing Fas on the cell surface (Gremion et al., 2004). This experimental system used was a coculture system that mimicked the cellular composition of the infected liver and this phenomenon of “bystander killing” has been suggested to be an important mechanism by which liver injury may be caused. A similarly modeled study using a PBMC based model may help to (1) explain the interaction between the virus and the immune system at this extra-hepatic interface and (2) clarify the role of the Fas/Fas ligand system in PBMC.

The average sFas value for the CH-C patient group was significantly higher as compared to the HIV infected patients and higher than that seen in normal healthy controls. Increased levels of sFas have been observed in HCV positive patients with liver cirrhosis (Seishima et al., 1997). The authors did not provide an explanation for the increased levels observed in cirrhosis but speculated that it could be due to any of the following reasons: (1) the sFas in serum may simply reflect a sloughing away of Fas from the dying liver cells, (2) if sFas in serum is formed as a result of alternative splicing of the Fas mRNA transcript then the increased levels of sFas in patients with liver cirrhosis could indicate that there is an enhancement of this alternate splicing mechanism in cirrhosis through as yet undescribed mechanisms, (3) the clearance rate of the sFas molecule may be suppressed in cirrhosis. The authors speculated that sFas by itself may be involved in the pathogenesis of liver cirrhosis but did not provide any explanation of how this may happen. Significantly higher levels of sFas have also been observed in patients with hepatocellular carcinoma (HCC) as compared to patients with cirrhosis and healthy controls (Jodo et al., 1998). The results of this study showed that surgical removal of the tumour reduced sFas to undetectable levels leading the authors to suggest that detection of sFas may be used as one of the clinical parameters for HCC. Studies have also shown that the levels of soluble Fas
correlate with the degree of hepatic inflammation (Hayashi and Mita, 1997; Iio et al., 1998). These observations were further substantiated in the current study where sFas levels showed a significant correlation to another marker of hepato-cellular injury, ALT and a marker of inflammation namely, TNF-alpha. Based on these findings it may be speculated as to whether a panel of tests that would include quantification of sFas, TNF-alpha, and ALT inclusive of other markers such as gamma glutamyltransferase and alpha fetoprotein may constitute a noninvasive means of staging disease without resorting to a liver biopsy.

In this study, significantly higher levels of soluble TNF-alpha were seen in CH-C infected patients as compared to healthy controls and HIV infected patients. A majority of patients with CH-C and HIV had shown plasma levels above the calculated cut off of 35 pg/ml indicating that this cytokine plays an important role in infection caused by these two viruses. A study that looked at the levels of TNF-alpha in chronic HCV infection reflected similar findings in that, patients with chronic HCV infection had higher levels of TNF-alpha as compared to healthy controls. It was also observed that levels of TNF-alpha correlated with serum ALT and alpha-glutathione-S-transferase (Nelson et al., 1997b). The presence of elevated levels of this immunomodulatory cytokine in persistent infection suggests that when the immune system is unable to mediate viral clearance it may contribute to hepatocyte injury (Rehermann, 2000).

To conclude the major findings of the study were that: Fas antibody induced cell death in the PBMC of CH-C patients was significantly lower than that seen in healthy controls. It can be speculated that with the establishment of chronic HCV infection a general down regulation of the Fas/Fas ligand system is a means by which the virus escapes immune surveillance and elimination. Plasma levels of sFas and TNF-alpha correlated significantly with ALT levels and further studies are needed to ascertain the implications of this finding in monitoring patients with chronic HCV infection.

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References


