Evaluation of Hemagglutinin Protein-Specific Immunoglobulin M for Diagnosis of Measles by an Enzyme-Linked Immunosorbent Assay Based on Recombinant Protein Produced in a High-Efficiency Mammalian Expression System

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Recombinant hemagglutinin (H) of the measles virus (MV) expressed in a mammalian high-expression system based on the Semliki Forest virus replicon was used in an enzyme-linked immunosorbent assay (ELISA) for the detection of specific immunoglobulin M (IgM) and IgG in patients with acute-phase measles. One hundred twelve serum specimens from 70 patients with measles were analyzed. Case definition was based on a commercial IgM ELISA that utilizes MV-infected cells (MV-ELISA) (Enzygnost; Behring Diagnostics); the clinical criteria of the Centers for Disease Control and Prevention (Atlanta, Ga.); and/or the increase in hemagglutinin test titers, neutralization test titers, and levels of MV-specific IgG whenever paired sera were available. The initial time courses of the IgM signal after the onset of rash are similar in the H- and MV-ELISAs. On days 0 to 19, both ELISAs detected IgM in 67 of 68 (98.5%) sera. Average maximal levels of IgM seem to persist, however, about 10 days longer in the MV-ELISA (up to day 25) than in the H-ELISA (day 15). From days 20 to 29 and 30 to 59, the H-ELISA detected only 64.3% (9 of 14) and 19.2% (5 of 26), respectively, of sera that were IgM positive by MV-ELISA. At least up to day 30, the performance of the H-ELISA seemed to be similar to that reported for commercial ELISAs based on whole MV. Our results demonstrate that MV H-specific IgM can be used to diagnose most measles cases from a single serum specimen collected within 19 days after the onset of rash and that the recombinant protein used in this study is suitable for this purpose.

Vaccination has reduced the worldwide morbidity and mortality of measles, and eradication of the disease within the next few decades has become a realistic goal. Despite high vaccination coverage, outbreaks continue to occur, and it is likely that a rapid intervention strategy after detection of measles cases will be required. However, rare and isolated cases tend to become more difficult to diagnose clinically. Incomplete protection after vaccination (vaccine-modified infections) can result in clinical measles with uncharacteristic symptoms (9, 22). Numerous diseases with similar skin involvement, such as allergies, add to the difficulties of measles diagnosis. These difficulties are compounded in patients with dark skin. Therefore, measles diagnosis relies increasingly on serological tests.

Diagnosis of measles may be confirmed by virus isolation, by the demonstration of a significant increase in specific immunoglobulin G (IgG) titers, or by the detection of anti-measles virus (MV) IgM antibodies by using radioimmunoassays (2, 18, 37), enzyme-linked immunosorbent assays (ELISAs) (27, 34), and direct or indirect fluorescence-antibody techniques (20, 24). MV IgM appears at the same time as rash (12, 21, 34) and can be detected 3 days after the onset of rash in most individuals (29, 32). IgM peaks on days 7 to 10 and wanes within weeks (28). Since IgM is transient, the demonstration of specific IgM corresponds to a recent primary measles infection (14, 21). A single serum specimen collected at the appropriate time (14) is now generally accepted to be sufficient to diagnose measles (10, 12, 14, 17, 26, 32, 34, 37).

Current IgG and IgM assays are based on whole MV or virus-infected cells as antigens (8, 10, 11, 33, 35, 36, 40). These antigens are possibly more costly and difficult to produce and preserve under standardized conditions than are recombinant proteins.

Rapid diagnosis of measles is essential for the timely implementation of control measures to prevent the spread of infection. Inexpensive, simple, and rapid tests which could be used under field conditions, as an alternative to whole-virus-based ELISA, would represent an important step towards measles control. Assays based on recombinant proteins would potentially benefit from the higher stability of their antigen. The nucleoprotein has been described as a suitable antigen to detect specific IgG and IgM (16, 38, 39). More recently we have shown that recombinant hemagglutinin (H) protein produced in a high-yield mammalian expression system can be used for the surveillance of measles immunity in late convalescents (3) and in vaccinees (4). The high sensitivity and specificity of this assay was due to high levels of detectable H-specific IgG antibodies in both of these cohorts.

In the present study, we have investigated whether this recombinant assay could also be used for detection of H-specific IgM antibodies and for the diagnosis of measles. (This work was done by Fabienne B. Bouche in partial ful-
MATERIALS AND METHODS

Serum panel. From 1996 to 1997, 112 serum samples were collected from 70 patients (age range, 1.1 to 35.4 years; median, 8.2 years) during a major outbreak and from several isolated cases of measles in Luxembourg. Paired sera were obtained from 31 patients; 4 patients were bled three times, and 1 patient was bled four times within 59 days after the onset of rash. A total of 70 first serum samples (50 on or before day 19), 36 second samples (17 on or before day 19), 5 third samples (4 on or before day 19), and 1 fourth sample (before day 19) were collected. All patients had confirmed cases of measles, based on the detection of specific IgM antibodies in a certified commercial ELISA (MV-ELISA) (Enzygnost; Behring Diagnostics, Marburg, Germany). In some patients measles was also confirmed by an increase in specific antibodies in paired sera (n = 36) and/or the clinical case definition of the Centers for Disease Control and Prevention (CDC) (Atlanta, Ga.) (n = 24) (Fig. 1). The CDC criteria include (i) generalized maculopapular rash for 3 days or more; (ii) fever of 38.3°C, if measured; and (iii) at least one of the following symptoms: cough, coryza, or conjunctivitis (7). All patients presented with a typical rash. Four of the 112 samples were obtained between 2 and 14 days before the onset of rash; these cases were also confirmed by increased hemagglutination inhibition (HI) and neutralization (NT) test titers and by MV-specific IgG and IgM antibodies in paired sera drawn after the onset of rash.

Thirty-five sera that were IgG positive and IgM negative by MV-ELISA served to determine the threshold for positivity of the IgM H-ELISA. These sera were positive by HI and NT assays. They were collected from 13 individuals who were vaccinated at least 12 months before collection (median age, 5.9 years; median age of vaccination, 15.8 months) and from 22 late-convalescent donors.

Serological assays. Anti-MV IgG and IgM were measured by using a certified commercial MV-ELISA (Enzygnost for IgG and IgM; Behring Diagnostics) based on whole-MV-infected simian cells, following the supplier’s instructions. HI and NT titers were determined as described before (15). The titers are expressed as log2 dilutions, with values of 200 mOD for the MV-ELISA (IgM Enzygnost). The hatched zone (100 to 200 mOD) was defined with sera that were negative for HI and NT, as described previously (3). The POS/NEG test was applied to compare the proportions of two groups of values within a cohort, and the t test was used to evaluate the significance of the difference between the mean values of two groups.

RESULTS

Definition of threshold for positivity. Specific IgM was measured by H-ELISA with sera obtained from late-convalescent adults with measles and from vaccinees; all of these individuals were negative for IgM by MV-ELISA. The IgG levels of these donors were between 270 and 2,750 mOD by MV-ELISA. By H-ELISA, the means (± SD) of IgM values for the convalescents and the vaccinees were 39 ± 61 (range, −77 to 131) and 23 ± 49 (range, −45 to 105), respectively. The t test detected no significant difference between the means of vaccinees and convalescents. When the IgM values of sera with IgG levels of <1,000 mOD and of those with >1,000 mOD were compared, no difference was detectable (26 ± 58.2 versus 35 ± 57.2; P value, not significant).

Based on the above-mentioned IgM-negative sera from vaccinees and late-convalescent donors, the threshold for IgM positivity was defined as the mean + 2 SD [i.e., 33 + (2 × 57), or 147]. Experiments with unadsorbed sera sometimes gave false-positive IgM titers in the H-ELISA (data not shown). Thus, in the present format of the H-ELISA, preadsorption with anti-human IgG (GULLSORB) gave a more reliable estimation of IgM levels.

Detection of MV-specific IgM in patients with measles. In 108 serum samples (from 70 different patients) drawn at different time intervals after the onset of rash, levels of specific IgM were analyzed both by H-ELISA and by MV-ELISA. Figure 2 shows that the MV-ELISA detected 107 IgM-positive sera (99.1%) whereas the H-ELISA detected IgM in only 81 sera (99.1%) whereas the H-ELISA detected IgM in only 81

Data are expressed as milli-OD (mOD). The threshold for positivity was defined as the mean mOD (+ 2 standard deviations [SD]) of the IgM-negative sera, measured after 2 h.

(iii) IgG detection. Specific IgG was measured by H-ELISA following the procedure described previously (3). In this assay, the H antigen and the negative control antigen were directly coated onto microtiter plates. The threshold for positivity (210 mOD) was defined with sera that were negative for HI and NT, as described previously (3).

For IgA and IgG detection, OD was measured after 2 h at 405 nm, and data are expressed as net mOD by subtracting for each serum sample the background obtained with the control antigen from the H antigen.

Statistics. The data were analyzed with Sigmastat software (Jandel Scientific, Erkrath, Germany). The t test was applied to compare the proportions of two groups of values within a cohort, and the t test was used to evaluate the significance of the difference between the mean values of two groups.

FIG. 2. Comparison of IgM values by H-ELISA and MV-ELISA of 112 serum samples drawn from 70 patients with acute-phase measles. Thresholds for positivity are 147 mOD in the H-ELISA (dotted line [defined in the text]) and 200 mOD for the MV-ELISA (IgM Enzygnost). The hatched zone (100 to 200 mOD) is undefined, according to the manufacturer. Single and paired sera of patients A/A, A/B, and B/B, respectively, are indicated. The symbols represent sera drawn before the onset of rash (n = 41; •) and on days 0 to 19 (●) and days 20 to 59 (□) after the onset of rash (n = 108).
sera (75%) on the basis of the above threshold. All (additional) sera collected before the onset of rash (on days 214, 29, 28, and 22; n = 4) were IgM negative in both ELISAs (Fig. 3).

Most of the sera that were IgM positive in both the MV-ELISA and the H-ELISA were found positive before day 20 after the onset of rash. Among 68 serum samples collected from 50 individuals between days 0 and 19 after the onset of rash, 67 (98.5%) had detectable measles-specific IgM by MV-ELISA and by H-ELISA (Fig. 3B).

The one serum that was negative in the MV-ELISA was drawn on the day of the onset of rash (day 0) and was positive by H-ELISA (372 mOD) (Fig. 2, patient A). This patient was IgG negative by both ELISAs and developed measles according to CDC criteria. A second sample (Fig. 2, serum A'), drawn on day 12 from the same patient, was IgM positive by both ELISAs (791 and 1,142 mOD by MV-ELISA and H-ELISA, respectively). This patient experienced a significant increase in HI and NT titers and in specific IgG (from 6 mOD to 748 mOD by IgG MV-ELISA and from 228 mOD to 726 mOD by IgG H-ELISA). This suggests that on day 0 the IgM level was accurately measured as positive by the H-ELISA, in contrast to the MV-ELISA measurement.

The serum obtained on day 1 from patient B (Fig. 2), who was IgM negative by H-ELISA and weakly positive by MV-ELISA (266 mOD), was the first of paired sera. The second sample from this patient (serum B', drawn on day 13 [Fig. 2]) was positive by both IgM ELISAs, and this patient experienced a significant increase in HI and NT titers and in specific IgG (from 14 mOD to 1,077 mOD by IgG MV-ELISA and from 142 mOD to 1,533 mOD by IgG H-ELISA). This may suggest that serum B, measured on day 1 by H-ELISA, was a false negative.

An additional 40 sera were drawn from 37 patients after day 19 from the onset of rash. Twenty of these patients were bled twice or more and were independently confirmed at least by an increase in specific IgG. Nineteen of the 20 were found IgM positive by H-ELISA either before day 20 (17 sera) or after day 20 (4 sera) from the onset of rash. One patient (bled on days 20 and 32) was IgM negative twice by H-ELISA. Another patient, who was bled only on day 23 but was confirmed by CDC criteria, was also IgM negative by H-ELISA. Thus, among the 24 sera which were independently confirmed by MV-ELISA and at least one other criterion, only 9 sera (37.5%) were IgM positive by H-ELISA.

Among the 16 (single) sera drawn after day 19 that were IgM positive by MV-ELISA and were not independently confirmed clinically or by IgG increase, 5 (29.4%) were positive by H-ELISA. Thus, among the sera obtained after day 19, no significant difference was observed in the proportion of sera that were positive in the H-ELISA, whether IgM positivity in the MV-ELISA was independently confirmed or not (37.5% versus 29.4%; P value, not significant).

**DISCUSSION**

Currently the most reliable diagnostic assays for measles are based on the detection of IgM by ELISA with whole MV or
Ozanne and d’Halewyn (26), who reported a sensitivity of 75% while the sensitivity of the MV-ELISA did not (98.1%). When an even longer interval is considered, the sensitivity of the H-ELISA was 92.7% and that of the incidence, 90.5%), and 85.5% for the Incstar test (specificity, 98.2%), 93.3% for the Gull test (specificity, 98.5%) (27). In contrast, lower levels of H-specific antibodies were detected by immunoprecipitation (13), by competition ELISA with H-specific monoclonal antibodies (27), and by comparing complement fixation with immunodiffusion tests (25). In addition, these antibodies seemed to develop only after the onset of rash (13). These observations suggested that H-specific antibodies may not be sufficient for diagnosis, but the observations are in conflict with the rapid increase of HI and NT antibodies, both of which are predominantly directed against H protein after the onset of rash (reference 30 and unpublished results).

Our study demonstrates that there are considerable amounts of H-specific antibodies within the early days after the onset of rash which are readily detectable with an ELISA based on a mammalian expressed recombinant protein. The initial time courses of IgM in the H-ELISA and the commercial MV-ELISA seemed to be similar. Maximal average levels of specific IgM were reached between days 5 and 10 after the onset of rash. This is in agreement with earlier studies with total IgM isolated by sucrose gradient and tested by HI (day 10) (6) or by IgM Enzygnost (days 5 to 10) (28, 32).

The early appearance of H-specific IgM translates into a high sensitivity (98.5%) of the H-ELISA within the first 19 days, which matches that of the Enzygnost MV-ELISA (98.5%) for the same sera. When an interval from 0 to 30 days is considered, the sensitivity of the H-ELISA was 92.7% and that of the MV-ELISA was 98.8%. In another cohort the sensitivity of the H-ELISA rapidly deteriorated (days 0 to 59, 75%) while the sensitivity of the MV-ELISA did not (98.1%) because it served as a “gold standard.”

The high sensitivity seems to agree with the results of Ozanne and d’Halewyn (26), who reported a sensitivity of 91.9% for Enzygnost between days 1 and 56. However, the overall sensitivity strongly depends on the number of sera collected during the later time points. In some studies only 0, 8, 5, or 4.5% of the samples were collected after day 15 (32), day 19 (23), day 25 (26), or day 30 (1), respectively. In contrast, 37% of the samples in our study were drawn after day 20 and 25% of the samples were collected after day 30. It is therefore important to compare sensitivities later after the onset of rash.

In the H-ELISA, the sensitivity was 64.3 (n = 14) and 19.2% (n = 26) between days 20 and 29 and days 30 and 59, respectively. In the study of Arista et al. (1), the sensitivity of Enzygnost was 40% between days 31 and 35.

After day 19, less than half of the sera (16 of 40) were solely confirmed by MV-ELISA. Therefore, the assessment of the sensitivity of the H-ELISA after day 19 may depend on the performance of IgM Enzygnost. However, essentially no difference in the sensitivity of the H-ELISA was found whether the sera were confirmed by the commercial ELISA only (29.4%) or by at least one additional parameter (37.5%). Thus, in our cohort, the difference in sensitivity between the H-ELISA and the MV-ELISA cannot be explained by excessive false-positive results (low specificity) of the MV-ELISA but rather by the accelerated waning of H-specific IgM in comparison to that of MV IgM.

The threshold that gave a sensitivity of 98.8% in the H-ELISA (days 0 to 19) was associated with a specificity of 100% in the IgM-negative panel. However, this needs to be confirmed in a panel of IgM-negative sera which is independent of the definition of the threshold. Lowering the threshold of the H-ELISA would decrease the specificity but increase the sensitivity above 95% within the first 30 days. A high specificity, i.e., a low percentage of false-positive results, was also reported by several authors for the Behring test (1, 26, 32).

Measles is most contagious within 1 week before and 1 week after the onset of rash (19). Serological assays before the onset of rash are not available, but during the contagious period after the onset of rash the H-ELISA and the MV-ELISA perform equally well. Most studies which rely on IgM for measles diagnosis recommend that serum samples be drawn within about 3 weeks after the onset of rash (1, 10, 14, 21, 26, 32). Thus, an optimal performance within 19 days seems to be sufficient for measles diagnosis (14, 26). Nonetheless, we are currently investigating whether further optimizing the assay could increase the sensitivity beyond day 19.
We have also studied the development of H-specific IgG in patients with measles. No false-negative serum was detected, and increases between paired sera were as significant as they were with MV-ELISA. In light of these results and the overall high sensitivity and specificity of H-ELISA for IgG (3, 4), we conclude that this assay is as reliable for the diagnosis of measles in paired sera as an ELISA based on whole MV. Despite the longer persistence of maximal levels of IgM in MV-ELISA and the early waning of H-specific IgM, the initial time courses of H- and MV-specific IgGs are similar. Since the H protein can be efficiently produced in a mammalian system (3, 4), and since recombinant proteins may benefit from enhanced stability, an ELISA based on this antigen seems like an interesting alternative in the search for a low-cost, rapid diagnostic system for measles.

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