Antibody Response to Epstein-Barr Virus in Infectious Mononucleosis

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Altogether 171 serum specimens from 58 patients with heterophil antibody-positive infectious mononucleosis were studied for antibody response to Epstein-Barr virus (EBV). The sera were tested for fluorescent immunoglobulin G (IgG) and IgM gel-precipitating (GP) and complement-fixing (CF) antibodies to EBV. All 58 patients had IgG and IgM antibodies to EBV. Both IgG and IgM antibodies developed rapidly; the IgM antibodies disappeared within 8 to 10 weeks, whereas the IgG antibodies remained at an almost constant level. The development of IgG antibodies was so rapid that a fourfold or greater rise in titers was noted only in 22% of the patients. Both GP and CF antibodies to EBV (crude P3HR-1 Burkitt cell antigen) developed slowly; the mean titers kept rising for more than 12 weeks. The micro GP technique seemed to be more sensitive than the CF method, because 86% of the patients with infectious mononucleosis had GP antibodies compared with 72% having CF antibodies. In patients with infectious mononucleosis, a seroconversion or significant rise in GP antibodies was noted in 57%, whereas only 19% had a similar change in CF antibodies. The most promising of these antibody assays in the diagnosis of recent infections was the EBV-specific IgM antibody technique, which enables one to make the diagnosis on the basis of only one serum specimen. In cases where the acute-phase serum specimen is missing, the diagnosis can be made later by using the GP and CF techniques.

Epstein-Barr virus (EBV) is now, with good reason, considered to be the etiological agent of infectious mononucleosis (IM) (10). The diagnosis of IM can be made, in most cases, from heterophil antibodies reacting in the Paul-Bunnel test or in the rapid slide tests. The heterophil antibody tests can, however, give false-positive reactions in some cases (14) or remain negative (15, 20). Especially children with EBV infections very often have negative reactions to heterophil antibodies, and the clinical picture is also usually atypical of IM. Fluorescent immunoglobulin [IgG] antibodies are also known to develop very rapidly in EBV infections, and the rise in antibody titers is seldom detected (9, 21). There are some reports of using the complement-fixing (CF) (24) and gel-precipitating (GP) antibodies in the diagnosis of recent infections with EBV (16), but neither of these techniques used alone is sufficient for diagnostic use. Schmitz and Scherer (21) have recently published promising results of the use of IgM antibodies to EBV in the diagnosis of recent infections. The results were later confirmed by Nikoskelainen and Hänninen (Scand. J. Clin. Lab. Invest. 30(Suppl 130):32, 1973). A new and useful test is the determination of EBV-neutralizing antibodies in the diagnosis of IM (11). This test is, however, very demanding and is applicable only to special laboratories.

The aim of this work was to study the antibody response to different EBV antigens in patients with IM. An evaluation of the suitability of different techniques for determination of antibodies in recent infections was also made.

MATERIALS AND METHODS

Patients and sera. Altogether 171 serum specimens from 58 patients who had heterophil antibody-positive IM were collected from 1970 to 1972 from southwest Finland. Twenty-three of the patients from the Department of Infectious Diseases, University of Turku, were observed with repeated serum specimens for up to 1 year after the onset of illness. The clinical picture of these patients was studied carefully and compared with the serological findings. Twenty-one of the patients had a pharyngeal and two had a glandular type of disease according to the classification of Klemola et al. (15). The rest of the patients were chosen from the routine serum specimens sent to the Department of Virology, University of Turku, for vi-
rus antibody titrations. All serum specimens with a positive reaction to heterophi antibodies by the sheep erythrocyte agglutination with differential absorption (Paul-Bunell) were chosen. This material also included control serum samples from 60 Finnish Red Cross blood donors collected in Turku.

Tests for antibodies to EBV. All serum specimens were tested for fluorescent IgG and IgM antibodies and also for GP and CF antibodies to EBV.

Test for IgG antibodies to EBV. In this study a modification of Henle and Henle’s indirect immunofluorescence technique was used (8). The Burkitt cell line, Shilfere, was applied. This line gave no fluorescence when stained directly with anti-human IgG or IgM conjugate. The cell cultivation, antigen slide preparation, and antibody titration, a modification of the Henle test, are described in detail elsewhere (13).

Test for IgM antibodies to EBV. The Burkitt cell line P3HR-1 was used in the titrations of IgM antibodies to EBV. The cells were cultivated in BME diploid, with a double amount of arginine and 10% newborn calf serum obtained from M. D. Wesselen, Department of Virology, University of Uppsala, Sweden. The cultures were usually kept with antibiotics: penicillin (100 U/ml); streptomycin (100 μg/ml); and kanamycin (200 μg/ml). Occasionally amfotericin B in a concentration of 2.4 μg/ml was included. After being cultivated at 35 C with 5% CO2 for 7 days, the cell concentration was adjusted from 105 to 1.5 × 106 cells/ml. The bottles were transferred to 32 C and kept without refedding for 14 days before being used for slides as described elsewhere (13). The P3HR-1 line was completely negative to direct IgG and IgM fluorescence but elicited a clear EBV-specific fluorescence in about 15 to 25% of the cells. This line has optimal EBV-specific IgG fluorescence in approximately 50 to 85% of the cells (12).

Test procedure. The cell slides were overlaid by serum dilutions in the same way as for the determination of IgG antibodies to EBV, but the incubation of the cells with serum was performed in a moist chamber at 37 C for 3 h instead of 45 min. Schmitt and Scherer (21) have found that a long incubation period with serum is essential for detection of IgM antibodies to EBV. This is perhaps due to the blocking effect of IgG antibodies, which have a smaller molecular weight and thus a faster diffusion rate. With long incubation, a partial exchange takes place between the IgM and IgG molecules, which are fixed to the antigen. Also, in this study a clear increase in IgM fluorescence was noted after incubation with the sera for 3 h instead of 45 min. After incubation of the cells with sera, the slides were washed with phosphate-buffered saline (PBS) for 15 min on a magnetic stirrer with three buffer changes, dried, overlaid with anti-human IgM conjugate (Wellcome) at a dilution of 1:20, and incubated again for 45 min. After being washed with PBS for 10 min and dipped in distilled water, the slides were mounted, a cover glass was placed over them, and the IgM antibody reading was carried out.

Reproducibility and sensitivity of the test. A serum pair from a child with infectious mononucleosis was tested for IgM reactivity against P3HR-1 cells on 35 different days. A titer of 1:80 was obtained twice, 1:160 29 times, and 1:320 four times in the first serum specimen. The second specimen taken 6 months after the onset of illness was negative in each test for IgM antibodies to EBV. Both serum specimens had a 1:160 IgG antibody titer to EBV.

Specificity of the test. To separate the IgM antibodies from other serum components, the sera were fractionated on a 37.5 to 12.5% (wt/vol in PBS) sucrose density gradient (2). The SW39 rotor was spun in Spinco model L ultracentrifuge for 18 h at 35,000 rpm. Ten fractions were collected from each gradient and stored at −20 C until testing. Three sera were fractionated in this way. The first had a very high IgG antibody titer to EBV, but it had a negative response to IgM antibodies to EBV and to heterophil antibodies. The second serum had a positive response to all these antibodies. The third specimen had a positive response to IgG antibodies to EBV and a negative one to heterophil antibodies, but gave a nonspecific IgM fluorescence to EBV due to the rheumatoid factor (6). This factor belongs to the IgM group and forms complexes with IgG antibodies, thus giving the nonspecific IgM fluorescence. For detection of the rheumatoid factor, Hyland’s latex agglutination slide test was used in all sera with positive reactions to IgM antibodies to EBV. The testing of fractions for all different types of antibodies is described. The same sera, with positive reactions to EBV IgM fluorescence but negative to latex agglutination, were absorbed with heat-aggregated human gamma globulin (Finnish Red Cross) by the method of Shirodaria et al. (23). After overnight incubation at +4 C, the aggregate was centrifuged away and the supernatant was tested for IgM fluorescence again. This was done in order to eliminate the possible anti-IgG giving false IgM fluorescence but not reacting in latex agglutination (23).

GP test. The micro GP test (19) was used to measure fluorescent antibodies to EBV. The EBV antigen for this test was made from a Burkitt lymphoma cell line P3HR-1. The crude antigen made from Tween 80-treated Burkitt cells was used. The antigen preparation and test procedure is described elsewhere (19).

CF technique. The same antigen as in the GP assay also seemed to be useful in the CF antibody test. Sever’s modified microtechnique (22) as described by Vihma (25) was used. Veronal-buffered saline with 0.1% gelatin was the diluent. The sera were heated at 56 C for 30 min, and the twofold dilutions of sera beginning from 1:5 were tested for CF reactivity with P3HR-1 antigen. The hemolytic system consisted of equal parts of 2% sheep erythrocytes and a dilution of hemolysin containing 2 full units. Four antigen units and about 1.6 units of complement were used in the test. After overnight incubation at +4 C, the hemolytic system was added and the plates were incubated at +37 C for 1 h. Then the plates were placed at +4 C for 1 to 2 h and read by visual estimation of the degree of hemolysis. The inhibition of hemolysis was recorded from 0 to 4, and readings 4+ and 3+ were considered positive. Serum titers were expressed as the reciprocal of the highest serum dilution still giving...
a positive reaction. Positive and negative sera from a patient with IM were included as controls in each test.

The control antigen for the CF test was made from human fetal fibroblasts in the same way as from P3HR-1 cells.

**Determination of heterophil antibodies.** The sheep erythrocyte agglutination technique with differential absorption (Paul-Bunnel) (3) was used to determine antibodies found in infectious mononucleosis.

**Handling of the data.** Exact details about the onset of illness given by the patients were available from all except four patients with a recent EBV infection. These four gave eight serum specimens, which are not included in the antibody curves.

The geometric mean titers used in the antibody curves were calculated from the logarithms of the respective titer values. The value 1 was chosen for titers 10 and 5.

**RESULTS**

**Fluorescent IgG antibodies to EBV.** All 58 patients with IM had fluorescent IgG antibodies to EBV, and these developed very rapidly. Only one patient had serum specimens taken so early that there was a lack of antibodies in the first specimen. Information about the day of onset of illness was available from 54 patients with IM. The IgG antibodies to EBV in their sera are shown in Fig. 1. The curve represents the geometric mean titers, which remained fairly stable throughout the observation period. The titers rose so rapidly that only 13 patients (22%) had a significant fourfold or greater rise in titers in the paired serum specimens taken in the acute phase and 10 to 20 days later. A significant fall in titers was detected in only 3 patients (5.2%).

**Fluorescent IgM antibodies to EBV.** All 58 patients with IM had IgM antibodies to EBV. These also developed rapidly, and the titer was usually at maximal level even in the first specimen (Fig. 2). Seven patients (12%) had a significant rise in IgM antibody titers in the serum pairs taken in the acute and convalescent phase. A significant fall in the titer of the paired serum specimens was noted in 11 patients (19%). The IgM antibodies to EBV disappeared very rapidly after the onset of illness, and after 12 weeks no patients had IgM antibodies to EBV.

**GP antibodies to EBV.** A seroconversion or a clear rise in GP antibodies was noted in sera from 33 patients (57%) with IM, and the GP titer was at maximal level even in the first specimen taken from 17 patients (29%). Altogether 50 patients (86%) had GP antibodies, and these developed slowly in most cases (Fig. 3 and 4). The GP antibodies kept rising for up to 10 weeks on the average, and none of the patients had a fall in GP antibody level. All patients who were observed for more than 12 weeks had GP antibodies (Fig. 5), but the patients with negative reactions were followed only up to maximum of 4.5 weeks after the onset of illness.

**CF antibodies to EBV.** The CF antibodies also developed slowly and the titer kept rising on the average for up to 10 weeks, the same as that of the GP antibodies (Fig. 6). A seroconversion or a significant rise in CF titers was detected in 11 patients (19%), 4 of them (6.9%) having the rise in the paired serum specimens. The rise in CF titer was not significant or the titer was at the maximal level already in the first specimen taken from 30 patients (52%), and only one patient had a significant fall in CF antibodies during the observation period. Altogether 42 patients with infectious mononu-
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Fig. 3. Development of gel-precipitating antibodies to EBV in sera from a patient (U.G.) with infectious mononucleosis. (1) 1.5 weeks after onset of illness; (2) 3 weeks after onset of illness; (3) 7 weeks after onset of illness; (4) 44 weeks after onset of illness; (5) 60 weeks after onset of illness; (6) positive control serum with weak reaction; (P3) Burkitt cell antigen.

Heterophil antibodies. In consequence of selection of study patients, all had heterophil antibodies, which were demonstrable soon after the onset of illness. They also disappeared rapidly, but slightly slower than IgM antibodies to EBV (Fig. 7).

Rheumatoid factor. Seven patients (12%) with IM had some rheumatoid factor-related activity in their sera tested with Hyland’s latex agglutination test. The agglutination was, however, much weaker than that of the positive control serum included in the test set. This latex test was made because the rheumatoid factor gives false-positive IgM fluorescence when present together with the specific IgG antibodies. Two patients had only a transient reactivity. Five patients had a similar reactivity in both the acute and early convalescent sera. The producer of the reagent states that about 4% of the normal population has some latex agglutinating reactivity in their sera with the Hyland latex agglutination slide test. The five patients with positive reactions in this study had, however, a clear EBV infection, since two of them had a significant fall in IgM titer; one had a significant rise in all types of antibodies. One of the remaining two had a significant rise in GP antibodies, and the other had a significant fall in IgG antibodies.

Control sera. The 50 control serum specimens all had positive reactions to EBV IgG antibodies. Three specimens also had positive reactions to EBV IgM fluorescence, the titers being 10, 20, and 40. All these specimens had negative reactions to latex agglutination and thus were absorbed by heat-aggregated human gamma globulin. When tested after absorption, only one serum gave IgM fluorescence at a titer of 20. This absorption procedure did not seem to affect the EBV-specific IgM fluorescence but removed almost completely the false IgM reactivity.

Comparison between the different EBV
Fig. 4. Development of gel-precipitating antibodies to EBV in serum specimens from a patient (A.H.) with infectious mononucleosis. (1) 0.5 week after onset of illness; (2) 1.5 weeks after onset of illness; (3) 16 weeks after onset of illness; (4) 18 weeks after onset of illness; (5) 38 weeks after onset of illness; (6) positive control serum from another patient with infectious mononucleosis; (P3) Burkitt cell antigen.

Fig. 5. Gel-precipitating antibodies to EBV in 54 patients with infectious mononucleosis. The curve represents the mean titers.

antibodies and the heterophil antibodies. All the individual EBV antibody and heterophil antibody curves can be seen in Fig. 8. There was a rapid, almost simultaneous development of the IgG and IgM antibodies to EBV and the heterophil antibodies, and the antibody curves had similar slopes. Two to three weeks after the onset of illness there were already differences,

Fig. 6. Complement-fixing antibodies to EBV in 54 patients with infectious mononucleosis. The curve represents the mean titers.
because the IgM antibodies to EBV and the heterophil antibodies began to disappear, whereas the IgG antibodies remained. Both the GP and CF antibodies developed slowly and kept rising for up to approximately 10 weeks, by which time the IgM antibodies to EBV and the heterophil antibodies had disappeared.

**Comparison of the GP and CF antibodies.** Both GP and CF antibodies developed slowly after the onset of illness. Eight patients (14%) had negative reactions to GP antibodies, but six of them had CF antibodies to EBV. Sixteen patients (28%) had negative reactions to CF antibodies, but 14 of them had GP antibodies to EBV. Only two patients had negative reactions to both CF and GP antibodies, but these patients were observed for only 3.5 weeks after the onset of illness.

**Clinical picture and the antibody status.** The 23 patients who were treated in the Department of Infectious Diseases, University of Turku, were studied carefully for the clinical signs of IM. These were typical in every patient. The peripheral blood smear, erythrocyte sedimentation rate, blood chemistry for detection of liver damage as well as the antistreptolysin O and antistaphylolysin titers, urinalysis, routine bacteriological cultures from throat, sputum (if available), feces, and urine, and the Mantoux reaction were checked. The patients with rising or falling EBV IgG antibody titers as well as those with exceptionally high EBV IgG and IgM titers were studied with special care. The antibody titers showed no correlation to the clinical symptoms and signs or to the clinical and bacteriological laboratory findings.

**Control tests on the specificity of the serological assays for EBV antibodies: fluorescent IgG antibody technique.** In some earlier studies of fluorescent IgG antibodies to EBV in sera from patients with systemic lupus erythematosus, the antinuclear antibodies have also been detectable with Burkitt cell antigen (4). These two types of antibodies have, nevertheless, been easily distinguishable. If antinuclear antibodies have been detected with the Burkitt cell antigen, a nuclear fluorescence has been observed in about 80% of the cells. The specific EBV fluorescence was detectable in only 1 to 3% of Silfere cells (Fig. 9) and in 15 to 30% of P3HR-1 cells (Fig. 10). A serum with a positive reaction to fluorescent antinuclear antibodies at a titer of 1:1,280 tested with mouse liver antigen was also titrated with Silfere and P3HR-1 antigens for EBV IgG antibodies with the same conjugate as in the antinuclear antibody test. The serum had with both Burkitt cell antigens an antibody titer of 1:320 to EBV. The fluorescence was specific for EBV, and no nuclear fluorescence, typical of antinuclear antibodies, was noted.

**Fluorescent IgM antibody technique.** To study the EBV-specific IgM antibodies (Fig. 11), three serum specimens were fractionated in sucrose gradient ultracentrifugation (Table 1). The first serum had only IgG antibodies to EBV, the second contained IgG and IgM antibodies to EBV and also heterophil antibodies, and the third had IgG antibodies to EBV and rheumatoid factor and gave a false-positive IgM fluorescence (6). After the sucrose gradient ultracentrifugation, 10 fractions were collected from each specimen and tested for fluorescent IgG and IgM antibodies to EBV, heterophil antibodies, and rheumatoid factor (Table 1). The IgG and IgM antibodies to EBV were detected in different fractions with no overlapping; IgM antibodies appeared near the bottom of the gradient below the IgG antibodies. Heterophil antibodies were found in the same fractions as IgM antibodies to EBV. After the
complete absorption of heterophil antibodies in fractions 3 and 4 of serum 2 (Table 1) with bovine erythrocytes, the IgM antibodies were left and the titer was unchanged. Thus the IgM antibodies to EBV are not dependent on heterophil antibodies. The serum 3 having rheumatoid factor did not give any false-positive fluorescence after fractionation, because the IgG antibodies to EBV and the rheumatoid factor were in different fractions. When the
**FIG. 11.** Fluorescent IgM antibodies as detected with the P3HR-1 antigen. Left, Positive serum; right, negative serum. Both sera have positive reactions to IgG antibodies to EBV.

### TABLE 1. Sucrose gradient fractionation of serum specimens with EBV IgG and IgM antibodies combined with heterophil antibodies or rheumatoid factor

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<tr>
<th>Fraction no. (from bottom to top)</th>
<th>Serum 1</th>
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* Serum 1:EBV IgG+, EBV IgM−, heterophil antibodies (HA)−, rheumatoid factor (RF)−. Serum 2:EBV IgG+, EBV IgM+, HA+, RF−. Serum 3:EBV IgG+, EBV IgM+ (nonspecific), HA−, RF+. The dilutions indicate the antibody titers obtained in the respective fractions.

**rheumatoid factor-containing serum was absorbed with aggregated human gamma globulin, both the rheumatoid factor and the false-positive IgM fluorescence were eliminated.**

**GP technique.** It is known that false-positive serological reactions of a transient nature sometimes develop in the course of infectious mononucleosis (5). Because the development of gel-precipitating antibodies to P3HR-1 crude antigen was almost a constant finding in this disease, some control GP tests were also made. An adequate control antigen should have been an EBV-free lymphoblastoid cell line. Since all established lymphoblastoid cell lines nevertheless contain EBV genome and also have EBV-specific CF activity (1), an identical control crude antigen for GP tests was made from diploid human fibroblasts. No positive GP reactions were obtained with this control antigen in 66 sera from 13 patients with infectious mononucleosis who developed strong precipitations to P3HR-1 antigen. A serum from a patient with some connective tissue disease, however, gave a positive GP reaction with this antigen.
CF technique. The complement fixation antigen for determination of CF antibodies to EBV was the same as GP antigen but diluted 1:40 with PBS. The control antigen for this test was also the same as for the GP assay and diluted 1:40. No positive reactions were obtained with this control antigen in 26 sera from five patients who had a strong rise in CF titer.

DISCUSSION

The fluorescent antibody technique for IgG antibodies to EBV (anti-VCA antibodies) is the most specific and sensitive of all techniques for detection of EBV antibodies. But this technique is not an ideal method for determination of antibodies in recent infections, because these antibodies develop very rapidly and probably remain throughout life (7) and the fall in titer is seldom detected. Therefore, no conclusions can be drawn from the high IgG antibody titers as an indication of a recent infection. This technique is ideal for seroepidemiological studies and for detection of recent infections if the pre-illness serum is available.

The micro GP antibody technique using the crude P3HR-1 Burkitt cell antigen is also sensitive and suitable for routine diagnosis. There are, however, patients who remain negative towards GP antibodies, because the sensitivity is lower than with the indirect immunofluorescence technique. The slow development of GP antibodies also makes it possible to confirm the diagnosis later if the acute-phase serum specimen is missing. Some of the patients in this study could not be observed for more than a few weeks, during which time the GP antibodies had not yet developed. The same crude P3HR-1 antigen was used for determination of CF antibodies to EBV. The CF technique was not, however, as sensitive as the GP method, since 28% of the IM patients had negative reactions to CF as compared with 14% with negative reactions to GP antibodies. A similar observation about the sensitivity of these two tests has been made for human wart virus antibodies (18). One explanation for this may be the nonreactivity of the IgM antibodies in the CF test but reactivity in the GP assay. This explanation will not, however, clarify the discrepancy between the GP and CF tests.

The most promising and useful of these tests in diagnosis of recent infections was the IgM antibody technique. All patients with a recent EBV infection according to other serological tests had IgM antibodies to EBV, but these disappeared very rapidly. The determination of IgM antibodies directly from the serum without fractionation was practical and suitable for routine use. The latex agglutination test for rheumatoid factor must, however, be made on all positive sera in order to confirm the specificity of the IgM fluorescence. It is known that patients with heterophil antibody-positive IM can sometimes have transient rheumatoid factor (27), which can render the IgM test from the whole serum useless. In those cases the conventional sucrose gradient ultracentrifugation can be used because it seems to separate the EBV IgG rheumatoid factor complexes. If then the fractions with or without latex agglutination indicate IgM fluorescence, a diagnosis of an EBV infection can be made. Absorption of the rheumatoid factor with heat-aggregated human gamma globulin also seemed to be a good method. This completely eliminated the false-positive fluorescence as well as the latex agglutinating activity. This method may be more suitable for routine use than the sucrose gradient ultracentrifugation.

With the EBV IgM antibody technique, a diagnosis also can be made from only one serum specimen containing IgM antibodies to EBV. The rheumatoid factor must, however, be excluded. Shirodaria et al. (23) have recommended that all serum specimens being used for virus-specific IgM fluorescence be absorbed with aggregated human gamma globulin. The control material of this study, in which some sera with negative reactions to latex agglutination gave false-positive IgM fluorescence that was eliminated by gamma globulin absorption, provides evidence for this.

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LITERATURE CITED


