Application of Virus-Specific Immunoglobulin M (IgM), IgG, and IgA Antibody Detection with a Polyantigenic Enzyme-Linked Immunosorbent Assay for Diagnosis of Epstein-Barr Virus Infections in Childhood

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The Enzygnost anti-Epstein-Barr virus enzyme-linked immunosorbent assay (ELISA) system, which is based on a defined antigen mixture and on detection of antibodies of the immunoglobulin G (IgG), IgM, and IgA classes, was evaluated for its reliability in diagnosing Epstein-Barr virus infections in childhood. With samples from 66 children, the Epstein-Barr virus status and the infection phase were defined by indirect immunofluorescence and anticomplement fluorescence assays: 11 children were seronegative, 8 had a primary infection, 20 had a recent primary or past infection, and in 27 a reactivated Epstein-Barr virus infection was diagnosed. When applying the Enzygnost ELISAs, 15 serum samples (22.7%) were not interpretable due to indeterminate results in at least one of the assays used and were therefore excluded from further evaluation. The respective sensitivities and specificities for the diagnosis of seronegativity were 100 and 100%, those for the diagnosis of primary infection were 100 and 97%, those for the diagnosis of recent primary or past infection were 100 and 52%, and those for the diagnosis of reactivated infection were 10 and 100%. This poor performance of the Enzygnost system with reactivated infections is due to the prerequisite of an IgG antibody value of >650 IU/ml for the diagnosis of viral activity, which was fulfilled in only two of the children. Despite the high rate of indeterminate results, the Enzygnost system is useful in diagnosing acute and past Epstein-Barr virus infection in childhood. For serological diagnosis of viral activity in childhood, a supplementary assay is necessary.

In childhood, the diagnosis of typical infectious mononucleosis is based on clinical findings plus a confirmatory serological test. In the setting of a pediatric university hospital, however, a number of children suffer from atypical or hazardous manifestations of acute and prolonged or reactivated Epstein-Barr virus (EBV) infections, while heterophile antibodies are often absent in childhood (5). A specific assay for the detection of anti-EBV antibodies is mandatory for the diagnosis of these atypical or heterophile antibody-negative pediatric cases.

Determination of the serology for antibodies against EBV by indirect immunofluorescence (IDIF) and anticomplement immunofluorescence (ACIF) is regarded as the reference method (12). Antibodies to viral capsid antigen (VCA) and early antigen (EA) are detected by IDIF, and antibodies to EBV nuclear antigen (EBNA) are detected by ACIF. This standard serology is well documented (16) and is an appropriate tool for the diagnosis of EBV infections in childhood (1, 6, 7, 13). Recently, an enzyme-linked immunosorbent assay (ELISA) system for the diagnosis of EBV infections was developed (Enzygnost Anti-EBV; Dade-Behring, Marburg, Germany). The test utilizes a defined mixture of the relevant EBV antigens EA, VCA, and EBNA-1. Diagnosis of the different stages of EBV infection is based on the determination of EBV-specific immunoglobulin M (IgM), IgG, and IgA antibodies with this assay. The detection of anti-EBV antibodies of the IgM and IgG classes is specific and sensitive for the identification of primary or past EBV infections (2, 3, 8, 9, 15, 19), and determination of IgA anti-EBV levels in patients with enhanced IgG anti-EBV antibody values (>650 U/ml) enables chronic or reactivated EBV infections to be diagnosed (4). However, no evaluation of the Enzygnost-based diagnosis of primary, recent, and prolonged or reactivated EBV infections in childhood is available. The aim of the present study was to evaluate the application of virus-specific IgM, IgG, and IgA antibody detection with the Enzygnost anti-EBV ELISA for the diagnosis of the different stages of EBV infections in childhood in comparison with the IDIF and ACIF reference assays.

MATERIALS AND METHODS

Patients. Samples (n = 66) from children (age range, 1 to 12 years; mean age, 6.5 ± 3.5 years) were analyzed. All specimens had been submitted by physicians from the Department of Pediatrics for routine diagnosis, either to confirm a primary EBV infection presenting with the typical clinical picture or to rule out an acute, prolonged, or reactivated infection with an atypical clinical presentation.

IDIF and ACIF. IDIF and ACIF were performed with Merifluor assays (Meridian Diagnostics, Bad Homburg, Germany). The EBV IgG and IgM IFA for detection of IgG and IgM anti-VCA, the EBV EA IgG IFA for detection of IgG anti-EA-D and -EA-R, and the EBV Ab ACIF for detection of anti-EBNA were performed according to the manufacturer’s recommendations. All serum samples were preabsorbed with anti-IgG antibodies prior to testing for IgM antibodies. Antibodies specific for VCA and EBNA with a titer exceeding 1:10 were considered positive. Anti-EA antibodies with a titer greater than 1:40 were considered indicative of an reactivated infection (10). The antibody patterns were interpreted as previously described (16): IgG anti-VCA, IgM anti-VCA, IgG anti-EA, and anti-EBNA negative, EBV negative; IgG anti-VCA positive,
RESULTS

The concordance of EBV serodiagnosis with both test systems and the accuracy are summarized in Table 1.

Seronegative patients. Of the 66 children, 11 were seronegative for EBV infection according to IDIF; 10 of these children were also negative in Enzygnost IgG and IgM ELISAs. One sample was negative in the IgG ELISA but indeterminate in the IgM ELISA.

Acute primary EBV infection. In eight cases, an acute primary EBV infection was diagnosed by IDIF when low IgM anti-VCA antibody titers (≤1:20) were detectable in the presence of positive IgG anti-VCA antibody titers and in the absence of anti-EBNA antibodies. On this basis, recent primary EBV infection was diagnosed in five children. All five samples were also correctly classified as past EBV infection by the Enzygnost IgG-IgM ELISA interpretation scheme; however, this scheme does not discriminate between the categories recent primary EBV infection and past EBV infection.

Past EBV infection. Of the 66 children, 15 were EBV seropositive without serological signs of EBV reactivation in the IDIF. With the Enzygnost ELISAs (IgG and IgM), eight of these samples were also correctly interpreted as past EBV infection. In two samples the IgG antibody result was indeterminate while the IgM result was negative. The IgG and IgM antibody results were indeterminate in two samples, while the IgG result was positive and the IgM result was indeterminate in another two samples. In one final sample the IgG result was negative and the IgM result was indeterminate.

Reactivated EBV infection. Reactivated EBV infection was diagnosed when anti-EA (IDIF) was detectable at a titer of >1:40 either in the presence of anti-EBNA (ACIF) or if a positive IgG anti-VCA (IDIF) or IgG anti-EBV (ELISA) status was documented for more than 3 months in anti-EBNA negative cases. Of the 66 children, 27 showed serological signs of reactivated EBV infection by IDIF. Using the Enzygnost ELISA system, IgA anti-EBV values were determined only in cases with an IgG anti-EBV value of >650 IU/ml, because only this antibody constellation is indicative of EBV reactivation according to the manufacturer’s evaluation. Only two of the children were correctly diagnosed by the Enzygnost system as having reactivated EBV infection, fulfilling the criteria of IgG level of >650 IU/ml and IgA OD of >0.6. In a further 17 cases the IgG anti-EBV value was positive below 650 IU/ml and the IgM anti-EBV value was negative. In three samples the IgG result was positive and the IgM result was indeterminate. In four further samples the IgG result was indeterminate and the IgM result was negative, and in one sample both the IgM and IgG results were positive.

The IgG anti-EBV values are crucial to the diagnosis of reactivated EBV infection using the Enzygnost system. The ELISA IgG levels of the patient group with reactivated EBV infection according to IDIF and those of the group with past infection according to IDIF are compared in Fig. 1A. The IgG levels of the two groups do not differ significantly (Wilcoxon test; P = 0.18). The receiver operating characteristic curve analysis shown in Fig. 1B demonstrates that an IgG anti-EBV cutoff point discriminating sufficiently between children with past EBV infection and children with reactivated EBV infection cannot be defined.

Relationship between age and Enzygnost IgG anti-EBV value. The IgG anti-EBV value determined by the Enzygnost ELISA depends on the age of the children. The IgG anti-EBV values were not significantly different (Wilcoxon test; P = 0.25) between children older than 15 years and those younger than 15 years.

### Table 1: Evaluation of the Enzygnost ELISA system for the diagnosis of EBV infections in pediatric patients (n = 66) compared to diagnosis by reference IDIF pattern

<table>
<thead>
<tr>
<th>Enzygnost system result</th>
<th>No. of patients with the following diagnosis by reference IDIF pattern:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Seronegative (n = 11)</td>
</tr>
<tr>
<td>IgG anti-EBV Positive</td>
<td>0</td>
</tr>
<tr>
<td>IgG anti-EBV Indeterminate</td>
<td>0</td>
</tr>
<tr>
<td>IgG anti-EBV Negative</td>
<td>0</td>
</tr>
<tr>
<td>IgM anti-EBV Positive</td>
<td>0</td>
</tr>
<tr>
<td>IgM anti-EBV Indeterminate</td>
<td>0</td>
</tr>
<tr>
<td>IgM anti-EBV Negative</td>
<td>0</td>
</tr>
<tr>
<td>Diagnosis</td>
<td>1</td>
</tr>
<tr>
<td>Seroconversion</td>
<td>10</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>100</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>100</td>
</tr>
</tbody>
</table>

a A subset of the patients whose results were positive had IgG anti-EBV levels of >650 IU/ml.
b EBV IgA OD of >0.6 in both serum samples.
c Calculation of test characteristics after exclusion of indeterminate results (n = 15).

The diagnosis of a recent primary EBV infection was established by IDIF when low IgM anti-VCA antibody titers (≤1:20) were detectable in the presence of positive IgG anti-VCA antibody titers and in the absence of anti-EBNA antibodies. On this basis, recent primary EBV infection was diagnosed in five children. All five samples were also correctly classified as past EBV infection by the Enzygnost IgG-IgM ELISA interpretation scheme; however, this scheme does not discriminate between the categories recent primary EBV infection and past EBV infection.

The Enzygnost Anti-EBV ELISA. The Enzygnost Anti-EBV assays for the detection of EBV-specific IgG, IgM, and IgA (Dade-Behring) were performed using an automated system (ELISA Processor III, Dade-Behring) according to the manufacturer’s protocol. Indeterminate results were not reanalyzed. Serum samples were presorbed with anti-IgG antibodies prior to analysis with the IgG and IgM ELISAs. Using the IgG and IgM assays, samples with a final optical density (OD) ranging between 0.1 and 0.2 were considered indeterminate, while samples with an OD of greater than 0.2 were considered positive. The cutoff OD for the detection of IgA anti-EBV was 0.6. IgG anti-EBV antibody values were quantified with the method as recommended by the manufacturer, and the results were expressed as international units per milliliter according to the Medical Research Council Reference Standard A, 66/235, for infectious mononucleosis (National Institute for Biological Standards and Control, World Health Organization, United Kingdom). According to the manufacturer’s protocol, all sera were tested in a first step with the IgG and IgM ELISAs. Subsequently, samples suspected of EBV reactivation due to an IgG value of >650 IU/ml were analyzed with the IgA ELISA. The results were interpreted as recommended by the supplier: IgG OD of <0.1 and IgM OD of <0.1, EBV negative; IgG OD of >0.2 and IgM OD of <0.1, past EBV infection; IgG OD of <0.1 and IgM OD of >0.2, acute EBV infection; IgG level of <650 IU/ml and IgM OD of >0.2, acute EBV infection or polyclonal response; and IgG level of >650 IU/ml, IgA OD of >0.6, and any IgM OD result, chronic or reactivated EBV infection.

### Statistical analysis

For all statistical tests and graphs, a statistical software package (MedCalc, Mariakerke, Belgium) was used.

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**Table 1.** Evaluation of the Enzygnost ELISA system for the diagnosis of EBV infections in pediatric patients (n = 66) compared to diagnosis by reference IDIF pattern.
levels (ELISA) of 55 IgG anti-VCA (IDIF)-positive children were correlated with their ages. The related scatter diagram in Fig. 2 demonstrates a significant positive correlation between the two parameters (non parametric Spearman’s rank coefficient; \( P = 0.025 \)).

**DISCUSSION**

The Enzygnost EBV ELISA system for the diagnosis of EBV infection is an accurate system for the determination of virus-specific IgG and IgM antibodies (2, 3, 8, 15, 19) and has also been shown to detect reactivated EBV infection in non-age-defined populations (4). However, according to our findings, the application of these ELISAs in a pediatric population is characterized by a high rate of indeterminate test results (15 of 66 samples [22.7%]), which points to a relevant imperfection in test performance, because clear-cut interpretation and diagnosis are not possible for these patients.

With these samples excluded from further evaluation, the specificity and sensitivity of the Enzygnost system are excellent for the diagnosis of EBV seronegativity and acute primary infection. The sensitivity for the diagnosis of past EBV infection and the specificity for the detection of reactivated infection are also excellent.

The Enzygnost system allows sufficient differentiation between seronegativity, acute primary infection, and recent primary or past EBV infection. However, the Enzygnost system does not allow distinction between recent primary and past infection and is therefore not suitable for the retrospective diagnosis of acute EBV infection in the previous few weeks in patients suffering from prolonged symptoms. In contrast, the specificity for the diagnosis of recent or past infection is 52%, while the sensitivity for the detection of reactivated infection is only 10%. This inadequate performance with these two test characteristics is due to poor discrimination between past and reactivated EBV infections. According to the manufacturer, reactivated infection is defined by an IgG anti-EBV level of \( >650 \) IU/ml and an OD of \( >0.6 \) for IgA. These criteria were fulfilled in only 2 of the 27 samples with reactivated EBV infection according to IDIF.

The failure of the Enzygnost system to detect reactivated EBV infection in childhood may be due to a low IgG anti-EBV starting level in the very young and the slow age-dependent progression of IgG antibodies values against the utilized antigen mixture. A generally lower production of IgG against these antigens in reactivated EBV infection in childhood may be responsible for this effect. The EA component included in the Enzygnost ELISA may be of importance. Because reactivated EBV infections are usually characterized by IgG antibodies against EA-D (16), the D component of the EA but not the R component is included in the antigen mixture (18) besides the antigens EBNA-1 and VCA. In childhood, however, a predominant antibody production against EA-R has been described, which may be due to immaturity of the immune system (6).

The recalibration of the IgG cutoff value for the diagnosis of reactivated EBV infection with the Enzygnost system failed to provide adequate results because the IgG levels for past and reactivated EBV infections did not differ significantly.
The failure to detect reactivated EBV infection in childhood is of clinical importance in immunocompromised patients. However, even in otherwise healthy pediatric patients, reactivated EBV infection should not be ignored by a serological test system, because it is an important aspect of differential diagnosis in persisting and atypical illnesses (11, 14, 17).

We conclude that diagnosis with the Enzygnost ELISA system is suitable for the diagnosis of seronegativity and acute EBV infection. The applicability for the diagnosis of recent primary and past EBV infections is limited by the failure to distinguish between the two infection phases as well as by a high rate of indeterminate results.

The Enzygnost system is not applicable to the diagnosis of reactivated EBV infection. As ELISA systems are advantageous in regard to standardization and automation in the clinical virology laboratory, we suggest the additional detection of anti-EA by IDIF to overcome the problems in the detection of reactivated EBV infections using the Enzygnost ELISA system.

REFERENCES