Isolation of recombinant fragments of the major outer-membrane protein of *Chlamydia trachomatis*: their potential as subunit vaccines

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Recombinant fragments of the major outer-membrane protein (MOMP) of *Chlamydia trachomatis*, expressed at high levels in *Escherichia coli*, were isolated and purified. Antisera to the recombinant proteins reacted preferentially with overlapping synthetic peptides covering the immunoaccessible variable segments of MOMP. These sera also reacted in a species-specific manner with the surface of intact infectious elementary bodies, and in a *Chlamydia* genus-specific manner in assays using denatured or bound chlamydial antigens. The ability of recombinant MOMP preparations to elicit antibody to the surface of chlamydial elementary bodies raises the possibility that these proteins may be useful for chlamydial vaccine development.

Introduction

Chlamydiae are obligate intracellular bacterial pathogens. In developing countries *Chlamydia trachomatis* serovars A, B and C are a major infectious cause of preventable blindness (trachoma). Worldwide, serovars D to K are an important cause of sexually transmitted disease and associated infertility (Robertson & Ward, 1988). Early trachoma vaccine trials using whole chlamydiae were partially successful in that short-term serovar-specific protection was achieved, but disease of enhanced severity was reported in some individuals reinfected with heterologous serovars (reviewed in Schachter & Dawson, 1978). In the ensuing search for a subunit vaccine which excludes these complications much attention has been focused on the major outer-membrane protein (MOMP) of *C. trachomatis*.

MOMP represents up to 60% of the surface protein of the infectious elementary body (EB) and the intracellularly replicative reticulate body (RB; Caldwell et al., 1981). MOMP carries serovar-, subspecies-, species and genus-specific epitopes (Terho et al., 1982; Batteiger et al., 1986; Conlan et al., 1988). Antibodies reactive with these epitopes form the basis for the serological classification of *C. trachomatis* (Wang & Grayston, 1982). The location of these epitopes has been determined using monoclonal antibodies (mAbs) in conjunction with expressed recombinant DNA (Baehr et al., 1988) or synthetic peptide (Conlan et al., 1988; Stephens et al., 1988) probes. The gene and inferred amino acid sequences of MOMP for all *C. trachomatis* serovars have been either wholly or partially determined (Pickett et al., 1987; Stephens et al., 1987; Baehr et al., 1988; Yuan et al., 1989). The MOMP sequence for *C. psittaci* ewe abortion agent has also been determined (Pickett et al., 1988a). Comparisons of these sequences has provided a molecular rationale for the serological diversity of chlamydiae.

mAbs (Lucero & Kuo, 1985; Zhang et al., 1987) to the surface-exposed serovar- and subspecies-specific epitopes of MOMP neutralize chlamydial infection. Species-specific antibody to MOMP generally fails to neutralize chlamydiae (Zhang et al., 1987) because the corresponding epitope is less well exposed (Kuo & Chi, 1987). However, Collett et al. (1989) recently described a species-specific mAb which reacted with a surface-exposed epitope of MOMP. This raises the possibility that a single component vaccine based on MOMP might be effective against all serovars of *C. trachomatis*.

Using recombinant DNA techniques, Pickett et al. (1988b) achieved high-level expression of recombinant serovar L1 MOMP (rMOMP) fragments in *Escherichia coli*. The present paper describes: (i) the facile purification of these recombinant protein fragments; (ii) the ability of these antigens to generate antibodies which
react at the surface of viable chlamydiae in a broad species-specific manner.

Methods

Organisms. The production of stable recombinants of E. coli strain K12-JM109 expressing approximately 1/4- or 1/2-length fragments of C. trachomatis serovar L1 MOMP as insoluble cytoplasmic inclusions has been described previously (Pickett et al., 1988). C. trachomatis serovar A (strain SA1), serovar B (strain B/Jul20/0T), serovar C (strain UW1), serovar D (strain 1883), serovar F (a recent clinical isolate), serovar G (strain IOL 238), serovar L1 (strain L1/440/LN) and C. psittaci (strain EAE A22/M) were grown and purified as previously described (Sahari & Ward, 1981).

Isolation of rMOMP. Stocks of recombinant bacteria were stored in liquid nitrogen. Thawed stock cultures were revived on Luria agar (16 h at 37 °C) containing ampicillin (50 mg l⁻¹) and IPTG (0·1 mm) as inducer. These cultures were used to inoculate 100 ml volumes of Luria broth (as above but with the agar at 10°C) which were incubated aerobically at 37 °C for 16 h in 500 ml conical flasks on an orbital shaker. Cells were harvested by centrifugation and treated with lysozyme-EDTA (Osborn & Munson, 1974). The resulting spheroplasts were lysed by sonication (MSE Soniprep, 3 × 15 s at maximum amplitude) and the insoluble rMOMP inclusions were harvested by centrifugation (12000 r.p.m. for 5 min, microcentrifuge) and washed twice in 20 mM-Tris/HCl, pH 7·0. Few intact cells remained as determined by interference microscopy. The washed pellet was treated with RNase A and DNAase for 1 h at 37 °C in the presence of MgCl₂ (50 mm), washed again, then solubilized at 30 °C for 30 min in 20 mM-Tris/HCl, pH 7·0 containing 5% (w/v) SDS. β-Mercaptoethanol was added to 5% (v/v), the mixture heated to 100 °C for 5 min, cooled and applied to a Sephadex G50 column (18 × 28 cm). The column was eluted with 50 mM-Tris/HCl, pH 7·0, containing 10 mM-EDTA, 1 mM-phenylmethylsulphonyl fluoride (PMSF), 0·1% (w/v) SDS and 0·05% (w/v) sodium azide at a flow rate of 22 ml h⁻¹ and 5 ml fractions were collected. Fractions were analysed individually by dot immunoblot using a mAb specific for C. trachomatis MOMP. Protein content of the fractions was determined with the Bradford reagent (Bio-Rad). The yield of rMOMP was 10–30 mg (protein) per litre of culture.

Antisera. For antibody production pooled fractions from the Sephadex column were dialysed against distilled water, the resulting pellet reabsorbed in PBS and emulsified with incomplete Freund’s adjuvant (IFA). Alternatively, the pooled fractions were adsorbed directly to alhydrogel without prior dialysis and washed twice in phosphate-buffered saline (Dulbecco A). Half-lap rabbits were bled then immunized at two intramuscular and two subcutaneous sites on days 1, 14, 28, 45 and 63, 114 and sera obtained on day 140. Rabbit antiserum against E. coli strain JM109 was kindly provided by Dr John Heckels of this department. This was produced by immunization with 100 μg (as protein) antigen at multiple subcutaneous sites on days 1, 14, 28, 45 and 80; primary immunization was in incomplete Freund’s adjuvant and all booster injections were in IFA. Antiserum was obtained on day 92. mAbs 6E and 2-3 were produced in Balb/c mice by standard techniques (Terho et al., 1982) using purified C. trachomatis serovar L1 organisms as antigen.

ELISA for antibody. Antiserum were titrated by an indirect ELISA in 96-well PVC microtitre trays (high activated, Flow Laboratories) using a published method (Robertson et al., 1987). For the detection of antibodies against chlamydiae and E. coli, the wells were coated overnight at room temperature with whole organisms (5 μg protein ml⁻¹, 100 μl per well) in 0·05 M-carbonate buffer, pH 9·6, containing 0·01% (w/v) sodium azide. For the detection of antibodies to rMOMP, washed inclusions were solubilized in 8 M-urea and diluted to 1 μg ml⁻¹ in coating buffer immediately prior to use. The test sera were titrated through a threefold dilution series in assays with rMOMP fragments or C. trachomatis and through a twofold series in assays with E. coli. Endpoint titres were defined as the last well to give a positive reaction compared to controls.

Immunoblotting. Immunoblotting of chlamydia was done as previously described (Conlan et al., 1989b). Primary rabbit antisera were used at 1:200 dilution; mAbs were used at 1:1000 dilution and were detected with goat anti-mouse IgG (H+L), alkaline phosphatase conjugate (Bio-Rad, 1:3000). Immunogold-labelling. Surface-exposed epitopes of MOMP on intact chlamydiae were detected by transmission electron microscopy (TEM) using immunogold as previously described (Conlan et al., 1989b). Test antisera were used at dilutions of 1:10 and 1:50; the protein G–gold conjugate was used at 1:10.

Epitope mapping. Solid-phase peptides for epitope mapping were synthesized according to a published method (Greyson et al., 1984) using a commercial kit (Cambridge Research Biochemicals). Synthesis was done according to manufacturer’s instructions and as previously described (Conlan et al., 1988). A series of duplicate decapptides spanning the entire serovar L1 MOMP sequence (Pickett et al., 1987) was synthesized. Each peptide overlapped the preceding decapptide by five amino acids.

Results

Analysis of rMOMP inclusions

Cytosplasmically located, insoluble rMOMP inclusions were isolated, solubilized and analysed by SDS-PAGE (Fig. 1). Major proteins with apparent molecular masses of 13·5 and 32·7, 28·2 and 13·2 kDa were obtained from the E. coli recombinants expressing respectively 1/2-, 3/4- or 3/2-length fragments of serovar L1 MOMP (molecular mass approx. 40 kDa). All samples contained numerous faint minor bands, several of which reacted on Western blots with a mAb specific for C. trachomatis MOMP (Fig. 2). These were not removed by gel filtration on Sephadex G50. This mAb did not react with the 13·5 kDa band of the rMOMP preparation nor with normal E. coli (not shown). The identity of this 13·5 kDa band, which was not present in the other recombinant samples, is unknown.

Production of antisera against rMOMP fragments

Immunization with recombinant proteins in both alhydrogel and Freund’s adjuvants produced high-titre antibodies reactive with all MOMP fragments on ELISA (Table 1). All of the antisera reacted in a genus-specific manner when whole chlamydiae were used as ELISA antigen. Pre-immune sera were negative for both assays. Antiserum raised against E. coli strain JM109 reacted with all rMOMP preparations, presumably due to the
Recombinant MOMP of C. trachomatis

Fig. 1. SDS-PAGE analysis of rMOMP fragments. Lanes: 1, molecular mass markers; 2, E. coli expressing rMOMP; 3, isolated rMOMP inclusions; 4, rMOMP from Sephadex G50; 5, blank; 6, E. coli expressing rMOMP; 7, rMOMP inclusions; 8, rMOMP from Sephadex G50; 9, blank; 10, E. coli expressing rMOMP; 11, rMOMP inclusions.

Fig. 2. Western blot of rMOMP fragments. Lanes: 1, rMOMP purified inclusions; 2, rMOMP purified inclusions; 3, rMOMP purified inclusions. Lanes 1–3 probed with mAb reactive with a species-specific epitope on C. trachomatis MOMP.

reactivity of these sera with different antigen preparations were noted. In particular, the lower reactivity of antisera 2 and 4 with rMOMP fragments was not matched by an equal decrease in reactivity to whole C. trachomatis antigens. Whether this is a reproducible phenomenon reflecting differences in antigen presentation or is a result of variability in the immune response of individual animals is unknown.

Reactivity of antibodies to rMOMP with chlamydiae

The reactivity of antisera to rMOMP with reduced and denatured antigens of chlamydiae on Western blots was exemplified by As1 (Fig. 3, lanes 1–4). This antiserum reacted with multiple bands from C. trachomatis serovars L1, B (B-complex), C (C-complex) but with far fewer bands for C. psittaci; the positions of the 'native' MOMP bands are indicated. One explanation for this might be the presence of cross-reactive epitopes on multiple chlamydial antigens. All antisera reacted in an identical manner. The most significant difference between the antisera was a marked decrease in the staining intensity of C. psittaci bands with antisera raised using alhydrogel as adjuvant. mAbs which were specific for MOMP reacted only with a single band on Western blot (Fig. 3, lane 5). The position of chlamydial LPS was determined using a Chlamydia genus-specific mAb (lane 6). All of the sera also reacted with E. coli strain JM109 on Western blots (Fig. 3, lanes 8 and 9). As1, 2 and 5 gave similar banding patterns with E. coli whilst the different blotting pattern of As6 was similar to that obtained with As3 and presence of E. coli contaminants, since the same serum did not react with whole chlamydial antigens. Likewise rMOMP antisera reacted with whole E. coli antigens. This reactivity was abrogated by adsorption of the rMOMP antisera with whole E. coli cells. This procedure did not affect the titre of the rMOMP antisera with whole C. trachomatis antigens. Apparent variations in...
Table 1. Reactivity of antisera (ELISA titre) with rMOMP fragments and whole chlamydiae

<table>
<thead>
<tr>
<th>Antiserum*</th>
<th>Recombinant fragments</th>
<th>C. trachomatis serovars:</th>
<th>C. psittaci (EAE)</th>
<th>E. coli (JM109)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>½-MOMP</td>
<td>½-MOMP</td>
<td>½-MOMP</td>
<td>L1</td>
</tr>
<tr>
<td>As1</td>
<td>437000</td>
<td>437000</td>
<td>437000</td>
<td>24000</td>
</tr>
<tr>
<td>As2</td>
<td>16000</td>
<td>16000</td>
<td>16000</td>
<td>2700</td>
</tr>
<tr>
<td>As3</td>
<td>437000</td>
<td>437000</td>
<td>437000</td>
<td>24000</td>
</tr>
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<tr>
<td>E. coli antiserum</td>
<td>1800</td>
<td>5400</td>
<td>1800</td>
<td>&lt;100</td>
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</table>

* As1, 3 and 5 were raised to ½, ½ and ½-rMOMP respectively using Freund's adjuvant. As2, 4 and 6 were raised to ½, ½ and ½-rMOMP respectively using alhydrogel as adjuvant.

Surface accessibility of MOMP epitopes

To avoid the possibility that binding to the solid phase of ELISA trays might alter chlamydial topography, the surface accessibility of MOMP epitopes was determined by immunogold-labelling of intact chlamydiae in suspension prior to TEM. The purified chlamydial preparations used were a mixture of EB with intermediate bodies and some RB of varying size. Between 15 and 30 organisms of each morphology were counted for each antiserum and chlamydial serovar examined. In general, the test sera reacted significantly (P < 0.05, t-test of sample means) with EB and RB of all C. trachomatis serovars examined when compared to pre-immune sera or to the antiserum produced against E. coli (Table 2). In all cases RB showed a greater degree of labelling than did EB. This may be a reflection of the increased surface area of the RB compared to EB, and developmental differences in the surface exposure of common epitopes. None of the test sera reacted with EB or RB of C. psittaci.

Epitope mapping

The molecular specificity of recombinant antisera was examined against a series of solid-phase peptides spanning the entire serovar L1 MOMP sequence (Fig. 4). None of the test sera reacted with peptides corresponding to the N-terminal ½ of L1 MOMP which is not present in the recombinant antigens (not shown). Antisera raised using Freund's adjuvant showed a wider spectrum of reactivity against peptides than did sera which employed alhydrogel as adjuvant. Pre-immune sera and antiserum to E. coli were negative in these assays. All antisera, with the exception of As5, reacted only with peptides which occur naturally in the respective immunizing MOMP fragments. We speculate that
mimics (mimotopes) of natural epitopes present elsewhere in the protein and recognized by low stringency antibodies. Antibodies reactive with peptides spanning the variable loop domains of MOMP were predominant.

Discussion

The immunopathological side effects of immunization with whole-cell chlamydial preparations has emphasized the need for a subunit vaccine. One difficulty with this approach, is the large-scale production of distinct, purified antigens from this fastidious intracellular bacterium. For MOMP, one approach has been the identification of continuous B cell epitopes using either lgt11-expressing fragments or synthetic peptides in conjunction with mAbs of known specificity (Baehr et al., 1988; Conlan et al., 1988; Stephens et al., 1988). This rationale may lead to a synthetic peptide vaccine. Indeed, antibodies raised to synthetic peptide homologues of MOMP, recognize the native structure on EB (Conlan et al., 1989b). However, the presentation of suitably adjuvanted synthetic peptides to the immune system and the probable requirements for additional T cell epitopes as well as carrier molecules remains problematic, especially as regards human usage (Shinnick et al., 1983). In these respects, larger recombinant proteins may be particularly useful in providing their own T-helper epitopes, thus making them inherently more immunogenic.

The ideal chlamydial vaccine should protect against infection with a wide spectrum of serovars. To date, neutralization studies have shown that antibodies reactive with serovar- and subspecies-specific epitopes exposed at the surface of the EB prevented chlamydial infectivity in vivo (Zhang et al., 1987) and in vitro (Caldwell & Perry, 1982; Lucero & Kuo, 1985). By contrast, antibodies directed against a hidden species-specific epitope did not abrogate infectivity (Zhang et al., 1987). Whether protection is a result simply of antibody binding to the MOMP surface is not known. The finding that antibody to the surface-accessible, genus-specific LPS failed to neutralize chlamydial infectivity in vivo (Zhang et al., 1987) might suggest that binding alone is insufficient for effective neutralization. In in vitro studies, MOMP-specific neutralizing antibodies did not prevent the attachment and uptake of EB, but did halt their differentiation to replicating RB (Caldwell & Perry, 1982). This differentiation has been shown to be dependent upon relaxation of the sulphydryl cross-linkages in EB MOMP (Bavoil et al., 1984; Hatch et al., 1984). Neutralizing antibodies might also function by cross-linking the MOMP porin structure, thus preventing differentiation events. If this is so then antibodies solely directed to linear epitopes may not be protective. Although type-, subspecies- and species-specific MOMP domains consisting of short segments of

Table 2. Labelling of intact chlamydiae with rMOMP antiseras by immunogold TEM

<table>
<thead>
<tr>
<th>Organism</th>
<th>Pre-bleed*</th>
<th>As1</th>
<th>As2</th>
<th>As3</th>
<th>As4</th>
<th>As5</th>
<th>As6</th>
<th>anti-JM109</th>
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<td>Serovar A EB</td>
<td>0-19</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>11-9</td>
<td>8-2</td>
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<tr>
<td>Serovar B EB</td>
<td>0-69</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>43-2</td>
<td>24-7</td>
<td>ND</td>
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<tr>
<td>Serovar C EB</td>
<td>0-69</td>
<td>14-9</td>
<td>1-4</td>
<td>14-1</td>
<td>11-7</td>
<td>6-7</td>
<td>11-7</td>
<td>0-04</td>
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<tr>
<td>Serovar D EB</td>
<td>0-60</td>
<td>40-6</td>
<td>35-2</td>
<td>38-3</td>
<td>58-7</td>
<td>32-0</td>
<td>41-5</td>
<td>4-7</td>
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<tr>
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<td>5-9</td>
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<td>5-4</td>
<td>3-7</td>
<td>10-4</td>
<td>0-73</td>
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<td>Serovar F EB</td>
<td>0-31</td>
<td>24-6</td>
<td>23-0</td>
<td>ND</td>
<td>26-9</td>
<td>20-1</td>
<td>34-7</td>
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<td>ND</td>
<td>ND</td>
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<td>10-1</td>
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<td>ND</td>
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ND, Not determined.

* Pre-immune serum from rabbit As6. Essentially similar results were obtained for each serovar on at least two separate occasions.
continuous amino acid sequence have been identified, we have recently obtained evidence that more than one of these loop regions may be spanned by a single mAb paratope (Conlan et al., 1990). Thus, it is likely that these linear antigenic segments interact to form the paratope contact regions of a discontinuous epitope.

The rMOMP fragments used in the present study were expressed as insoluble inclusions located in the cyto-
plasm (Pickett et al., 1988b). This enabled substantial purification by the simple expedient of spheroplasting followed by lysis and centrifugation. Although immunochimical assays demonstrated the presence of contaminating E. coli antigens in the recombinant preparations, these appear to be minor components, possibly associated with host-cell ribosomes occluded within the rMOMP inclusions. Control experiments showed that antibodies raised against contaminating antigenic material were not responsible for the specific reactivity with chlamydial antigens to rMOMP. The multiple bands observed on Western-blotting the rMOMP preparations against a MOMP-specific mAb indicated the presence of MOMP aggregates and processing fragments. These may also occur in chlamydial organisms, which might explain the multiple bands of C. trachomatis antigens reactive with high titre antisera to the rMOMP.

Immunization with all three rMOMP fragments produced Chlamydia genus-reactive antibodies by ELISA and Western blotting. However, in immunogold assays, where intact chlamydiae were labelled in suspension, only C. trachomatis species-reactive antibodies were detected. This emphasizes the importance of using native bacteria whose topography is unaltered by binding to a solid phase when searching for immunoreactive surface epitopes. Whether those antibodies which reacted at the EB surface also possessed useful biological functions remains to be determined. It was encouraging, however, that even immunization with the smallest MOMP fragment produced antibodies to known neutralizing epitopes in VS IV, as this recombinant appeared to be the easiest to purify as assessed by SDS-PAGE and Western blots. Moreover, adsorption of recombinant fragments to hydrogel, a human-approved adjuvant, evoked antibodies to exposed epitopes of MOMP.

Epitope mapping studies with solid-phase peptides were employed in an attempt to explain the serological specificity of recombinant MOMP antisera in molecular terms. The antisera reacted almost exclusively with peptides spanning the variable segments of MOMP, as is the case for neutralizing MOMP mAbs produced by immunization with whole chlamydiae (Zhang et al., 1987). This was surprising given the harsh denaturing conditions to which the MOMP fragments were subjected prior to immunization, but it offers the possibility that rMOMP might be useful for vaccine development. It is possible that MOMP fragments refolded into native conformation following isolation and solubilization. Alternatively, the variable segments may contain epitopes which are immunodominant because they occur in mobile loop regions or because of the functional juxtaposition of strong T-helper epitopes. An examination of the peptides reacting with MOMP antisera did not provide a ready explanation for the serological reactivity observed in immunochimical assays with whole or denatured organisms. Thus, none of the antisera bound to peptides containing genus-specific sequences, despite their reactivity with denatured C. psittaci antigens on Western blot and in ELISA. All of the antisera reacted with peptides from VS IV, even though the species-specific peptide LNPTIAG located in this region is only poorly exposed, if at all, on the EB surface of most serovars (Conlan et al., 1989a; Kuo & Chi, 1987; Zhang et al., 1987). Hence, this reactivity is unlikely to account for the observed species-specific labelling of intact chlamydiae. However, VS IV appears to be immunologically complex, as two surface-exposed, sub-specific epitopes flank each side of the species-specific region (Conlan et al., 1988, 1989b).

MOMP is currently the main candidate for the development of a chlamydial vaccine. Monospecific polyclonal antibodies to MOMP, with their high binding affinity, may also be useful for the development of diagnostic tests for chlamydial antigen. The observation reported here that rMOMP preparations in conjunction with human-acceptable alhydrogel adjuvant generate high-titre antibodies, of similar epitope-specificity to mAbs produced by immunization with whole chlamydiae (Conlan et al., 1988), is therefore of major interest. Collaborative studies in progress also indicate that these same rMOMP preparations can be presented, via dendritic cells, to stimulate primary and secondary T cell responses (Dr A. Stagg, personal communication). The ability of rMOMP fragments to stimulate protective immunity in vivo is currently being investigated using a mouse model of human C. trachomatis infection.

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References


