Detection of Antiviral Antibodies with Predetermined Specificity Using Synthetic Peptide–β-Lactamase Conjugates: Application to Antibodies Specific for the preS Region of the Hepatitis B Virus Envelope Proteins

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SUMMARY

Amino acid sequences coded for by the preS region of the hepatitis B virus (HBV) envelope gene are present both in HBV and in subviral hepatitis B surface antigen (HBsAg) particles. Consequently, anti-preS-specific antibodies are elicited during the course of HBV infection. Such antibodies are virus-neutralizing. Therefore, it is important to determine whether or not vaccination with HBsAg also induces an anti-preS-specific immune response. We describe here an enzyme-linked immunosorbent assay applicable for the screening of sera from vaccinated individuals for anti-preS antibodies. IgG from serum specimens was adsorbed to staphylococcal Protein A on a superparamagnetic support and subsequently mixed with a synthetic peptide analogue [preS(120-145)] covalently linked to β-lactamase. The presence of anti-preS in serum specimens resulted in binding of the conjugated β-lactamase to the magnetic support. The adsorbed enzyme was quantified colorimetrically.

INTRODUCTION

The major protein component of the hepatitis B virus (HBV) envelope (env) and of subviral hepatitis B surface antigen particles (HBsAg) is a relatively hydrophobic, cysteine-rich sequence of 226 amino acids having a molecular weight of $25 \times 10^3$. The antigenicity and immunogenicity of this protein (designated S-protein) depends on the maintenance of disulphide bonds (Vyas et al., 1972; Sukeno et al., 1972; Dreesman et al., 1973). The open reading frame on HBV DNA coding for S protein (Charnay et al., 1979; Peterson et al., 1977) has the capacity to code for a protein consisting of 389 to 400 amino acids (depending on the antigenic subtype of HBV). The DNA sequence corresponding to this reading frame and preceding the gene for S-protein (i.e. S-gene) has been designated the preS region (Tiollais et al., 1981). Proteins larger than the S-protein [in its non-glycosylated ($25 \times 10^3$) or glycosylated ($29 \times 10^3$) form] have indeed been conclusively identified in the HBV envelope and in HBsAg. Antigenic determinants specific for these proteins, but absent on S-protein, have been clearly discerned (Machida et al., 1984; Neurath et al., 1984a; Heermann et al., 1984). Work with synthetic peptide analogues and with recombinant DNA containing portions of the preS region nucleotide sequence have established that the protein moieties of HBV (HBsAg) components of mol. wt. over $29 \times 10^3$ correspond to the following sequences: (i) the 'middle' protein (Tiollais et al., 1985) representing S-protein with 55 additional amino acids at the N terminus and coded for

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by the preS region [amino acid residues preS(120–174)] and (ii) the ‘large’ protein (Tiollais et al., 1985) consisting of the ‘middle’ protein with an additional 108 to 119 N-terminal amino acids [depending on the antigenic subtype; amino acid residues preS(1–119) or preS(12–119) (Neurath & Kent, 1985)], and containing all amino acids encoded by the HBV env gene (preS and S regions) (Neurath et al., 1984a, 1985a; Okamoto et al., 1985; Michel et al., 1984; Wong et al., 1985; Offensperger et al., 1985; Persing et al., 1985).

The preS sequences of the middle and large HBV env protein have the following properties distinct from S-protein: (i) a high proportion of charged amino acid residues and high hydrophilicity; (ii) an absence of cysteine residues; (iii) the highest HBV subtype-dependent amino acid sequence variability among HBV DNA gene products and minimal homology with preS sequences corresponding to non-human hepadnaviruses (Neurath et al., 1985b). These properties indicate that the preS sequences: (i) are exposed on the surface of HBV (HBsAg), (ii) may determine the host range of HBV, in accordance with their involvement in attachment of the virus to hepatocytes (Neurath et al., 1985a), (iii) have a disulphide bond-independent antigenicity and immunogenicity confirmed experimentally (Neurath et al., 1984a), and (iv) are recognized by the host's immune system (Neurath et al., 1984a, 1985a). Antigenic determinants located on preS sequences are more immunogenic than S-protein determinants located on the same HBsAg particle (Milich et al., 1985). The presence of preS sequences enhances the immune response to S-protein and circumvents the immunological non-responsiveness to S-protein in non-responder mouse strains (Neurath et al., 1985d; Coursaget et al., 1985; Milich et al., 1985). Antibodies with anti-preS specificity are virus-neutralizing (Neurath et al., 1985e; Kent & Neurath, 1985). These findings indicate that an immune response to preS sequences may play an important role in protection against HBV infection. Therefore, it is important to determine whether or not humans vaccinated against hepatitis B develop antibodies to preS-specific determinants. However, sufficiently sensitive assays for such antibodies have not been available. This report describes an enzyme-linked immunosorbent assay (ELISA) for anti-preS antibodies utilizing the synthetic peptide preS(120–145) conjugated with β-lactamase.

**METHODS**

*Sera from persons vaccinated with hepatitis B vaccines.* Sera mostly preselected on the basis of a high level of antibodies to the HBV S-protein (anti-HBs) were obtained from individuals immunized with one of the following hepatitis B vaccines: Merck, Sharp & Dohme Hepatavax-B (MSD) (Hilleman et al., 1982), Pasteur Hevac-B (Pasteur) (Guesry et al., 1982) and The Central Laboratory of the Netherlands Red Cross Blood Transfusion Service HB-vaccine (CLB) (Reerink-Brongers et al., 1982).

Additional sera were used in the course of the development of the ELISA test: rabbit antisera to HBV from which antibodies to the S-protein had been removed (Neurath et al., 1976, 1985a); rabbit antisera to the synthetic peptides preS(120–145) and preS(12–32) (Neurath et al., 1984a, 1985a) and pooled serum from individuals vaccinated with an experimental vaccine (McAuliffe et al., 1982). The latter serum pool contained sera of individuals positive for antibodies with anti-preS specificity as determined by double antibody radioimmunoassays (RIA; Neurath et al., 1985a).

For comparison, sera from individuals who were infected with HBV or acquired anti-HBs as a result of HBV infection were also tested for anti-preS-specific antibodies.

Human sera were tested for anti-HBs by RIA (AUSAB test; Abbott Laboratories, North Chicago, Ill., U.S.A.) and the results were expressed in international milliunits (mIU)/ml calculated from a calibration curve relating c.p.m. to serial dilutions of an international anti-HBs standard.

**Preparation of synthetic peptide-β-lactamase conjugates.** Procedures for the conjugation of antibodies with β-lactamase (Yolken et al., 1984; Neurath et al., 1985c) were followed. One mg of either synthetic peptide preS(120–145) or preS(12–32) (Neurath et al., 1984a, 1985a) in 250 μl 0.1 M-phosphate buffer pH 6.8 was each mixed with 2 mg (250 μl) β-lactamase (Type I, Sigma) pre-dialysed against the same buffer. Fifty μl of 0.2% glutaraldehyde was added to the mixture in 5 μl aliquots. After incubation for 2 h at room temperature, the conjugate was dialysed at 4°C against phosphate-buffered saline pH 7.4, changed three times. Ethanolamine (0.05 M) was added to the conjugate after the first dialysis buffer change.

**ELISA tests.** Ten μl aliquots of serum samples were mixed with 400 μl 0.14 M-NaCl, 0.01 M-Tris–HCl, 0.02% NaN₃, pH 7.2 (TS) containing 10 mg/ml bovine serum albumin (TBS). To determine the dilution endpoints of various sera, serial dilutions (400 μl) were made in TSB containing 0.5% (v/v) normal rabbit serum. Twenty μl of
magnetic Protein A (Biomag M4600; Advanced Magnetics Inc., Cambridge, Ma., U.S.A.) was added to the diluted specimens. After 30 min at room temperature, the magnetic Protein A with immunoglobulins adsorbed from the serum specimens was separated in a magnetic field using the M4700 magnetic separator (Advanced Magnetics). Excess fluids were aspirated from the magnetic particles which were subsequently washed twice with TSB. The synthetic peptide β-lactamase conjugate (400 μl; diluted 2.5 × 10^4-fold in TSB) was added to the magnetic particles and the mixtures were incubated 30 min each at 37 °C and 20 °C. The particles were separated in a magnetic field and washed twice with TS and once with the substrate solution (1 mg/ml soluble starch, 15 μg/ml penicillin G, 30 μg/ml I2, and 0.8 mg/ml KI in 0.14 M-NaCl, 0.05 M-phosphate buffer pH 7.2). Finally, 400 μl of the substrate solution was added and 90 min later, the absorbance (A570) of the samples was read using the MR600 Microplate Reader (Dynatech Laboratories, Alexandria, Va., U.S.A.) after transfer into wells of 96-well plates. The specimens were considered positive for antibodies if the corresponding absorbance was < 1/2 of that controls [1/5000 and 1/1000 diluted rabbit antiserum to the peptides preS(120–145) and preS(12–32), respectively].

RESULTS

Establishment of conditions for the ELISA test

Double antibody RIA tests and ELISA tests using either synthetic peptide–β-galactosidase (β-gal) conjugates (Neurath et al., 1984a, b, 1985a) or a β-gal fusion protein expressed in Escherichia coli (Offensperger et al., 1985) have been used to detect antibodies recognizing synthetic peptide analogues of the HBV env proteins. Using the RIA test, anti-preS-specific antibodies were detected in sera of seven out of 12 persons immunized with an experimental hepatitis B vaccine (Neurath et al., 1985a; McAuliffe et al., 1982). These antibodies were not detected in recipients of the MSD vaccine lacking preS sequences due to the conditions used for vaccine manufacture (Neurath et al., 1985a). When the RIA test was applied to sera from recipients of two other vaccines (Pasteur and CLB), known to contain small amounts of preS sequences at least in some lots of vaccine (Neurath et al., 1985f), only marginally positive results were obtained (RIA ratio units between 2.1 and 3.0) with a portion of vaccine recipients. Negative results were obtained with ELISA assays based on β-gal conjugates or the corresponding fusion protein. We continued our efforts to demonstrate with confidence the presence of anti-preS-specific antibodies in vaccinated individuals by attempting to develop more sensitive assays.

First an assay was developed differing from the test finally adopted (see Methods) in two aspects. Firstly, staphylococci bearing Protein A were used instead of magnetic Protein A, and all separations were done by centrifugation rather than by a magnetic field and secondly, diluted serum samples were first incubated with the synthetic peptide–β-lactamase conjugates and the immune complexes were subsequently adsorbed on the staphylococcal particles. Excellent results were obtained with this test applied to anti-HBV, to anti-peptide sera and to a group of sera from recipients of the Pasteur vaccines. However, screening of normal human and chimpanzee sera surprisingly revealed that a high proportion of sera gave false positive results. This unexpected problem was avoided by adsorbing first the immunoglobulins from serum specimens to Protein A and subsequently adding the peptide–β-lactamase conjugate to the washed Protein A–immunoglobulin complexes. Replacement of staphylococci by magnetic Protein A further simplified the performance of the assays.

The dilution endpoints of anti-preS(120–145), anti-HBV and pooled sera from hepatitis B vaccine recipients in this assay were 1/3 × 10^6, 1/3 × 10^5 and 1/2.5 × 10^4, respectively, using preS(120–145)–β-lactamase (Fig. 1). With preS(12–32)–β-lactamase, the respective endpoints were 1/1.25 × 10^4, 1/300 and 1/130 (Fig. 2). The comparatively lower endpoints for antibodies recognizing preS(12–32) in anti-HBV and the human serum pool probably reflects the lower content of the large HBV env protein in HBsAg (HBV) in comparison with the content of the middle protein (Heerman et al., 1984; Neurath et al., 1985a). The assays were sequence-specific, i.e. anti-preS(12–32) did not react with preS(120–145)–β-lactamase and anti-preS(120–145) did not react with preS(12–32)–β-lactamase. The assay utilizing preS(120–145)–β-lactamase, because of its higher sensitivity for detection of anti-preS-specific antibodies, was selected for
Fig. 1. Results of ELISA tests with fivefold serial dilutions of rabbit antisera to HBV (○) (Neurath et al., 1976, 1985a), and to the synthetic peptide preS(120–145) (●) (Neurath et al., 1984a), and with dilutions of a serum pool from recipients of a hepatitis B vaccine (□) (McAuliffe et al., 1982). The enzyme-labelled antigen was preS(120–145)-β-lactamase. Dashed horizontal line indicates A570 corresponding to substrate. Note that decreased preS(120–145)-β-lactamase binding to magnetic Protein A at higher serum dilutions results in higher A570 readings, indicating a decreasing decolorization of the substrate.

Fig. 2. Results of ELISA tests with fivefold serial dilutions of rabbit antisera to HBV (○) and to the synthetic peptide preS(12–32) (●), and with dilutions of a serum pool from recipients of a hepatitis B vaccine (□). The enzyme-labelled antigen was preS(12–32)-β-lactamase. For further explanations see legend to Fig. 1.
Table 1. Results of screening selected sera from persons who had been infected with HBV or were vaccinated with distinct hepatitis B vaccines for anti-preS-specific antibodies

<table>
<thead>
<tr>
<th>Characterization of population</th>
<th>Source of vaccine* and lot number (if available)</th>
<th>Number of anti-preS-positive sera/total number of sera tested</th>
<th>Level of anti-HBs (anti-S-protein antibodies) (mIU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Healthy individuals after receiving 3 doses of vaccine</td>
<td>Pasteur† (4) 02</td>
<td>10/10</td>
<td>2250-10500</td>
</tr>
<tr>
<td>2. As 1</td>
<td>Pasteur† (4) 1005</td>
<td>0/12</td>
<td>600-12000</td>
</tr>
<tr>
<td>3. Haemodialysis personnel 1 month after 4th dose of vaccine</td>
<td>Pasteur† (3)</td>
<td>15/15</td>
<td>53000-533000</td>
</tr>
<tr>
<td>4. Haemodialysis patients 1 month after 4th dose of vaccine</td>
<td>Pasteur† (3)</td>
<td>14/20</td>
<td>49-15520</td>
</tr>
<tr>
<td>4a. Subdivision of group 4</td>
<td>Pasteur† (3)</td>
<td>12/12</td>
<td>364-15250</td>
</tr>
<tr>
<td>4b. As 4a</td>
<td>Pasteur† (3)</td>
<td>2/8</td>
<td>49-291</td>
</tr>
<tr>
<td>5. Haemodialysis personnel 1 month after 4th dose of vaccine</td>
<td>CLB‡ (3)</td>
<td>11/15</td>
<td>50000-690000</td>
</tr>
<tr>
<td>6. Haemodialysis patients 1 month after 4th dose of vaccine</td>
<td>CLB§ (3)</td>
<td>2/20</td>
<td>3200-88700</td>
</tr>
<tr>
<td>7. Healthy individuals after receiving 3 doses of vaccine</td>
<td>CLB‡ (3)</td>
<td>0/20</td>
<td>10400-119600</td>
</tr>
<tr>
<td>8. Haemodialysis personnel 1 month after 4th dose of vaccine</td>
<td>CLB‡ (5)</td>
<td>10/25</td>
<td>610-7800</td>
</tr>
<tr>
<td>9. Haemodialysis personnel 1 to 5 months after 3rd dose of vaccine</td>
<td>MSD (1)**</td>
<td>0/10</td>
<td>–</td>
</tr>
<tr>
<td>10. Homosexual men who acquired anti-HBs after HBV infection</td>
<td>– (1)</td>
<td>13/20</td>
<td>280-1020</td>
</tr>
<tr>
<td>11. Haemodialysis patients in the course of transient hepatitis B</td>
<td>– (1)</td>
<td>10/10††</td>
<td>–</td>
</tr>
</tbody>
</table>

* Indicates manufacturer of vaccine and, in parentheses, laboratory from which the sera were obtained, indicated by numbers identical to those given on the title page for the authors' affiliations.
† Vaccine dose 5 μg.
‡ Vaccine dose 3 μg.
§ Vaccine dose 27 μg.
‖ Different batches of vaccine were used.
¶ The pool of 10 sera was assayed by the AUSAB test.
** Vaccine dose 20 μg.
†† Six of these patients had received the MSD vaccine but were not protected.

screening of sera from humans who had been infected with HBV or were immunized with distinct hepatitis B vaccines.

Screening of sera for anti-preS-specific antibodies

Comparative tests with serial dilutions of pooled sera from recipients of a hepatitis B vaccine (McAuliffe et al., 1982) revealed that ELISA tests with preS(120-145)-β-lactamase are approximately 50- to 100-fold more sensitive than double antibody RIA tests or ELISA assays with preS(120-145)-β-gal (data not shown). For this reason, it was possible that sera previously screened by the two latter methods, and found to be negative, may actually be positive for anti-preS-specific antibodies. Results summarized in Table 1 show that this was indeed the case.
The Pasteur vaccine (except group 2, Table 1) elicited anti-preS antibodies in both staff members and patients of haemodialysis units more efficiently than the CLB vaccine, although the latter induced higher levels of anti-S-protein (= anti-HBs) in patients (compare groups 3 and 4 with groups 5 to 7). The antibody responses to either vaccine were higher in personnel than in patients of haemodialysis units. These conclusions were confirmed by determining the anti-preS(120–145) dilution endpoints (for comparison, see Fig. 1) of anti-preS-positive sera from groups 3, 4 and 5, respectively. The corresponding values were 1/5000, 1/200 and 1/50. Within a single group (compare 4a and 4b) the prevalence of anti-preS-positive samples was correlated to the level of anti-HBs. In accordance with data reported earlier, the MSD vaccine did not elicit anti-preS antibodies. A portion (65%) of homosexual men who acquired anti-HBs as a result of HBV infection had detectable anti-preS in their serum. All individuals tested (group 11) who had transient hepatitis B became anti-preS-positive in the course of disease in accordance with published data (Neurath et al., 1985a). Interestingly, six of these preselected haemodialysis patients had been vaccinated with the MSD vaccine (all but one patient developed anti-HBs) but were not protected against hepatitis B (Stevens et al., 1984b). The anti-preS responses were not related to the gender of the vaccine recipients.

The results presented, obtained with preselected serum specimens, indicate qualitative differences between distinct vaccines presumed to contain similar levels of the immunogen. They also suggest differences between distinct lots of vaccines produced by the same manufacturer. The availability of anti-preS assays for screening of sera from vaccine recipients and of tests for direct determination of preS sequences in hepatitis B vaccines (Neurath et al., 1985f) should contribute to the standardization of hepatitis B vaccines and to improved quality control during vaccine manufacture.

DISCUSSION

Antibodies recognizing preselected regions of a protein can be generated by immunization with appropriate synthetic peptides. The application of such antibodies, having predetermined specificity, in biology and medicine is becoming increasingly attractive (Lerner, 1982). Synthetic peptide analogues also offer the opportunity to serve as reagents for discerning antibodies of predetermined specificity among the repertoire of antibodies produced by immunization with complex proteins, viruses and their subunits. This may be especially advantageous in the search for antibodies specifically recognizing biologically important epitopes.

Domains encoded by the preS region of the HBV env gene play a significant role in the life cycle of HBV (Neurath et al., 1985a). Therefore, it has become important to detect antibodies recognizing these domains in sera of humans who had been infected with HBV or who were immunized with hepatitis B vaccines. Earlier studies (Neurath et al., 1984a, 1985a) established that anti-HBV sera recognize at high dilutions a synthetic peptide preS(120–145) corresponding to the 26 N-terminal amino acids (encoded by the preS gene) of the HBV middle protein. Therefore, we assumed that this peptide could represent the basis for the design of an assay more sensitive than the tests used previously. Such an assay was developed using a preS(120–145)–β-lactamase conjugate. The reason why the use of this particular conjugate led to an assay 50- to 100-fold more sensitive than the preceding tests is not well understood. It is possible that the glutaraldehyde-induced polymerization between the peptide and β-lactamase led to products preferentially recognized by antibodies elicited by HBV (HBsAg) containing preS sequences. In this respect it should be mentioned that the assay described here is less sensitive (approx. 1/10 for detection of antibodies generated by immunization with preS(120–145) than double antibody RIA tests described before (A. R. Neurath et al., unpublished data). This is probably due to (i) diminished accessibility of the N-terminal end of the synthetic peptide involved in linkage to β-lactamase and (ii) the finding that immunization with HBV and preS(120–145), respectively, elicits antibodies recognizing distinct segments of the preS(120–145) sequence. Human anti-preS antibodies preferentially recognize the C-terminal portion of preS(120–145) (Kent et al., 1985). Therefore, the diminished accessibility of the N-terminal portion, involved in the
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glutaraldehyde-mediated attachment to β-lactamase, is not expected to affect the binding of human anti-preS antibodies to the conjugated peptide.

An assay for antibodies specifically recognizing the HBV large protein was also developed. This assay is based on the synthetic peptide preS(12–32) linked to β-lactamase. Since the content of the HBV large protein in HBsAg is usually much lower than the content of the middle protein (Heermann et al., 1984; Neurath et al., 1985a), the dilution endpoints of sera from vaccinated persons were by two orders of magnitude lower in this assay as compared to endpoints in ELISA utilizing preS(120–145)–β-lactamase (Fig. 1 and 2). For this reason, the large protein-specific assay cannot be recommended for screening sera from recipients of current hepatitis B vaccines. However, this assay is useful for studying the antibody repertoire in sera of persons with hepatitis B, and potentially important for immunogenicity studies on future hepatitis B vaccines (based on synthetic peptides or on recombinant DNA) containing the sequences preS(1–119) or its portions.

The tests described are specific for antibodies reacting with Protein A. If needed, similar tests can be developed for immunoglobulin subclasses not recognized by Protein A by replacing Protein A linked to a superparamagnetic carrier with anti-immunoglobulin antibodies bound to similar carriers having active groups suitable for covalent linking of proteins. (Such carriers are available from Advanced Magnetics Inc.)

Trials of different hepatitis B vaccines have proven their good efficacy in immunocompetent recipients (Stevens et al., 1984a). However, considerable differences between the Pasteur, CLB and MSD vaccines with respect to their efficacies in haemodialysis patients were reported (Desmyter et al., 1983; Desmyter & Colaert, 1984; Stevens et al., 1984b). It is possible that these differences are due to the distinct capacities of these vaccines to elicit anti-preS-specific antibodies. The MSD vaccine lacks preS sequences (Neurath et al., 1985a); therefore, it cannot elicit the corresponding antibodies. As demonstrated here, the other two vaccines have the potential to elicit these antibodies (unless a ‘wrong’ lot of vaccine is used?). It has to be recognized that the results described in this report were obtained with serum specimens diluted (for technical reasons) 1:40. Therefore, specimens scored as negative actually may have contained very low levels of anti-preS-specific antibodies. Recipients of the Pasteur vaccine generally developed higher levels of anti-preS-specific IgG than did recipients of the CLB vaccine. Unfortunately, the lots of vaccine used in efficacy trials were not available so that their levels of preS sequences could not be evaluated. However, results with other vaccine lots suggested that the Pasteur vaccine contained 100-fold higher levels of preS sequences than the CLB vaccine (Neurath et al., 1985f, and unpublished data). The content of preS sequences even in the Pasteur vaccine was low (20 ng/μg HBsAg). This indicates that preS sequences are highly immunogenic in man, in accordance with previous reports concerning humans (Neurath et al., 1984a, 1985a) and mice (Milich et al., 1985). These findings augur well for future vaccines, based on recombinant DNA and/or synthetic peptide technologies, designed to contain higher levels of preS sequences.

It is of interest that some haemodialysis patients who received the MSD vaccine, yet became infected with HBV, developed anti-preS-specific antibodies as a consequence of infection (Table 1, group 11). It is possible that these patients might have been protected after receiving a vaccine containing preS sequences since they were immunocompetent to respond to these sequences.

The availability of sensitive immunoassays for preS sequences (Neurath et al., 1985f) and the corresponding antibodies will allow determination of the contribution of antibodies with anti-preS specificity to protective immunity against hepatitis B.

The use of synthetic peptide–β-lactamase conjugates in combination with magnetic Protein A offers a good opportunity for the development of screening assays for detection of antiviral antibodies in general. The availability of Protein A expressed in E. coli (Repligen, Cambridge, Mass., U.S.A.) makes such tests economically feasible. Such assays should permit the detection of antibodies specifically reacting with defined functional domains of a virus if sequences corresponding to these domains are determined. The described test design may also contribute
to the solution of recent problems associated with the diagnosis and prevention of the acquired immunodeficiency syndrome.

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REFERENCES


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