Dynamics of Anti-VAR2CSA Immunoglobulin G Response in a Cohort of Senegalese Pregnant Women

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Background. Pregnancy-associated malaria (PAM) is precipitated by the accumulation of parasites in the placental intervillous spaces and causes maternal anemia and low birth weight. In PAM, placental parasites adhere to chondroitin sulfate A (CSA) through a unique set of variant surface antigens (VSA PAM). Several studies have shown that 1 var gene, var2csa, is transcribed at high levels and expressed in CSA-binding Plasmodium falciparum parasites.

Methods. Plasma levels of anti-VAR2CSA immunoglobulin G (IgG) in Senegalese women were measured during pregnancy by enzyme-linked immunosorbent assay, using 3 recombinant proteins representing 3 domains of the var2csa gene product.

Results. The 3 recombinant proteins were specifically recognized by plasma from pregnant women but not by control plasma. A parity-dependent recognition pattern was observed with 2 of the 3 VAR2CSA antigens. A kinetic study demonstrated that a single Plasmodium falciparum infection was able to trigger a VAR2CSA-specific antibody response. Among women with infected placentas, women with high anti-VAR2CSA IgG levels at enrollment were more likely to present with a past infection than with an acute/chronic infection.

Conclusions. Anti-VAR2CSA IgGs are involved in clinical protection against pregnancy-associated malaria and strengthens the hope for making a VAR2CSA-based vaccine.
The corresponding VAR2CSA protein was shown to be expressed on the surface of infected erythrocytes, and anti-VAR2CSA IgGs were specifically found in pregnant women [13, 14]. In the present study, we measured the anti-VAR2CSA IgG response in a cohort of Senegalese pregnant women and analyzed the acquisition of VAR2CSA-specific IgGs during the course of pregnancy, in relation to clinical and parasitological follow-up.

SUBJECTS, MATERIALS, AND METHODS

Study site, field surveillance, and plasma sample collection. The present study was conducted in Thiadiaye Hospital (Thiadiaye is a town situated 130 km east of Dakar, the political capital of Senegal) and in 2 corresponding health centers in the neighboring villages of Fissel and Sandiara. Malaria there is seasonally transmitted, during the rains from September to December, with an estimate of 10 infected bites/individual/year [15].

Pregnant women were enrolled in a cohort study during an antenatal consultation (ANC) during the period 30 July–15 October 2001. Women <6 months pregnant were enrolled if (1) they were not infected with malaria parasites at the time of inclusion and declared not to have had malaria since being pregnant and (2) they were likely to be exposed to infective mosquito bites during their pregnancy. A total of 306 pregnant women (found to be negative for malaria by immunochromatographic test and thick blood smear) with a mean ± SD age of 24.1 ± 6.1 years were followed for evidence of malaria by active and passive detection through monthly ANC visits and through weekly home visits until delivery. At each ANC visit, a blood sample was obtained from all women, regardless of whether they presented with fever. During home visits, a capillary blood sample was collected from women presenting with fever (axillary temperature >37.5°C). All women presenting with fever and a positive thick blood smear were given curative treatment with chloroquine, the first-line antimalarial drug in Senegal at that time. However, most (72/119) of the malarial infections were symptomless. At delivery, peripheral and placental blood was investigated by microscopy for the presence of malaria parasites. Placental biopsy specimens were also collected and processed. Malarial infections were classified as described elsewhere [16]. Plasma was isolated and stored at −20°C until use. Blood samples from 9 nulligravid women and 8 men living in the same area were also collected.

The ethical committee, Ministry of Health, Senegal, provided ethics approval for this research. All participants gave informed consent.

Flow cytometry. Six *P. falciparum* isolates were collected in November 2003 from infected placentas from women in maternity wards in Guediawaye, a suburb of Dakar, who had type O red blood cells [17]. Antibodies (IgG) to VSAs expressed by these 6 placental isolates were measured in the plasma samples collected at enrollment and delivery by flow cytometry. Samples were coded and subjected to blinded testing and analysis, using a FACSCalibur cytometer (Becton Dickinson) [17]. The level of IgG recognizing VSAs was expressed as median fluorescence intensity (MFI) in a channel of ethidium bromide–labeled infected erythrocytes. For each isolate, the threshold for positivity was defined as 2 SDs above the mean MFI from 30 nulligravid women from Thiadiaye.

Cytoadhesion inhibition assays. CSPG purified from human placental tissue was coated as circular spots onto Falcon Petri dishes (Becton Dickinson), as described elsewhere [17]. Serum samples from 30 pregnant women, selected for their VSA recognition ability, were used in CSPG binding competition assays against the 6 placental parasite isolates. Antiadhesion activity was expressed as the percentage of binding compared with the adhesion control, defined by the mean adhesion occurring with plasma from 6 Senegalese nulligravid women. This binding did not differ significantly from the binding obtained in assays without plasma.

Recombinant antigens. Three domains (DBL1-x, DBL5-x, and DBL6-e) of the synthetic *var2csa* gene previously generated [14] were cloned into the pBAP-TOPO vector (Invitrogen) by polymerase chain reaction. Recombinant proteins from these constructs were produced in baculovirus-infected *S.9* cells and purified as described elsewhere [18].

ELISA. After the optimal coating concentration for each VAR2CSA antigen was determined, plasma levels of specific IgG were measured by ELISA. A recombinant MSP1 (yPfMSP1-19) protein was used for measuring antibodies against a *P. falciparum* antigen not related to PAM. The optical density was converted into arbitrary units (AUs), as described elsewhere [19]. Antibody responders were defined as those having an antibody level (in AUs) >2 SDs above the mean absorbance yielded by 55 negative control plasma samples from unexposed French pregnant women. A pool of these plasma samples was used as a negative control, whereas a pool of plasma samples from multigravid pregnant Senegalese women, previously demonstrated to have high levels of anti-VSA IgGs against placental isolates, was used as a positive control. A group of 20 negative control samples from unexposed French men were also included in the study.

Statistical analysis. Differences between groups were tested by the appropriate nonparametric Mann-Whitney test. The χ² test for unpaired samples (or Fisher’s exact test, when required) was used to test for differences between categorical variables. Correlations (ρ) were assessed by Spearman test. Associations between the level of antibodies and placental infection when controlling for parity were tested using multivariate logistic regression. For paired analysis, absolute differences in antibody levels between time points were calculated for each pair of samples. The variations in antibody levels between time points

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were tested by the Wilcoxon signed rank test for matched pairs. The significance limit was $P<.05$. SAS (version 8.2; SAS Institute) software was used.

RESULTS

Study population. Of the 306 women enrolled, 12 were lost to follow-up, and the delivery serum sample was lacking for 19. Plasma samples were available from 275 pregnant women at delivery, of whom 60 were primigravid, 52 were secundigravid, and 163 were multigravid. Plasma samples were available from 265 of these 275 women at enrollment, making paired analysis possible. Placental histological data were available for 226 women; of these women, 172 were found to be negative for infection, whereas 54 presented with signs of placental infection.

Malarial infection during follow-up and at delivery. During follow-up, 119 women experienced at least 1 positive peripheral parasitemia episode between enrollment and delivery. Of these women, 47 also presented with fever at this time and received a curative regimen of chloroquine (the drug advocated in Senegal at the time of study for both prophylaxis and treatment). At delivery, placental infection was observed in 15% of women by microscopy and in 24% by a combination of microscopy and histologic analysis. The prevalence rate of placental infection was highest in primigravid women (28%), gradually decreased to the 15%–22% range between the second and fourth pregnancies, then reached very low rates (2%) after the fifth pregnancy. Of the 54 women with a placental infection, 25 presented with an acute infection, 14 presented with a chronic infection, and 15 presented with a past infection. A single peripheral thick blood smear was positive during the entire follow-up for 54 of the 119 women; 14 of the 119 also presented at delivery with an active placental infection, and 5 presented with a past infection. Twenty-five other women presented with an active placental infection but either never were found to be infected ($n = 10$) or were found to be positive more than once ($n = 15$) before delivery.

Sero logical reactivity to VAR2CSA domains. The 3 recombinant VAR2CSA domains were specifically recognized by ELISA in plasma samples from pregnant women (figure 1), and the
Table 1. Levels of antibody to 3 VAR2CSA domains (DBL1-x, DBL5-x/H9255, and DBL6-x/H9255) in a cohort of pregnant women from Senegal.

<table>
<thead>
<tr>
<th>Group, period</th>
<th>Antibody level, mean AU (% of subjects)</th>
<th>DBL1-x</th>
<th>DBL5-x</th>
<th>DBL6-x</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected during pregnancy (n = 156)</td>
<td>Enrollment</td>
<td>17.9 (44.0)</td>
<td>26.3 (51.0)</td>
<td>28.1 (60.0)</td>
</tr>
<tr>
<td></td>
<td>Delivery</td>
<td>19.9 (41.0)</td>
<td>20.1 (28.0)^a</td>
<td>27.3 (52.0)</td>
</tr>
<tr>
<td>Infected during pregnancy (n = 119)</td>
<td>Enrollment</td>
<td>23.1 (46.0)</td>
<td>27.2 (47.0)</td>
<td>28.8 (60.0)</td>
</tr>
<tr>
<td></td>
<td>Delivery</td>
<td>27.3 (53.5)^a</td>
<td>38.4 (52.0)^a</td>
<td>38.7 (77.0)^a</td>
</tr>
</tbody>
</table>

**NOTE.** AU, arbitrary units.

^a P< .02, enrollment vs. delivery.

plasma antibody levels were higher in pregnant women than in control groups of European adults not exposed to malaria (P< .0001 for all comparisons). In the individual pregnant women, there was a tight correlation between the antibody levels to the 3 VAR2CSA domains (0.6 < r < 0.8; P< .0001 for all comparisons). The relationship between pregnancy and VAR2CSA expression was demonstrated by the markedly higher levels of anti-DBL5-x and anti-DBL6-x antibodies in pregnant women than in the men and nulligravid women from Senegal (P< .001 for all comparisons). The plasma levels of anti-MSP1 antibodies were higher in the malaria-exposed individuals than in the donors from France, but there were no statistically significant differences in the plasma levels of anti-MSP1 antibodies between the different groups of donors from Senegal (data not shown).

A parity-dependent recognition profile of IgG against 2 of the 3 recombinant domains was seen both at enrollment (DBL5-x, \( \rho = 0.23 \), \( P = .03 \); DBL6-x, \( \rho = 0.20 \), \( P = .03 \)) and at delivery (DBL5-x, \( \rho = 0.27 \), \( P = .03 \); DBL6-x, \( \rho = 0.25 \), \( P = .03 \)) (figure 2). No parity dependency was seen with anti-MSP1 IgG levels. Plasma samples from these pregnant women were also assessed for PAM-specific anti-VSA IgG, using 6 freshly collected placental isolates. The levels of specific anti-VSA IgG, as measured by flow cytometry, correlated with all 3 VAR2CSA-specific IgG levels (0.3 < \( r < 0.7 \); P< .0001 for all comparisons).

To examine the relationship between antibody level and placental infection, women were divided into those who presented with an infected placenta at delivery and those in whom there was no evidence of placental infection. At delivery, levels of antibody to the recombinant VAR2CSA domains were higher in women with infected placentas than in those with uninfected placentas (P< .001 for all comparisons). No such relationship was observed in plasma samples collected early during pregnancy. However, of the women presenting with a placental infection, women with anti-VAR2CSA IgG levels at enrollment that were above the median were more likely to present with a past infection than with an acute/chronic infection (DBL1-x, \( P = .03 \); DBL5-x, \( P = .13 \); DBL6-x, \( P = .03 \) [\( \chi^2 \) test]). Similarly, the mean levels of antibodies at enrollment were lower when parasites were present in the placenta (acute/chronic infection) than when they were absent (past infection) (DBL1-x, \( P = .09 \); DBL5-x, \( P = .07 \); DBL6-x, \( P = .06 \)) (figure 3). This pattern was not observed with anti-MSP1 antibody. No sig-
Anti-VAR2CSA IgG Response

Figure 4. Acquisition and decay of anti-VAR2CSA IgGs after a *Plasmodium falciparum* infection during the course of pregnancy. Day 0 corresponds to the time that peripheral parasitemia was detected. Squares denote DBL1-x, triangles denote DBL5-x, and Xs denote DBL6-x. The first point of each graph corresponds to enrollment, and the last point corresponds to delivery. a, b, and c, Representative examples of how antibody levels changed in time in individual women. In panel a, antibody levels increase sharply at the time of infection and remain high (representative of 16 women). In panel b, antibody levels increase and decrease (representative of 26 women). In panel c, antibody levels are stable (representative of 7 women). AU, arbitrary units.

Significant relationship was found between plasma levels of anti-VAR2CSA IgGs and maternal anemia or birth weight of the offspring either at enrollment or at delivery.

Among the 119 women who experienced at least 1 *P. falciparum* infection during their pregnancy, the levels of anti-VAR2CSA IgGs were higher at delivery than at enrollment (*P* < .001 for all comparisons, Mann-Whitney paired samples rank sum test), even after parity was controlled for (DBL1-x, *P* = .02; DBL5-x, *P* < .0001; DBL6-x, *P* = .004). The levels of antibodies against all 3 VAR2CSA domains were similar at delivery among the 47 women who had received chloroquine treatment and among the 72 women who did not (*P* > .74 for all comparisons). In women who never presented with a positive thick blood smear during follow-up, anti-VAR2CSA IgG levels did not change in a systematic pattern (table 1).

**VAR2CSA-like immunogenic epitopes carried by PAM parasites.** The longitudinal study design made it possible to monitor anti-VAR2CSA IgG levels before and after the detection of parasites in the 49 women in whom 1 peripheral infection was detected during pregnancy. Three general patterns were detected. In some women, the antibody levels increased after detection of the infection and subsequently remained fairly stable (16 women; figure 4a). In other women, there was an increase in levels at the time of infection, followed by a marked decrease later during pregnancy (26 women; figure 4b). The last pattern was found in 7 women (figure 4c), who had rather high anti-VAR2CSA IgG levels before infection, and there was no change in the anti-VAR2CSA IgG levels in connection to the demonstration of parasitemia. It is possible that these women were infected with non-PAM parasites, because none presented with a placent al infection at delivery. In general, antibody levels decreased more often when the infection occurred during the second trimester, but this was probably the consequence of the disappearance of parasites, because decreasing VAR2CSA levels were not observed in women presenting with an active placental infection (table 2). The peak in antibody levels often coincided with the detection of the parasites (figure 4b) but also occurred after this point in time (figure 4a). This delay in the occurrence of the boost was more frequent in primigravid women than in multigravid women, probably indicating a slower development of the primary response to VAR2CSA in these women.

**Relationship between anti-VAR2CSA IgGs and antibodies inhibiting the in vitro adhesion of some placental isolates.** All 6 placental isolates bound to CSA and CSPG and transcribed high levels of the var2csa gene [13, 17]. In plasma samples from 30 pregnant women, selected according to their VSA recognition, the level of anti-VAR2CSA IgGs correlated with those of antibodies inhibiting the adhesion of 2 of these 6 isolates (table 3). The lack of correlation found for the other 4 isolates suggests a possible heterogeneity between placental isolates. Similar associations were observed between cytometry-based data and adhesion inhibition activity of the plasma samples studied [17].

**DISCUSSION**

Plasma antibodies from pregnant women recognize VSA of CSA-selected lines or placental isolates in a parity-dependent manner [17, 20]. These responses were measured toward the entire repertoire of VSA present on the surface of infected erythrocytes. PAM parasites express antigenically distinct VSA, and recent studies have shown that parasites expressing VAR2CSA,
a member of the PfEMP1 family, on the surface of infected erythrocytes have the VSA<sub>pam</sub> phenotype, which is characteristic of placental parasites [11, 13, 14]. Unlike many <var>var</var> genes, the <var>var2csa</var> gene shows a high level of homology in genetically distinct isolates [12, 21]. In the present study, 3 individual domains of the VAR2CSA protein were used to measure specific antibodies present in plasma samples from pregnant women. The recognition profiles of all 3 domains correlated with those of the VSA expressed by 6 placental isolates. The difference in levels of antibody to DBL5-<var>e</var> and DBL6-<var>e</var> between Senegalese pregnant women, on the one hand, and Senegalese nulligravid women and men, on the other, suggests that antibodies against VAR2CSA are acquired during pregnancy; this is in line with previous data regarding parity dependency of PAM-specific and protective antibodies [6, 7]. However, the present study is the first, to our knowledge, to investigate the development of anti-VAR2CSA IgGs over the course of pregnancy, and the results further strengthen the hypothesis that VAR2CSA is specifically expressed by placental parasites. Anti-VAR2CSA IgG responses increased between enrollment and delivery in women who had a <i>P. falciparum</i> infection during their pregnancy, suggesting that these women had been infected with parasites expressing epitopes similar to those expressed on VAR2CSA domains. The decay of anti-VAR2CSA IgG after infection in the subgroup of women with a single peripheral infection during pregnancy and with no active placental infection (table 2) suggests that anti-VAR2CSA IgGs are involved in parasite clearance—a notion supported by the finding that high levels of anti-VAR2CSA IgGs were associated with reduced subsequent risk of active placental infections.

VSA<sub>pam</sub>-specific IgGs seem to mediate protection against PAM [6, 7, 8, 22], and women with chronic placental infection and low VSA<sub>pam</sub> IgG levels are much more likely to be anemic and to have babies with low birth weight than are women with high antibody levels [7]. In a subsequent study of the same women, similar associations were found between the anti–DBL5-<var>e</var> VAR2CSA IgG levels at delivery and birth weight [14]. The lack of association between the levels of anti-VAR2CSA IgGs at enrollment and clinical consequences of PAM in this study is probably due to the limited number of women presenting with chronic placental infection. The lack of protective association with antibody levels at delivery may also be due to epidemiological differences in malaria between study sites, because kinetics of anti-VSA antibodies is likely to differ between areas of stable/intense or low/seasonal transmission. Antibody levels at delivery in our study were mostly markers of an active placental infection, because infections occurring close to the time of delivery were more often associated with an acute infection in the placenta. In these women, especially in women with lower parity who are experiencing primary immune responses, the kinetics of the antibody response may be still in the ascending period. Delivery may, thus, not be the best time point at which to assess the protective role of antibodies, particularly in low-transmission areas. In contrast,

### Table 2. Changes in the levels of anti-VAR2CSA IgGs during pregnancy, measured in 49 Senegalese women in whom a single thick blood smear was found to be positive during follow-up, grouped according to placental histologic assessment at delivery.

<table>
<thead>
<tr>
<th>Group, antibody</th>
<th>Change in antibody level, mean AU (&lt;i&gt;P&lt;/i&gt; value&lt;sup&gt;a&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Between enrollment and antibody peak</td>
</tr>
<tr>
<td>No active placental infection at delivery (n = 40)</td>
<td></td>
</tr>
<tr>
<td>DBL1-&lt;var&gt;x&lt;/var&gt;</td>
<td>11.8 (.005)</td>
</tr>
<tr>
<td>DBL5-&lt;var&gt;e&lt;/var&gt;</td>
<td>25.8 (.001)</td>
</tr>
<tr>
<td>DBL6-&lt;var&gt;e&lt;/var&gt;</td>
<td>12.2 (.004)</td>
</tr>
<tr>
<td>Active placental infection at delivery (n = 9)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>DBL1-&lt;var&gt;x&lt;/var&gt;</td>
<td>13.7 (.04)</td>
</tr>
<tr>
<td>DBL5-&lt;var&gt;e&lt;/var&gt;</td>
<td>27.7 (.03)</td>
</tr>
<tr>
<td>DBL6-&lt;var&gt;e&lt;/var&gt;</td>
<td>15.1 (.02)</td>
</tr>
</tbody>
</table>

**NOTE.** AU, arbitrary units.

<sup>a</sup> Wilcoxon matched pairs signed rank test.

<sup>b</sup> Five women were excluded because of an insufficient no. of plasma samples.

### Table 3. Association between plasma levels of anti-VAR2CSA IgGs and the ability of plasma to inhibit the binding of placental isolates (47, 48, 49, 51, 53 and 55) to chondroitin-sulfate proteoglycans.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>47</th>
<th>48</th>
<th>49</th>
<th>51</th>
<th>53</th>
<th>55</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBL1-&lt;var&gt;x&lt;/var&gt;</td>
<td>0.02</td>
<td>0.32</td>
<td>−0.21</td>
<td>−0.01</td>
<td><strong>0.37</strong></td>
<td>0.2</td>
</tr>
<tr>
<td>DBL5-&lt;var&gt;e&lt;/var&gt;</td>
<td>0.03</td>
<td>0.03</td>
<td>0.02</td>
<td>−0.02</td>
<td><strong>0.51</strong></td>
<td><strong>0.39</strong></td>
</tr>
<tr>
<td>DBL6-&lt;var&gt;e&lt;/var&gt;</td>
<td>−0.07</td>
<td>−0.04</td>
<td>−0.09</td>
<td>−0.01</td>
<td><strong>0.50</strong></td>
<td><strong>0.43</strong></td>
</tr>
</tbody>
</table>

**NOTE.** Data are Spearman correlation coefficients (<i>r</i>). Significant (<i>P</i>&lt; .05) results are shown in bold.
the level of antibodies at the beginning of pregnancy may yield relevant information concerning the protectiveness of the antibodies. Women with high levels of anti-VAR2CSA IgGs at enrollment were likely to better control placental infections than were those with lower levels. Anti-VAR2CSA IgGs can persist for several months, as is shown by the parity dependency of the prevalence rates of such antibodies in women at enrollment (a period free of transmission). The presence of detectable amounts of such antibodies in some primigravid women at enrollment was surprising. It may be hypothesized that, once the placenta appears, it is able to select parasites with the PAM phenotype from the parasite populations normally present at the submicroscopic level in most individuals in areas in which malaria is endemic [23, 24], even during periods of nontransmission. This selection may generate small waves of parasite populations that probably have low fitness but are able to stimulate the immune system, since the measures yielded parity-dependent profiles. Evidence from in vitro adhesion inhibition of some, but not all, placental isolates suggests that anti-VAR2CSA IgGs can interfere with CSA-binding sites of these isolates. The lack of a relationship between anti-VAR2CSA IgGs and antiadhesion antibodies in 4 of 6 of these isolates suggests the existence of heterogeneity among placental isolates. This heterogeneity may involve multiple antigenic VSAs with affinity to CSA or simply a polymorphism within the var2csa gene product. VAR2CSA protein from CSA-adherent parasites possesses multiple CSA-binding domains, and a domain carrying this property in 1 strain may not have the same ability in another strain, as was recently shown for the DBL3-X domain of the A4 and 3D7 strains [25]. This functional heterogeneity among VAR2CSA domains may explain differences and similarities among placental isolates. Since it is likely that isolates can use different VAR2CSA domains for binding CSA, epitopes involved in CSA binding may differ between isolates. In the Gamain et al. [25] study, DBL2-x, DBL3-x, and DBL6-e were shown to be able to bind CSA. In the present study, adhesion-inhibitory antibody levels correlated with those of anti-DBL5-e and anti–DBL6-e antibodies for 2 isolates, suggesting that both domains carry CSA-binding sites. Alternatively, only 1 domain may carry the ligand, but the reactivities of both domains may be linked because of their architectural proximity in the protein. The finding that one particular var gene (var2csa) is highly transcribed by placental isolates [13] and by several CSA-selected strains [11, 12, 26] indicates that the repertoire of PIEMP1 molecules involved in PAM is probably restricted, although isolates may use similar or different domains for interaction with CSA. This is in line with the increasing protection that corresponds to increasing gravidity [27]. Taken together, because clinical consequences of PAM are likely to be attributable to active chronic infection in the placenta [7], high levels of antibodies to VAR2CSA early during pregnancy are likely to contribute to the mechanisms of protection, possibly by limiting parasite adhesion to the placenta or by a cytophilic process clearing the parasites that sequestered in the placenta through other putative mechanisms. An expanded panel of VAR2CSA domain constructs representing different epitopes will allow a detailed study of human antibodies to polymorphic or conserved regions of the VAR2CSA protein, as well as an analysis of their relative importance in protective immunity. Such analyses will be useful for the logical design of a VAR2CSA vaccine that will be suitable for efficient use in different malaria-endemic populations.

Acknowledgments

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References


