

Antibody Response to Human Parvovirus B19 in Patients with Primary Infection by Immunoblot Assay with Recombinant Proteins

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Human parvovirus B19 recombinant VP1 and VP2 capsid proteins were produced by a procaryotic pGEX expression plasmid to evaluate the humoral response by immunoblot assay in 14 patients with primary infection. The same concentrations of VP1 and VP2 recombinant proteins were used. This demonstrates that VP1 immunoglobulin M detection and/or VP1 immunoglobulin G seroconversion is a reliable marker of primary infections. Consequently, detection of antibodies to B19 VP1 might be helpful for identifying patients at risk for chronic B19 infection or patients who are susceptible to viral reinfection.

Specific immunoglobulin M (IgM) and IgG antibodies appear 10 to 12 days and 2 weeks, respectively, after experimental and natural human parvovirus B19 infection (1). IgM antibodies may be found in serum for 3 to 6 months, and IgG antibodies presumably persist for life, with an increase in their level after reexposure (1, 4). Human parvovirus B19 capsids are composed of two structural polypeptides, the minor (4%) VP1 protein (83 kDa) and the major (96%) VP2 protein (58 kDa) (9). Several regions containing neutralizing epitopes have been localized to linear sequences of genes encoding B19 VP2 and VP1 (14). These genes were demonstrated to be functionally important (7). Previous immunoblot assay data were obtained with B19 virus as an antigen and therefore with the two capsid proteins in strikingly different quantities (9, 12). We have produced VP1 and VP2 proteins with a procaryotic expression system and used them in equivalent quantities to evaluate by immunoblot assay the humoral response in patients with primary infections.

The pEMBL-B19 plasmid was used to obtain human parvovirus B19 capsid protein pGEX expression plasmids. This plasmid contains the 5 kb of the viral genome, but because of a cloning artifact, the adenine at nucleotide 3940 is missing (13). The VP1 gene (nucleotides 2444 to 4786) and the VP2 gene (nucleotides 3125 to 4786) were amplified by PCR as described before (8). *EcoRI* restriction sites were synthesized as part of the PCR primers to facilitate subsequent cloning manipulations (5' VP-1 G GGG GAATTC ATG AGT AAA AAA AGT GGC AAA, 5' VP-2 G GGG GAATTC ATG ACT TCA GTT AAT TCT GC, and 3' VP-1-VP-2 G GGG GAATTC CAA TGG GTG CAC ACG GCT). In order to construct an intact coding region, the missing adenine was reintroduced by in vitro mutagenesis performed with the T7-GEN in vitro mutagenesis kit (U.S. Biochemical Corporation). Therefore, we obtained VP genes with sequences strictly identical to those of the wild-type B19 isolate B19-Wi (13). Afterward, amplified DNA was cloned into the pGEX-1AT plasmid expression vector (Pharmacia). The resultant plasmids, designated pGSTVP-1 and

pGSTVP-2, were used to transform *Escherichia coli* JM-109 by standard procedures (14). Induction of transformed cultures with 0.1 mM isopropyl- β -D-thiogalactopyranoside (Serva) resulted in the production of 109-kDa (GSTVP-1, VP-1 [83 kDa] plus glutathione *S*-transferase [GST; 26 kDa]) and 84-kDa (GSTVP-2, VP-2 [58 kDa] plus GST [26 kDa]) fusion proteins.

These proteins were produced in bacteria as insoluble aggregates and could not be purified by affinity chromatography even after denaturation in 8 M urea and soft refolding by dialysis. So, 1 g of induced bacteria was harvested and suspended in 1 ml of lysis buffer (50 mM Tris-HCl [pH 8.0], 25% [wt/vol] sucrose, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride), and the mixture was then treated with lysozyme (2 mg, 4°C for 30 min; Sigma). MgCl₂, MnCl₂, and DNase I (Boehringer-Mannheim) were added to the resulting lysate to final concentrations of 10 mM, 1 mM, and 10 μ g/ml, respectively. After incubation at 20°C for 30 min, 2.5 ml of detergent buffer (0.2 M NaCl, 1% [wt/vol] deoxycholic acid, 1% [vol/vol] Nonidet P-40, 20 mM Tris-HCl [pH 7.5], 2 mM EDTA) was added to the lysate, which was then centrifuged at 5,000 \times g for 10 min. The pellet was washed three times in 0.5% Triton X-100–1 mM EDTA solution.

GST-human parvovirus B19 capsid fusion proteins were used in the immunoblot assay on separate strips. Samples containing 1 μ g of the GSTVP-1 or GSTVP-2 fusion protein were boiled in Laemmli sample buffer, electrophoresed through a sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gel, and electrotransferred (Phast-System; Pharmacia) onto nitrocellulose filters (Schleicher & Schuell) (Fig. 1). As a control, unfused GST (0.5 μ g) was mixed with the GSTVP-1 fusion protein before electrophoresis. The membranes were blocked with 5% skim milk–phosphate-buffered saline (BioMerieux) overnight at 20°C. For IgG antibody detection, the membranes were incubated at 37°C with agitation for 2 h with untreated sera diluted 1:100. After three washes for 10 min each time with PBS–0.05% Tween 20, the filters were incubated with alkaline phosphatase-conjugated goat anti-human IgG (Sigma) at 37°C for 1 h and were then washed as described above. The substrate solution contained nitroblue tetrazolium and bromochloroindolyl phosphate (Sigma). Anti-B19 IgM detection was done after pretreatment of sera with RF-Absorbant (Behring). Final 1:100 dilutions of the sera were incubated overnight at 20°C before soaking them for 1 h at 37°C with alkaline phos-

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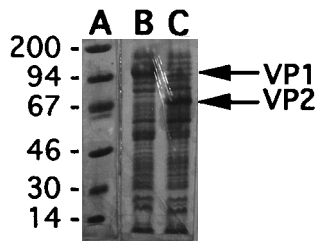


FIG. 1. Coomassie-stained SDS-12.5% polyacrylamide gel showing electrophoretic migration of nonpurified bacterial extracts. Lanes: A, molecular size markers (in kilodaltons); B, bacterial extract containing pGSTVP-1 plasmid (109 kDa); C, bacterial extract containing plasmid pGSTVP-2 (84 kDa).

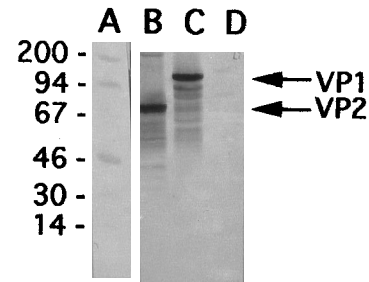


FIG. 2. SDS-12.5% polyacrylamide gel electrophoresis and Western blot analyses of bacterial extracts revealed with sera from patient G and alkaline phosphate-conjugated goat anti-human IgG. Lanes: A, molecular size markers (in kilodaltons); B, bacterial extract containing plasmid pGSTVP-2 (84 kDa); C, bacterial extract containing plasmid pGSTVP-1 (109 kDa); D, bacterial extract containing plasmid pGEX (26 kDa).

phatase-conjugated anti-human IgM (Sigma). Positive controls were obtained with rabbit polyclonal antibodies to the unique region of VP1 and polyclonal antibodies to the common region of VP1-VP2. A semiquantitative interpretation of the Western blot (immunoblot) assay signal was used as follows: weak (+), medium (++), strong (+++).

The results of immunoblot assay are summarized in Table 1 and Fig. 2. The population studied consisted of 14 patients (8

males and 6 females; median age, 11 years) presenting with primary infection (positive antigenemia at day 0). Six presented with aplastic crisis, two presented with arthralgia, one presented with aplastic anemia, one presented with erythema

TABLE 1. Anti-B19 parvovirus IgM and IgG antibodies to VP-1 and VP-2 capsid proteins determined by immunoblot assay in 14 patients with primary infections

Patient	Sex, ^a age (yr), condition	Time after antigenemia (days), CIE ^b	DNAemia (PCR) result	RIA (P/N) ^c		Western blot VP-1		Western blot VP-2	
				IgM (≥ 1.5)	IgG (≥ 6.1)	IgM	IgG	IgM	IgG
A	F, 44, arthralgia	5	-	17.0	10.0	+++	-	+	-
		40	-	10.0	22.0	++	++	-	-
		100	-	2.0	16.0	+	+++	-	-
		114	-	1.6	14.0	+	+++	-	-
		153	-	1.0	15.0	+	++	-	-
B	F, 30, arthralgia	8	-	15.0	19.0	++	+	+	-
		150	-	3.0	23.0	-	+++	-	+
		210	-	1.5	28.0	-	+++	-	+
C	M, 16, erythema infectiosum	14	-	12.0	10.0	-	+	-	+
D	F, 31, aplastic crisis	6	+	7.6	10.0	+	-	-	-
E	M, 9, aplastic crisis	6	+	12.6	7.5	++	-	+	-
		10	+	11.0	7.0	++	-	+	-
		39	+	22.0	170.5	+	+	+	+
		240	-	0.9	6.9	-	++	-	+
F	M, 6, aplastic crisis	540	-	1.0	16.0	-	+	-	+
		920	-	1.0	8.0	-	+	-	+
		19	+	16.0	15.7	+	++	+	++
		12	+	15.6	23.0	++	+	++	-
G	M, 5, aplastic crisis	42	+	14.4	25.0	+	+	+	-
		94	-	6.7	30.0	+	++	-	-
		142	-	5.8	30.0	+	++	-	-
		51	-	5.4	9.3	+	+	-	+
H	F, 27, aplastic crisis	100	-	7.5	12.0	+	++	-	+
		114	-	4.2	15.0	+	++	-	+
		133	-	3.9	12.0	+	++	-	+
		160	-	2.1	15.0	+	++	-	-
I	M, 9, aplastic crisis	175	-	1.5	16.0	-	++	-	-
		37	-	12.7	22.0	-	+	-	-
		60	-	4.1	14.0	-	++	-	-
		69	-	3.0	10.2	-	++	-	-
J	M, 6, aplastic anemia	30	-	6.0	6.1	+	+	+	+
		35	-	6.4	10.4	+	+	-	+
K	F, 35, pregnancy	7	+	38.0	32.0	+++	-	+++	-

^a F, female; M, male.

^b CIE, counterimmunoelectrophoresis.

^c P/N ratio, positive/negative ratio; values in parentheses are the cutoff values in the RIA.

^d NK, not known.

infectiosum, and one presented with fetal hydrops; no clinical data were available for three patients. For seven patients, sequential serum specimens were obtained, whereas for the remaining seven patients, only one serum specimen was tested. Altogether 33 serum specimens were collected and studied by quantitative radioimmunoassay (RIA) (IgM, IgG) and counter immunoelectrophoresis (antigenemia) (4, 5). Sera from each patient were tested for B19 DNA (DNAemia) by PCR as described previously (3).

In 12 of 14 patients, VP1 IgM antibodies were efficient markers for confirming primary human parvovirus B19 infection; conversely, VP2 IgM antibodies were present in only three patients and were absent from six patients. VP1 IgM antibodies were detected for as long as 5 months in two patients (patients A and J), but VP2 IgM antibodies disappeared within 1 month (patient F). VP1 IgG antibodies were also detected more often (11 patients) than VP2 IgG antibodies (8 patients). Eight serum specimens from six patients (patients D, E, F, G, H, and N), among which five presented with an aplastic crisis, were DNAemia positive during the first 42 days after antigenemia and VP1 IgM positive. For five patients (patients I, J, K, M, and K), DNAemia was negative, but the first sample was obtained late after positive antigenemia and VP1 IgM antibodies were always detected except in one patient (patient K). The remaining three patients with arthralgia (patients A and B) or erythema infectiosum (patient C) were DNAemia negative at days 5, 8, and 14, and 2 of them were VP1 IgM positive. No antibody to GST was detected in any of the 33 serum specimens. Some discrepancies between RIA and Western blot assay results were found. In patient A, the serum sample obtained at day 153 was IgM negative by RIA but IgM positive by VP1 immunoblot assay. Conversely, for patients C and K, all serum specimens were IgM positive by RIA but VP1 IgM negative by the Western blot assay, and the same discordant result was observed in patient B at days 150 and 210. This suggests that in these patients IgM or IgG antibodies to B19 recognize essentially conformational epitopes.

Our data show that the antibody response against VP1 in patients with primary human parvovirus B19 infection is more frequent, intense, and persistent than that against VP2. Other investigators (10, 12) reported that in patients with B19 infections, VP2 antibodies precede VP1 antibodies. Nevertheless, VP1 and VP2 represent 4 and 96% of the capsid proteins, respectively, explaining the possible misinterpretation of the results in those studies which have used B19 virus as an antigen in immunoblot assay. To avoid this artifact, we used VP1 and VP2 fusion proteins in the same quantities on separate strips. Because it is well established that VP2 amino acids are identical to VP1 amino acids except for 226 residues on the amino terminus of VP1, our results suggest that IgM antibodies are directed mainly against the specific portion of VP1. Moreover, sera from five patients (patients A, B, F, H, and J) collected 150 days after antigenemia showed antibodies (IgG) against VP1, but only two of the patients had an antibody response to VP2. These results are consistent with those of Kurtzman et al. (7), who have demonstrated that both anti-VP1 and anti-VP2 specificities are present in convalescent-phase antisera from healthy human subjects; however, VP1 is the major antigen recognized by late-convalescent-phase antiserum or commercial immunoglobulin preparations. It has been demonstrated that this VP1-specific region is exposed on the surface of the virion; this could explain its high degree of immunogenicity (6, 10). We cannot exclude the fact, however, that antibodies directed against the specific VP1 portion mainly recognize linear epitopes, whereas antibodies against VP-2 were directed against conformational epitopes (15).

Our study suggests that patients with human parvovirus B19-related diseases such as arthralgia or eruption have a brief DNAemia compared with that in patients with aplastic crisis, in whom B19 DNAemia lasts for as long as 42 days. Because our study population was so small, this trend is not statistically significant, but it needs further investigation. Irrespective of the clinical situation, VP1 IgM and/or IgG antibodies are always associated with the clearance of B19 virus from serum. In addition, neutralization correlates with anti-VP1 reactivity by immunoblot assay, as suggested recently (2). Therefore, our immunoblot assay which detects antibodies to VP1 may be useful for identifying patients at risk for reinfection or chronic infection, i.e., the ones who do not elicit an IgM-IgG response to VP1.

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