Antibodies to Meningococcal Class 1 Outer Membrane Proteins in South African Complement-Deficient and Complement-Sufficient Subjects

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Inhibition assays were used to investigate human serum antibodies to the meningococcal class 1 outer membrane proteins. We adapted the whole-cell enzyme-linked immunosorbert assay technique to determine the ability of sera to inhibit the binding of murine subtyping monoclonal antibodies. Serum samples from 33 South African subjects with a deficiency in the sixth component of complement as well as serum samples from various groups of complement-sufficient subjects were investigated. Subjects were subdivided according to whether they were (i) convalescent from Neisseria meningitidis infections, (ii) nonconvalescent, or (iii) controls. Preliminary subtyping investigations had shown that P1.2 was present on 36% of meningococcal clinical isolates from Cape Province, South Africa. Assays with the anti-P1.2 antibodies showed the presence of high antibody levels in many deficient sera and moderately elevated levels in some sera from the complement-sufficient convalescent patients. P1.2, P1.4, P1.15, and P1.16 are epitopes situated on loop 4 of the class 1 outer membrane proteins, whereas P1.7 is on loop 1. Inhibition assays showed that human sera that inhibited binding by P1.2 monoclonal antibodies tended to inhibit the other monoclonal antibodies directed to loop 4 epitopes. This suggests that the epitopes recognized by the human antibodies are not exactly the same as the epitopes recognized by the murine monoclonal antibodies and raises the possibility of the importance of other epitopes.

Neisseria meningitidis infections frequently result in life-threatening meningitis and/or bacteremia. In immunocompetent individuals, the disease seldom occurs more than once. Goldscheider et al. (8) presented convincing evidence of the role of bactericidal antibodies in preventing the development of the disease, although the role of opsonic antibodies in protection against meningococcal disease requires further investigation (6). The situation in individuals who have defects in the complement system, particularly of the terminal complement pathway, is somewhat different. These individuals frequently suffer recurrent infections, and the role that antibodies play both in eliminating the infections and in protecting subjects from further infections is still uncertain.

In the Western Cape Province, South Africa, meningococcal infections are endemic, with an incidence of approximately 20 cases per 100,000 population per year, rising to about 100 cases per 100,000 per year in epidemic periods (15). A relatively large number of individuals with a genetic deficiency of the sixth complement component (C6) have been diagnosed within this area, and many of the affected subjects suffered recurrent meningococcal infections (13). Preliminary data showed that some patients did develop antibodies to meningococcal outer membrane proteins (OMP), but the specificity of these antibodies was not investigated (15). Studies of serum antibodies in American complement-deficient subjects also confirmed the presence of potentially bactericidal and opsonic antibodies (4).

Class 1 meningococcal OMP are currently regarded as likely components of a meningococcal vaccine (16, 18). The class 1 proteins are porin proteins present on almost all meningococcal isolates (14), and antigenic diversity is limited and allows subtyping classification (2). Cloning and sequencing the genes for the class 1 proteins (3) have led to deduction of the amino acid sequences of four different strains (11, 18). Models for class 1 and other porins predict eight surface-exposed regions or loops, of which loops 1 and 4 are the most accessible in class 1 (18). Differences in the amino acid sequences between subtypes are mostly confined to the two variable regions coding for these loops. Epitope analysis with the relevant murine monoclonal antibodies (MAbs) showed that the anti-P1.7 MAb reacted with peptides from loop 1 of the P1.7 strain and the anti-P1.16 and anti-P1.15 MAb reacted with peptides from loop 4 of the relevant strains (11). The epitope for an anti-P1.2 MAb has now been ascribed to loop 4 (18). The epitope for P1.4 is also believed to be within loop 4, since many P1.4 strains also express the loop 1 P1.7 epitope.

Murine MAb are tools developed to recognize subtype differences, and their specificities will not necessarily be reflected by human anti-class 1 antibodies. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) studies combined with electroblotting (19) have shown the presence of anti-class 1 antibodies in immune sera, but this method does not allow any determination of epitope specificity. To address this problem, Mandrell and Zolllinger (10) developed an inhibition enzyme-linked immunosorbert assay (ELISA) and showed that the majority of serum samples from convalescent patients were able to inhibit the binding of appropriate subtyping MAbs to OMP preparations from the patient’s own infecting meningococcal strains. Neverth-
TABLE 1. MAb s used, subtyping antigens they recognize, and loop locations of these antigens

<table>
<thead>
<tr>
<th>MAb</th>
<th>Antigen</th>
<th>Loop</th>
</tr>
</thead>
<tbody>
<tr>
<td>MN14C11.6</td>
<td>P1.7</td>
<td>1</td>
</tr>
<tr>
<td>MN2B6G</td>
<td>P1.2</td>
<td>4</td>
</tr>
<tr>
<td>MN4C5E</td>
<td>P1.2</td>
<td>4</td>
</tr>
<tr>
<td>MN2B9.34</td>
<td>P1.4</td>
<td>4</td>
</tr>
<tr>
<td>MN3C5C</td>
<td>P1.15</td>
<td>4</td>
</tr>
<tr>
<td>MN5C11G</td>
<td>P1.16</td>
<td>4</td>
</tr>
</tbody>
</table>

less, inhibition of a murine bactericidal MAb gives limited information, and the critical question is still which antibodies, directed at which epitopes, are biologically relevant?

Preliminary epidemiological investigations of Cape meningococcal isolates indicated that class 1 subtype P1.2 was present on 36% of strains and represented the most prevalent subtype recovered from both complement-sufficient and -deficient individuals (12). Of the other antigens studied, P1.15 was found on 15% of isolates, whereas P1.16, P1.7, and P1.4 were rare and found in approximately 2, 2, and 0% of the isolates, respectively. We investigated the human response to natural infection by determining the development of antibodies that would inhibit the binding of the anti-P1.2 loop 4 murine MAb s. Because P1.4 is also situated within loop 4, an anti-P1.4 MAb was used in order to investigate the degree of specificity of the inhibition. The study also included inhibition assays with MAb s directed against P1.15, P1.16, and P1.7.

MATERIALS AND METHODS

Antibody assays. The antibody assays were a modification of the inhibition ELISA described by Mandrell and Zollinger (10). The anti-class 1 murine MAb s used were produced at the Rijksinstituut voor Volksgezondheid en Milieuhygiene, Bilthoven, The Netherlands. Table 1 lists the MAb s and the loop locations of the class 1 antigens that they recognize. Titertek polyvinyl chloride (PVC) flat-bottomed microplates (Flow) were sensitized as for the whole-cell ELISA (1). Suspensions (100 µl/well; optical density at 660 nm [OD<sub>660</sub>] of 0.05) in phosphate-buffered saline (PBS) of heat-killed (56°C for 45 min) meningococci of appropriate subtypes were added, and plates were left to dry overnight at 37°C. Plates were stored at room temperature, and Tween 80 (0.025%) in tap water was used to wash the plates three times just prior to use and also during the assays. The concentrations of MAb s to be used in the inhibition assays were determined by pretitration. For these titrations, antibodies were diluted with PBS containing 0.5% bovine serum albumin (BSA) and 0.01% Tween 80 (BSA-Tween); 100 µl of each dilution was added to the sensitized wells and incubated for 1 h at 37°C. Plates were washed, and 100 µl of horseradish-peroxidase-conjugated anti-mouse immunoglobulin G (IgG; Sigma, St. Louis, Mo.), diluted 1:1,000 in BSA-Tween, was added. After reincubation for 1 h at 37°C, the plates were washed, and color was developed with freshly prepared tetramethylbenzidine (Sigma) (90 µg/ml in 110 µM sodium acetate [pH 5.5] with 0.025% H<sub>2</sub>O<sub>2</sub> (30%). The reaction was stopped with 100 µl of 2 M H<sub>2</sub>SO<sub>4</sub>, and the OD was read at 450 nm on a Microplate reader (Bio-Rad). Antibody dilutions producing just less than maximum (plateau) antibody binding to the organisms were used as the final MAb concentration for the inhibition assays.

For the inhibition assays, human serum samples were diluted with BSA-Tween and heat inactivated at 56°C for 30 min. Samples were added, in duplicate, to appropriately sensitized plates to give final serum dilutions of 0.1, 0.05, and 0.025 in a total reaction volume of 100 µl. The plates were incubated for 30 min at 37°C prior to addition of the MAb, and the incubation was continued for another 60 min. Plates were then washed, incubated with conjugated anti-mouse IgG, and processed for color development as described above.

The percent inhibition for each sample was calculated from the OD readings minus the appropriate blanks from the following formula: [(OD of wells with monoclonal only – OD of wells with sample and MAb)/OD of wells with MAb only] × 100. Results are expressed either as percent inhibition or in units of antibody compared with a serum with high antibody levels. This serum, from subject N, produced high inhibition of binding of all the MAb s except the anti-P1.7 MAb (see Fig. 2), but it was only used as a standard for calculation of human antibody levels from the inhibition of the anti-P1.2 MAb MN2B6G. The standard was arbitrarily ascribed as having 100 U of anti-class 1 antibody, and levels in the other sera were determined relative to this standard serum. A standard curve was set up for every plate, with four duplicate dilutions of the standard serum. The duplicate results for the dilution of the unknown that produced inhibition that fell within the midrange of the standard curve were used for calculating the units of antibody present in the unknown. This calculation automatically adjusted the results for interpolate variation and adjusted for the nonlinear inhibition curves at high antibody concentrations (see Fig. 1). A control serum that was obtained from an individual with no history of clinical meningococcal infection and that produced low inhibition was also run on each plate.

Subjects. The subjects investigated were divided into eight groups according to their serum complement activity and clinical and epidemiological criteria (Table 2). All C6D subjects were discovered by meningococcal disease in themselves or in an immediate family member. The absence of the sixth complement component was diagnosed by means of a functional hemolytic assay described previously (9, 13). The C6D subjects were subdivided into convalescent, nonconvalescent but with clinical infection within the past year, and never clinically infected (groups 1, 2, and 3, respectively; Table 2). As meningococcal infection tends to occur at a later age in complement-deficient than in normal individuals (13), and as age may be related to immune responsiveness (15), we excluded data for all children who were less than 5 years old. Convalescence was taken as the period from 14 to 65 days after admission for the acute episode. This wide period was used for both the deficient group 1 patients and the sufficient group 5 patients. It was necessary because the deficient subjects were usually referred to the study after discharge from the hospital. Approximately 85% of the C6D subjects and the complement-sufficient group 5 and group 6 subjects were Cape coloreds (of mixed caucasoid, southeast Asian, and southern African [Hottentot] extraction); however, a few were Xhosa-speaking black Africans. The group of control African blood donors (group 7) were all black Africans, and the group of healthy British laboratory workers (group 8) were all caucasoid. For the purposes of the statistical analyses, any individual subject was investigated only once, and any C6D patient for whom we had a convalescent sample was investigated in group 1.

Laboratory diagnosis of meningococcal disease was made by cerebrospinal fluid or blood culture and occasionally by microscopy only. The complement-deficient patients were...
TABLE 2. Criteria used for categorizing subjects

<table>
<thead>
<tr>
<th>Group (no.)</th>
<th>Complement status</th>
<th>Age range (yr)</th>
<th>Meningococcal infection</th>
<th>Comments</th>
<th>Range (median) of anti-class 1(^a) antibody levels (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (19)</td>
<td>C6D</td>
<td>7–31 (mean, 20.1)</td>
<td>Convalescent</td>
<td>14–65 days postadmission</td>
<td>7–396 (48)</td>
</tr>
<tr>
<td>2 (9)</td>
<td>C6D</td>
<td>14–32 (mean, 20.2)</td>
<td>Previous, nonconvalescent</td>
<td>Infection &gt;1 yr previously</td>
<td>8–237 (18)</td>
</tr>
<tr>
<td>3 (4)</td>
<td>C6D</td>
<td>7–21 (mean, 14)</td>
<td>No history of infection</td>
<td>13–42 (20)</td>
<td></td>
</tr>
<tr>
<td>4 (29)</td>
<td>Complement sufficient</td>
<td>7–52</td>
<td>Family members of C6D subjects</td>
<td>4–191 (22)</td>
<td></td>
</tr>
<tr>
<td>5 (20)</td>
<td>Complement sufficient</td>
<td>5–58 (mean, 16.7)</td>
<td>Convalescent</td>
<td>14–65 days postadmission</td>
<td>2–81 (16)</td>
</tr>
<tr>
<td>6 (13)</td>
<td>Complement sufficient</td>
<td>5–32 (mean, 14.7)</td>
<td>Noninfected control</td>
<td>Comparable noninfected group</td>
<td>5–15 (8)</td>
</tr>
<tr>
<td>7 (13)</td>
<td>Complement sufficient</td>
<td>≥18</td>
<td>Noninfected control</td>
<td>Black African blood donors</td>
<td>2–83 (20)</td>
</tr>
<tr>
<td>8 (10)</td>
<td>Complement sufficient</td>
<td>21–41</td>
<td>Noninfected control</td>
<td>British laboratory workers</td>
<td>8–13 (11)</td>
</tr>
</tbody>
</table>

\(\text{a}\) The mean ages are shown when the ages of all members of the group were known. The range and median of the anti-class 1\(^a\) antibody levels are also shown.

RESULTS

The initial experiments for the study were carried out with the anti-P1.2 MAbs MN4C5E and MN2B6G (Table 1) in order to identify sera with anti-class 1 P1.2 antibodies. Forty-eight serum samples representative of subjects in the various groups were screened in this way, and inhibitory activity was found in a number of them. The inhibition patterns produced by three serum samples from one C6D case are illustrated in Fig. 1. The samples were taken 3 weeks, 9 weeks, and 3 years after her second meningococcal infection, and this infection was with a B:nt:P1.2 organism. She was 19 years old at the time of infection. The figure illustrates that the convalescent specimen had strong inhibitory activity for both MAbs, with stronger inhibition for MN4C5E than for MN2B6G. By 9 weeks postadmission, the inhibitory activity was very much lower and had disappeared 3 years later. Results for the control serum from plates run on different days showed up to 20% inhibition of MAb binding, and therefore up to 20% inhibition was considered as indicating that no relevant antibodies could be detected. The mean (range) inhibition of MN2B6G by the control MAb was 18% (5 to 16%).

In order to investigate whether the inhibition observed was specific for the binding of the MAbs to P1.2 epitopes, we tested the majority of the 48 serum samples for their ability to inhibit three other MAbs directed at the epitopes P1.4, P1.15, and P1.16 within the loop 4 region of the class 1 OMP and one MAB directed against P1.7, an epitope within the loop 1 region. The results for some of these serum samples, tested at a final serum dilution of 0.05, are shown in Table 2. Figure 2A shows the percent inhibition of MAb binding brought about by serum samples from representative group 1 subjects (C6D patients convalescing from meningococcal disease), Fig. 2B shows the results for C6D patients who had either suffered infection more than a year previously (group 2) or had never suffered clinical meningococcal disease (group 3), and Fig. 2C shows graphs for various C6-sufficient individuals, including the group 8 serum used as a negative control in all assays. (Groups are detailed in the legend.) Results for the inhibition of all six MAbs were obtained for the majority of the 48 serum samples, but results are illustrated only for 34 representative sera. The results for the other 14 sera confirm the results illustrated in Fig. 2.

With almost all of the positive sera, the highest inhibition was produced in assays with the anti-P1.2 MAb MN4C5E. Inhibition of the other anti-P1.2 antibody, MN2B6G, tended to be very similar to the inhibition of the anti-P1.4 antibody MN20B9.34, and the correlation coefficient for the results with those two MAbs on the 48 sera at a 0.05 final serum dilution was \(r^2 = 0.83\). Human sera capable of inhibiting the anti-P1.2 and anti-P1.4 MAbs also inhibited the anti-P1.15 and anti-P1.16 MAbs, although the degree of inhibition was not as closely correlated. The negative-control result is representative of the group 8 results.

The inhibition experiments showed that a number of sera that were able to produce marked inhibition of the MAbs directed against loop 4 epitopes did not significantly inhibit...
the anti-P1.7 MAb and that inhibition patterns for the anti-P1.7 MAb do not necessarily follow those of the other MAbs. The correlation coefficient of inhibition of MN2B6G and MN14C11.6 for the 48 sera was only $r^2 = 0.42$. These results led us to the conclusion that investigations of human antibody inhibition of murine MAbs directed against loop 4 epitopes do not determine only antibodies to the subtyping epitopes themselves. The situation and possible variability of the epitopes recognized by the human antibodies are not yet defined, and therefore we will refer to the human antibodies that we investigated by the loose term human anti-class 1 antibodies (with the superscript 4 indicating probable loop 4 region antigens).

FIG. 1. Inhibition-of-binding curves for three serum samples taken from one C6D patient. Whole organisms carrying P1.2 class 1 OMP were used to sensitize plates. The MAbs inhibited were the two anti-p1.2 MAbs MN2B6G (□) and MN4C5E (○). The conditions of the assay and the calculation of percent inhibition are described in the text. (A) Serum sample taken 3 weeks after the onset of the patient's second episode of meningococcal infection (the isolate was B:nt:P1.2). (B) Serum samples taken 9 weeks after the onset of the same episode. (C) Serum sample taken 3 years later; there were no intervening episodes of infection.

FIG. 2. Inhibition of binding of anti-class 1 MAbs by the indicated human sera at a 0.05 dilution. (A) Inhibition by sera from group 1 C6D patients convalescent from a recurrent attack of meningococcal disease. All patients were teenagers or adult. (B) Inhibition by sera from other C6D subjects. Six subjects were group 2 nonconvalescent subjects with a history of infection more than 1 year previously (M, N, O, P, Q, and R); of these, three (M, N, and P) had had recurrent disease. Four were group 3 with no history of meningococcal infection (S, T, U, and V). Results with two samples from a group 2 patient are illustrated (1 and 2); sample 2 was taken 2 years after sample 1, and the patient suffered no interim clinical meningococcal infection. Serum N was used as a standard for the standardized anti-class 1 ELISAs. (C) Inhibition by sera from complement-sufficient subjects. Three subjects were group 4 complement-sufficient family members of C6D subjects (a, b, and c), and seven subjects were group 5 convalescent patients (d, e, f, g, h, i, and j). k, black African control (group 7); l, British laboratory worker (group 8) control serum. For the results for all individuals illustrated in Fig. 2, the absence of a bar indicates that inhibition of the relevant MAb was not tested with that particular human serum.
DISCUSSION

The meningococcus carries a number of cell surface OMP, and many of these show antigenic variations which allow classification of the organisms. Among these are the class 2 and 3 OMP, which carry the serotype specificities, and the class 1 OMP, which carry the subtype specificities. In addition, there are many other cell surface proteins as well as polysaccharide and lipopolysaccharide antigens, and the situation is thus very complex. The majority of studies of antibody levels following meningococcal infection have looked at responses to polysaccharide or OMP preparations produced from organisms of known group and/or sero- or subtype (7, 15). Such studies have not allowed definition of the specificities of the antibodies determined. Although it is known that complement-deficient patients produce antibodies to OMP preparations following infection, very little is known of the biological role of these antibodies. The study of the functional relevance of antibodies in this situation would be greatly facilitated by determination of the antibody specificities.

We commenced the study with investigations of human anti-P1.2 antibodies, and as shown in Fig. 1, the assays measured infection-related antibodies. However, the results in Fig. 1 demonstrate that sera containing anti-P1.2 and not indeed of inhibiting the anti-P1.2 MAbs were also capable of inhibiting MAbs directed against other loop 4 region antigens. Results obtained with the anti-P1.4 MAb MN2909.34 showed a particularly strong correlation with the results obtained with the anti-P1.2 MAbs. Subtyping studies of approximately 140 isolates from the Cape have failed to find any carrying the P1.4 antigen (12), and therefore it appears extremely unlikely that the ability of the sera from the Cape subjects to inhibit the anti-P1.4 MAb arises from antibodies induced by exposure to the P1.4 antigen itself. We consider it probable that the close correlation of the inhibition of the MN2B6G and MN2909.34 indicates that human antibodies recognizing antigens that are spatially close but not identical to, the epitopes recognized by these two MAbs. Obviously, the specificities of these human antibodies may differ in different sera, and it may be that some are induced by infections with other organisms, such as nonpathogenic neisseriae. Estabrook and coworkers (5), using similar inhibition assays, were able to define meningococcal lipooligosaccharide epitopes recognized by human immune sera. It may be that murine and human antilipooligosaccharide antibodies have similar specificities or that the epitopes are easier to define on the smaller lipooligosaccharide molecules. Much important information is still needed about the class 1 epitopes recognized by human antisera, including: (i) what number of different epitopes are recognized by human sera, (ii) what are the amino acid compositions and tertiary structures of these epitopes, and (iii) where on the molecule are they situated. The information already available on the class 1 proteins (11, 18) suggests that variations between different class 1 proteins are found on the tips of the loop 1 and loop 4 regions. The greater part of the eight predicted surface loops, including loop 1 and 4, is, however, constant. Nevertheless, our data do present the possibility that the epitopes recognized by the human antibodies are not necessarily directed to the variable sequences themselves. Antibody binding to nearby regions during preincubation with human sera, as performed in our studies, could sterically
hinder the binding of the subtype-specific MAbs. The critical question of whether human antibodies to conserved class 1 OMP epitopes have bactericidal potential still needs to be addressed.

P1.7 is located within the loop 1 region, and therefore antibodies that inhibit MAb binding to loop 4 region epitopes are unlikely to inhibit the anti-P1.7 MAb, and this may account for the poor correlation between MN2B6G inhibition and MN14C11.6 inhibition. However, specific antibodies were not purified from the human sera, and whole sera with anti-class 1 MAbs are likely to also contain antibodies directed towards the loop 1 region.

We studied anti-class 1 MAbs in a number of sera from C6D and complement-sufficient patients who had suffered meningococcal infection. Figure 3 shows the results of anti-class 1 MAbs in the various groups of subjects. Convalescent C6D patients (group 1) tended to have the highest antibody levels and also had a very wide range of levels. For all except one of the patients, the relevant infection was a recurrent infection, which may explain why levels were significantly ($P < 0.01$) higher than found in the complement-sufficient convalescing patients (group 5); however, other factors, such as increased time of exposure to the organisms or differences in antigen processing, may play a role. The convalescent complement-sufficient patients (group 5) did have significantly ($P < 0.01$) raised levels compared with the control subjects (group 6). High levels were found in certain nonconvalescing deficient patients (group 2) and nondeficient immediate-family members (group 4), and this may indicate repeated or recent exposure to antibody-inducing organisms. The inhibition results illustrated in Fig. 2B show that deficient subjects who have already suffered recurrent disease can develop markedly increased antibody activity without the development of clinical disease. The African adult blood donor controls (group 7) had levels that were not statistically different from those of convalescent sufficient patients (group 5). Socioeconomic factors probably led to exposure to organisms that induced the high antibody levels. Unfortunately, we had no serum samples taken prior to the development of infection, but comparing the antibody results for groups 1, 2, and 5 with the low levels found in group 6 strongly suggests that the antibodies were infection induced.

The tendency to relapse has been reported for United States terminal complement component-deficient subjects (6), and although the Cape patients did not have overt relapses, some maintained very high antibody levels for several years. This would be most easily explained by continuous or repeated exposure to relevant antigens. The rapid decline in antibodies following recovery of the subject illustrated in Fig. 1 reinforces our concern as to the cause of these high antibody levels. The benzathine penicillin that high-risk susceptible subjects receive as prophylaxis (15) against recurrent clinical disease would not be particularly efficient in eliminating foci of infection from the nasopharynx. It may therefore be wise to use a course of rifampin or ciprofloxacin treatment to eliminate organisms from the subjects and their immediate family.

Further investigations are required on a number of aspects of this study. As outlined above, more direct measurements of the specificities of the antibodies are required to determine the important meningococcal epitopes for vaccine production. If the epitopes recognized by human antibodies are indeed less diverse than the subtyping epitopes, this may have important implications for vaccine production. Another question of importance to vaccine production is the duration of the antibody response. The indications from the data illustrated in Fig. 1 are that high antibody levels are not naturally maintained after recovery, and if the anti-class 1 measured here prove to be protective in normal individuals, it would obviously be beneficial to prolong the response. Unfortunately, we had few such sets of sequential samples, but we hope to extend this section of the study. Further investigations of the role of nonpathogenic neisseriae in inducing protective immunity might also be fruitful. The important question that we have not yet answered in this study is whether the antibodies we detect are either bacterial or opsonic. Another corollary that we wish to address is the possible role of antibodies of various specificities in protecting individuals with complement deficiency from further meningococcal infection.

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REFERENCES


