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New Oligopeptide Immunoglobulin G Test for Human Parvovirus B19 Antibodies

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A new, highly sensitive and specific enzyme immunoassay using oligopeptides as antigen (enzyme-linked immunosorbent assay [ELISA] B19-OP) for detecting parvovirus B19-specific immunoglobulin G (IgG) was established. As antigens, B19-specific oligopeptides of 24 and 30 kDa derived from a 196-kDa fusion protein of β-galactosidase and viral capsid protein (VP1) of B19 after CNBr cleavage and separation by high-pressure liquid chromatography were used. Of 139 serum specimens tested in parallel for anti-B19 IgG by standard ELISA using B19 particles as antigen and by ELISA B19-OP, 73 (52.5%) were positive and 63 (45.3%) were negative in both tests, and 3 (2.2%) were negative by standard ELISA but positive by ELISA B19-OP and by immunoblot. By using ELISA B19-OP, it was possible to detect anti-B19 IgG in an asymptomatic blood donor 4 weeks after acute infection, and anti-B19 IgG titers of 10^{-5} could be measured in convalescent-phase sera.

The human parvovirus B19, first discovered by Cossart et al. in 1975 (9), causes erythema infectiosum (3). In patients with chronic hemolytic anemia, B19 infection can cause aplastic crisis (4), and in pregnancy, B19 infection can lead to hydrops fetalis resulting in fetal loss (6, 18). Recently, it has been shown that chronic B19 infection can occur in immunodeficient patients (14). Transmission of B19 by clotting-factor concentrates in hemophilic patients has been reported (5, 16), although the clinical features of such infections have not been thoroughly investigated. B19 infection can also be associated with vascular purpura (15).

Until now, the serological diagnosis of acute or past B19 infection has been based on the detection of specific immunoglobulin M (IgM) or IgG by using viral particles partially purified from plasma obtained from patients during the viremic phase of B19 infection as antigen (1, 2, 8, 17). The scarcity of B19 antigen-positive plasma limited the possibilities of B19 antibody testing, and attempts to grow B19 in permanent cell cultures have been unsuccessful. Recently, B19 DNA was transfected into the genome of a Chinese hamster ovary cell (CHO) line which then expressed B19 capsid proteins as noninfectious virionlike particles to be used as antigen for serological assays (12). Also, the establishment of an enzyme-linked immunosorbent assay (ELISA) for the detection of B19-specific IgM and IgG by using a synthetic peptide has been recently reported (10). The gene of the structural viral protein (VP1) of B19 had been previously cloned into the expression vector pMLB 1115.216 and expressed in *Escherichia coli*, and thus a 196-kDa β-galactosidase B19 fusion protein was obtained which was reactive in immunoblot (20). However, until now, problems with solubility have prevented its use for ELISAs. In this paper, we describe the fragmentation of this B19 fusion protein by cyanbromide (CNBr) cleavage and the use of the resulting oligopeptides for detecting B19-specific IgG by an oligopeptide enzyme immunoassay (ELISA B19-OP).

MATERIALS AND METHODS

Clinical specimens. A total of 139 serum specimens with known B19 antibody status were tested. The 139 serum specimens were as follows. (i) The first group of serum specimens were acute-phase sera from an asymptomatic blood donor (A; $n = 8$) and a patient with erythema infectiosum (B; $n = 15$) collected 4, 6, 8, 12, 17, 19, 21, and 36 weeks after viremia (A) and up to 19 weeks after onset of clinical disease (B). These 23 serum specimens (A and B) were all anti-B19 IgG positive; up to week 19 (A) and week 14 (B), the sera were also IgM positive as determined by ELISA (17). Except for sera from weeks 21 and 36, sera had been titrated previously for anti-B19 IgG by ELISA (17), and the titers were 10^{-3} (4 weeks), 10^{-5} (6 weeks), 10^{-5} (8 weeks), 10^{-6} (12 weeks), 10^{-6} (17 weeks), and 10^{-5} (19 weeks), with a prozone effect up to a dilution of 1:100 in all sera taken after week 4 following viremia. (ii) The second group of serum specimens included 50 that were positive for anti-B19 IgG but negative for IgM by ELISA. (iii) The third group included 59 serum specimens negative for anti-B19 IgM and IgG. (iv) The last group included sera ($n = 7$) positive for rheumatoid factor by latex agglutination but negative for anti-B19 IgG by ELISA (kindly supplied by B. Wilske, Munich, Federal Republic of Germany). Additionally, as random samples, sera of blood donors ($n = 80$) from Chemnitz/GDR (kindly supplied by E. Gerike, Berlin, German Democratic Republic) with unknown B19 antibody status were tested to determine the cutoff of the ELISA B19-OP.

Antigen preparation. The 196-kDa fusion protein of β-galactosidase and VP1 of parvovirus B19 truncated for 51 amino acids at the C terminus (20) was cleaved by the CNBr method described by Gross and Witkop (11). According to sequence data of β-galactosidase and VP1 of B19, 23 oligopeptides of β-galactosidase (13) and 11 of VP1 (19) (Fig. 1) should be generated after CNBr cleavage. For the cleavage

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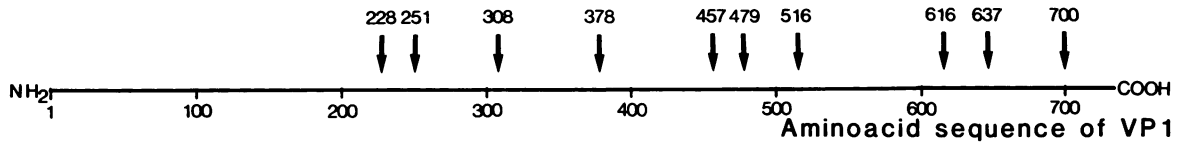

FIG. 1. CNBr cleavage sites of all methionyl peptide bonds of the structural viral protein (VP1) of parvovirus B19 (19).

reaction, 1 ml of 70% formic acid mixed with 0.1 mg of CNBr was added to 1 ml of the B19 fusion protein preparation (0.3 mg/ml in 6 M urea), vortexed, and incubated for 4 h at room temperature. A 1-ml volume of H_2O was then added. After freeze-drying (Speed Vac Concentrator; Savant Instruments, Farmingdale, N.Y.), the sample was dissolved in about 4 ml of 1% trifluoroacetate and 6 M guanidine-hydrochloride and was then fractionated by high-pressure liquid chromatography (HPLC) by using a C4 wide-pore reversed-phase column (Baker, Gross-Geran, Federal Republic of Germany). The flow rate was 2.0 ml/min when a gradient of from 0 to 80% acetonitrile in 0.1% trifluoroacetate and H_2O was used. Fifty fractions of 2 ml were collected, freeze-dried in a speed-vac centrifuge, and resuspended in 150 μl of phosphate-buffered saline (PBS) plus 35 μl of 6 M guanidine.

Detection of B19-specific oligopeptides. To identify oligopeptides of VP1 reacting with B19-specific IgG, the HPLC fractions were diluted 1:100 in 0.2 M carbonate buffer, pH 9.5, and 50 μl of each fraction was placed in duplicate wells of flat-bottomed microtiter plates (Greiner, Nürtingen, Federal Republic of Germany) and incubated overnight in a wet chamber at 4°C. After washing three times with PBS containing 0.5% Tween-20 (washing buffer), 50 μl of an anti-B19 IgG-positive but IgM-negative serum or an anti-B19 IgG- and IgM- negative serum (dilution of 1:100 in PBS containing 2% Tween-20 and 3% fetal calf serum [PBS-TF]) was added to each well and the wells were incubated for 2 h at 37°C in a wet chamber. The wells were washed three times, 50 μl of a peroxidase-conjugated anti-human IgG (Dako, Copenhagen, Denmark), diluted 1:1,000 in PBS-TF, was added, and the wells were then incubated for 1 h at 37°C. Finally, plates were washed three times, aspirated to dryness, and incubated with 100 μl of *ortho*-phenylenediamine (10 mg/ml) and H_2O_2 (1 μl/ml) diluted in 0.2 M phosphate buffer, pH 6.0, for 15 min at 4°C in the dark. The reaction was stopped with 100 μl of 1 M H_2SO_4, and the optical density (OD) was measured in a multichannel photometer (SLT, Salzburg, Austria) at 492 nm.

Oligopeptide ELISA. For testing clinical specimens, the ELISA B19-OP was performed as described above by using dilutions (1:300) of fractions containing VP1 oligopeptides reacting with anti-B19 IgG, a test serum dilution of 1:100, and a dilution of peroxidase-conjugated anti-human IgG of 1:1,000. As controls, two anti-B19 IgG-positive and IgM-negative and two anti-B19 IgG-negative and IgM-negative serum specimens were tested in duplicate in each microtiter plate. Test results were considered valid if the mean OD of the positive control serum specimens was ≥0.700 and if the mean OD of the negative control serum specimens was ≤0.160. Sera were regarded as positive for anti-B19 IgG if the mean OD of the test samples (S) was >2.1 the mean OD of the negative control (N).

ELISA. As a reference test, standard ELISAs based on the anti-γ or anti-μ capture assay technique were used as described previously (17). In brief, microtiter plates were coated with anti-γ or anti-μ and incubated with the test serum. After incubation with B19 particles derived from plasma of a blood donor, an anti-B19 monoclonal mouse antibody and a peroxidase-conjugated anti-mouse IgG were added.

Immunoblot. For determining anti-B19 IgG in sera, an immunoblot was performed as described previously (17) by using B19 antigen derived from a blood donor plasma. B19 viral proteins were transferred to nitrocellulose and incubated with test sera and peroxidase-conjugated anti-human IgG.

Oligopeptides of VP1 reacting with anti-B19 IgG were analyzed by immunoblot by a previously described technique (17). After electrophoresis on a 20% sodium dodecyl sulfate (SDS)-polyacrylamide gel, the oligopeptides were transferred to nitrocellulose, incubated first with a serum known positive for anti-B19 IgG but negative for IgM, and then incubated with peroxidase-conjugated anti-human IgG.

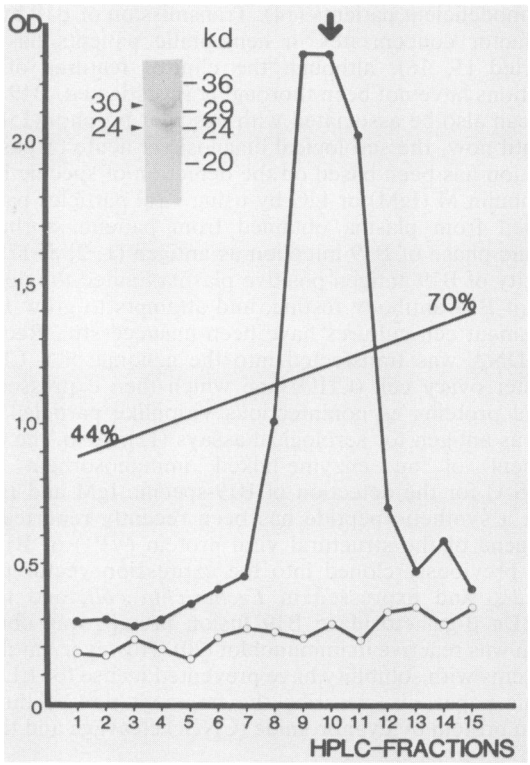

FIG. 2. Determination of B19-specific oligopeptides by enzyme immunoassay after CNBr cleavage of a recombinant β-galactosidase B19 fusion protein and separation by HPLC with an acetonitrile gradient. The sera used are anti-B19 IgG positive and IgM negative (●) and B19 IgG negative and IgM negative (○). Additionally, an immunoblot of fraction 10 is shown.

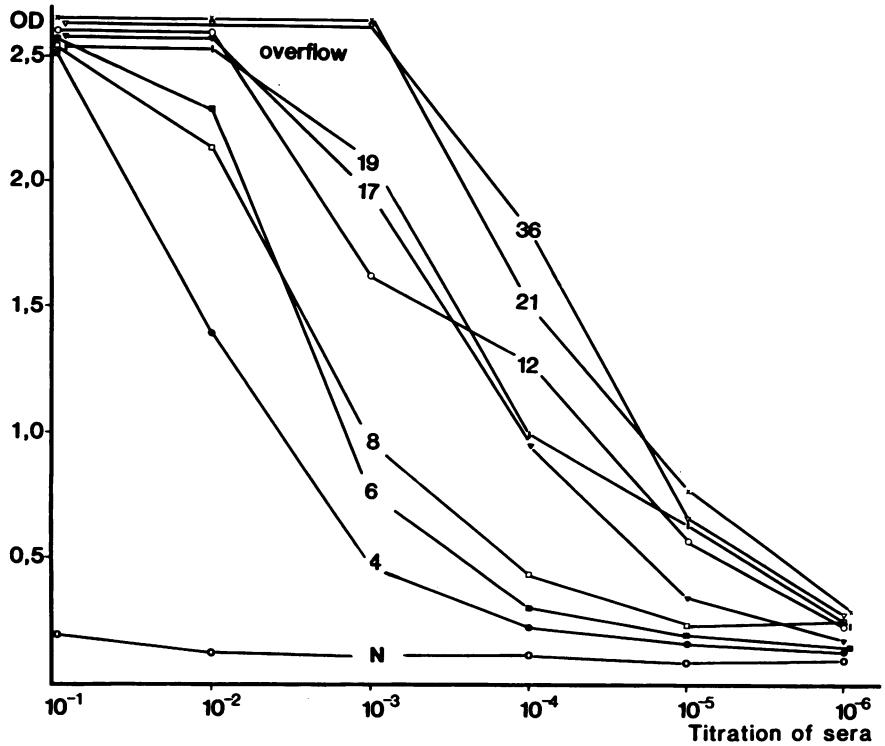

FIG. 3. Titration of sera taken consecutively from an asymptomatic patient (A) 4, 6, 8, 12, 17, 19, 21, and 36 weeks after donation of a B19-positive blood unit and a serum specimen negative for B19 marker N (○) tested by ELISA B19-OP.

RESULTS

Separation of oligopeptides. The HPLC fractions (Fig. 2) were analyzed in an ELISA for the B19 VP1 oligopeptides by using anti-B19 IgG-positive and IgG-negative sera. Fractions 9 to 11 reacted most strongly with anti-B19 IgG-positive serum but not with anti-B19 IgG-negative serum, indicating the presence of immunoreactive B19 oligopeptides in these fractions. Fractions 9 to 11 were titrated in an ELISA by using an S/N ratio of >2.1 with a standard positive control serum as an endpoint. The titers were 10^{-2} for fraction 9, 10^{-3} for fraction 10, and 10^{-2} for fraction 11. Fraction 10 was used, therefore, for further evaluation of the anti-B19 IgG test. Immunoblot analysis of fraction 10 revealed two B19-specific proteins of 24 and 30 kDa which reacted with an anti-B19 IgG-positive serum (Fig. 2).

Evaluation of the ELISA B19-OP. Different panels of sera were tested by ELISA B19-OP to evaluate sensitivity and specificity of the assay.

Sensitivity. Serum specimens (n = 8) taken consecutively at 4, 6, 8, 12, 17, 19, 21, and 36 weeks after B19 viremia in a blood donor (A) were titrated in $\log_{10}$ steps (Fig. 3). Sera in a dilution corresponding to the same dilution of a negative control serum were regarded as positive by ELISA B19-OP if the S/N ratio was ≥2.1. The ELISA B19-OP identified all eight serum specimens as anti-B19 IgG positive, with titers ranging from 10^{-3} (4 weeks) to 10^{-5} (12 to 36 weeks). A prozone effect, as observed in a previous titration of these sera by standard ELISA, was not observed by the ELISA B19-OP (17). For further testing, a serum dilution of 1:100 was chosen. Sera (n = 15) taken consecutively from another patient (B) up to 19 weeks after onset of clinical disease were all positive for anti-B19 IgG in a serum dilution of 1:100, and titration yielded titers of 10^{-3} to 10^{-5}.

Sera (n = 50) positive for anti-B19 IgG but negative for IgM by ELISA (dilution 1:100) from the diagnostic virology laboratories of the Max von Pettenkofer Institute were also anti-B19 IgG positive by ELISA B19-OP.

Specificity. To examine the possibility that false-positive reactions could be caused by rheumatoid factor, seven serum specimens positive for rheumatoid factor but negative for anti-B19 IgG by standard ELISA were tested by ELISA B19-OP; all were clearly negative. Sera (n = 59) which were anti-B19 IgG and IgM negative by standard ELISA were tested by ELISA B19-OP; 56 (94.9%) were negative and 3 (5.1%) were positive by ELISA B19-OP. These three sera (negative by standard ELISA but positive by ELISA B19-OP with an S/N ratio between 2.1 and 3.0) were positive for anti-B19 IgG by immunoblot analysis reacting against the major structural protein VP2. No antibodies were detected against VP1.

In total, results of all sera (n = 139), both anti-B19 IgG positive and negative, of 115 individuals tested previously by standard ELISA (17) were in agreement with results from ELISA B19-OP in 136 serum specimens (97.8%); 3 serum specimens (2.2%) of three individuals which were negative by standard ELISA were positive by ELISA B19-OP.

Reproducibility. For determining the reproducibility of ELISA B19-OP, one negative control serum specimen and two positive control serum specimens were tested repeatedly in eight assays of the same batch of VP1 oligopeptides in duplicate over 5 days. The OD variation of the negative control serum ranged from 0.110 to 0.165, and the two positive control serum specimens consistently gave OD values of >0.800. Only VP1 oligopeptide batches which discriminated between these standard control sera in the OD ranges mentioned above were used for testing.

Screening of blood donor. To investigate the OD distribution of a random sample, sera of blood donors (n = 80) with

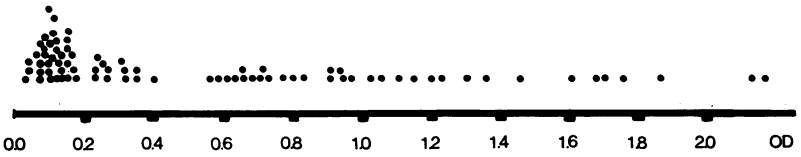

FIG. 4. Scattergram of the OD of sera (n = 80) of blood donors from Chemnitz/GDR, with unknown B19 immune status tested by ELISA B19-OP (dilution 1:100).

unknown B19 antibody status from Chemnitz/GDR were tested by ELISA B19-OP (Fig. 4). Of these 80 serum specimens tested in one assay, 34 (42.5%) gave an OD value of <0.18 (mean, 0.10), 34 (42.5%) gave an OD value of >0.55, and 12 (15.0%) gave an OD value of 0.21 to 0.40. Five serum specimens of the later group (OD of 0.21 to 0.40) were tested by immunoblot, and all five were positive for anti-B19 IgG reacting with VP2. An S/N ratio of >2.1 was therefore taken as cutoff between anti-B19 IgG-positive and -negative sera. Nevertheless, sera in the gray zone with an S/N ratio of 2.1 to 4.0 were regarded as borderline.

DISCUSSION

By CNBr cleavage (11) of the fusion protein and HPLC fractionation of the oligopeptides, we were able to eliminate previous problems with the solubility of a β-galactosidase VP1 B19 fusion protein so that some fractions could be used as an antigen for an enzyme immunoassay (ELISA B19-OP). As shown by immunoblot (Fig. 2), the oligopeptides reactive with B19-specific IgG in the ELISA B19-OP are two proteins of 24 and 30 kDa. Only one oligopeptide of that size (24 kDa) is located at the amino-terminal end at positions 1 to 228, according to the amino acid sequence of B19 VP1. We think there may be an incomplete cleavage at the methionine at position 251, resulting in the additional, larger oligopeptide of 30 kDa. As both oligopeptides were reactive in immunoblot, it is likely that an immunogenic region for the detection of anti-B19 IgG is located in amino acid positions 1 to 228.

It has been published recently that a synthetic peptide of the amino acids at positions 284 to 307 can be used as antigen for detecting anti-B19 IgM and IgG (10). This sequence is localized in the VP2 region. According to the immunoblot analysis, the oligopeptide which we use as antigen for the detection of anti-B19 IgG is localized in the VP1 region.

By using CNBr cleavage and HPLC fractionation of the β-galactosidase VP1 B19 fusion protein, we also tried to obtain VP1 oligopeptides reacting with anti-B19 IgM to establish an IgM test. However, the results obtained so far were inconsistent. Evaluation of the ELISA B19-OP showed that it is highly sensitive and specific for detecting anti-B19 IgG. Sera with known anti-B19 IgG titer by standard ELISA were also found positive with comparable titers by ELISA B19-OP. In addition, anti-B19 IgG could be detected by ELISA B19-OP in three serum specimens negative by standard ELISA, and the anti-B19 IgG positivity of these three serum specimens was confirmed by immunoblot. In using oligopeptides as antigen, it is possible that antibodies directed against the same epitopes present on whole virions are not detected. At present, it is not known whether this causes false-negative results.

With ELISA B19-OP, anti-B19 IgG could be detected in a serum specimen of a patient taken 1 week after onset of erythema infectiosum and 4 weeks after viremia occurred in a blood donor. This early detection of anti-B19 IgG is the same as that of previous observations by using standard ELISA or radioimmunoassay (1, 6, 8, 17). All convalescent-phase sera were anti-B19 IgG positive by ELISA B19-OP.

The OD distribution of a random sample of sera of blood donors (n = 80) showed that an S/N ratio of >5.0 can be determined in 34 serum specimens (42.5%). These sera can be regarded as positive. Thirty-four (42.5%) serum specimens gave an S/N ratio of <2.1 and can be regarded as negative. In 12 (15.0%) serum specimens, the S/N ratio was between 2.1 and 4.0. These sera should be regarded as borderline and retested in another test system to confirm the reactivity. Immunoblot analysis with B19 virus or recombinant B19 viral proteins as antigen should be performed in sera with borderline S/N ratios. However, as immunoblot analysis lacks sensitivity, false-negative results are still possible. Further studies are necessary to clearly define the cutoff value of the ELISA B19-OP. Positive and negative results of specimens in the assay were reproducible. However, as differences in the oligopeptide batches related to purification of crude β-galactosidase VP1 B19 fusion protein and to separation by HPLC were observed, only batches which discriminated between the negative and the positive control sera in the OD ranges mentioned above were used for testing.

Test systems for detecting anti-B19 IgG are still not widely available because of insufficient supplies of B19 virus (1, 2, 8, 17). Use of infectious B19 particles for antibody tests has led to several infections of laboratory staff (7); use of ELISA B19-OP avoids such handling of infectious materials.

In comparison with the standard ELISA and radioimmunoassay (1, 2, 8, 17), by using B19 particles and requiring an overnight step, the ELISA B19-OP demands little time (3.5 h) and makes possible rapid analysis of the B19 immune status. This can be clinically important in cases of contact with B19 in pregnant women, as those who are anti-B19 IgG positive at contact have no risk of infection.

With the B19 oligopeptide enzyme immunoassay (ELISA B19-OP), a test system has been established which can be used widely for anti-B19 IgG screening.

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