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Identification of the Region Including the Epitope for a Monoclonal Antibody Which Can Neutralize Human Parvovirus B19

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In this study, we identified a region in the human parvovirus structural protein which involves the neutralization of the virus by a monoclonal antibody and site-specific synthetic peptides. A newly established monoclonal antibody reacted with both viral capsid proteins VP1 and VP2. The epitope was found in six strains of independently isolated human parvovirus B19. The monoclonal antibody could protect colony-forming unit erythroid in human bone marrow cell culture from injury by the virus. The monoclonal antibody reacted with only 1 of 12 peptides that were synthesized according to a predicted amino acid sequence based on nucleotide sequences of the coding region for the structural protein of B19 virus. The sequence recognized by the antibody was a site corresponding to amino acids 328 to 344 from the amino-terminal portion of VP2. This evidence suggests that the epitope of the viral capsid protein is located on the surface of the virus and may be recognized by virus-neutralizing antibodies.

Human parvovirus B19 was discovered in normal human plasma in 1975 (7) and was shown to be the etiologic agent of erythema infectiosum (fifth disease) in normal children (2) and associated with rheumatoid arthritis-like disease in adults (31, 37). In patients with hemolytic anemia, it causes aplastic crisis of bone marrow (11, 24, 29, 30, 33), and in immunosuppressed hosts, it causes persistent pure red cell aplasia (10, 18, 19). Intrauterine infection of the fetus can result in hydrops fetalis and spontaneous abortion in early pregnancy (1, 3, 14). The virion has a single-stranded linear DNA molecule about 5.4 kb long, with hairpin structures at each extremity. It contains two major open reading frames, a 71-kDa nonstructural protein encoded by the left open reading frame and overlapping 83-kDa (VP1) and 58-kDa (VP2) structural proteins encoded by the right open reading frame (5, 8, 9, 34) of the B19 genome. The virus can replicate only in erythroid precursors in bone marrow (25, 28) and in fetal liver cells (38) in vitro. This extreme dependence on host cell differentiation stage is limiting to the replication of the virus. This also makes it difficult to diagnose the infection early and undertake vaccination for high-risk patients such as immunosupressed hosts, patients suffering from hemolytic anemia, and pregnant women.

In this study, we identified the site of the epitope of viral capsid protein (VP1 and VP2), which involved the neutralization of the virus by a colony-forming unit erythroid (CFU-e) assay and enzyme-linked immunosorbent assay (ELISA) with a monoclonal antibody.

MATERIALS AND METHODS

Preparation of the virus. A B19 virus was prepared from the serum of a patient in aplastic crisis and the serum of a normal blood donor which reacted to human antiserum against B19 virus by counterimmunoelectrophoresis as described previously (26). The B19 virus-containing serum was pelleted by centrifugation at 110,000 × *g* for 2 h (Hitachi RP42 rotor) and purified by centrifugation through a gradient of 20 to 65% sucrose prepared in buffer containing 20 mM Tris-HCl, (pH 7.5), 100 mM NaCl, and 1 mM EDTA at 150,000 × *g* for 18 h (Hitachi RSP40T rotor). The fractions corresponding to a density of 1.20 to 1.22 g/ml in which the B19 virus was detected by counterimmunoelectrophoresis and dot blot hybridization were pooled. The viruses were used as an immunogen for the production of a monoclonal antibody and as antigens for the immunoblotting assay described below.

Production of monoclonal antibody. On days 0, 7, and 14, a BALB/c mouse was immunized with a subcutaneous injection of purified B19 virus in Freund's complete adjuvant. Three days after the last immunization, spleen cells from the mouse were fused with plasmacytoma cell line X63-Ag8.653 by using polyethylene glycol 1500 (Boehringer, Mannheim, Federal Republic of Germany [FRG]) (12, 15). After 14 days, the specificity of the antibodies secreted from the hybridomas was screened by dot blot ELISA against B19 proteins. A nitrocellulose sheet (Schleicher & Schuell, Dassel, FRG) was placed in a 96-well microfiltration apparatus (Bio-Rad, Richmond, Calif.), and 0.1 μg of purified virus was added to each well. After the sheet was treated with 1% bovine serum albumin (Sigma, St. Louis, Mo.) in buffer containing 20 mM Tris-HCl (pH 7.5) and 0.5 M NaCl for 1 h at room temperature, the culture supernatant was transferred to each well and incubated for 2 h at 37°C. After each well was washed with buffer containing 20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, and 0.05% Tween-20 (TBS-Tween), alkaline phosphatase-labeled anti-mouse immunoglobulin G (IgG) (Promega, Madison, Wis.) was added as a second antibody, and the sheet was further incubated for 1 h at 37°C. Then the sheet was washed with TBS-Tween. Color development with Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate was carried out according to the manufacturer's protocol. The supernatant of dot blot-positive clones was confirmed by the immunoblotting procedure. The hybridoma cells in the antibody-positive well were recloned twice and passaged as an ascites tumor. Then the ascites fluid was collected and used as the source of monoclonal antibody.

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The specificities of the monoclonal antibodies were determined by the immunoblotting assay.

Immunoblotting procedure. The proteins of the viruses were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions with Laemmli's discontinuous buffer system (21). The separated polypeptides were elecrophoretically transferred to nitrocellulose sheets with an electrode buffer containing 25 mM Tris-HCl (pH 8.3), 192 mM glycine, and 20% (vol/vol) methanol at 0.6 A for 3 h. Then, the nitrocellulose sheets were treated with either seropositive or seronegative human sera or monoclonal antibody as the first antibody for 2 h at 37°C, biotinylated anti-human IgG or biotinyl anti-mouse IgG as the second antibody for 1 h at 37°C, and streptavidin-biotinylated horseradish peroxidase complex (Amersham International, Buckingham, U.K.) as an indicator of bound second antibodies for 1 h at room temperature. After each step, the sheets were washed with TBS-Tween. Polypeptides which reacted with antibodies were developed by horseradish peroxidase with 4-chloro-1-naphthol (Bio-Rad) as a substrate (13, 36).

CFU-e assay. The human sera containing B19 viruses were mixed with diluted monoclonal antibody, human serum of convalescent-phase erythema infectiosum, or medium and incubated at 37°C for 30 min. Bone marrow cells were obtained from a hematologically healthy donor who gave informed consent. Mononuclear cells separated by density gradient centrifugation with Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden) were incubated with the virus-containing mixture or medium at 4°C for 2 h and then added to the CFU-e assay system (25). Assay of CFU-e was done in a plasma clot containing 10^5 bone marrow mononuclear cells in 0.5 ml of Iscov's medium (Gibco Laboratories, Grand Island, N.Y.) containing 20% fetal calf serum, 1% bovine serum albumin (Calbiochem-Behring, La Jolla, Calif.), 10% beef embryo extract (Flow Laboratories, North Ryde, Australia), 1 U of human erythropoietin (Toyobo, Osaka, Japan) per ml, and 10% seronegative human AB plasma. The cultures were evaluated after 7 days of incubation at 37°C with 5% CO_2 in humidified air. After the clot was fixed and stained, the number of CFU-e (defined as colonies of eight or more benzidine-positive cells) were counted. All experiments were done in replicate plates.

Synthetic peptides. Peptides were synthesized according to the predicted amino acid sequence based on the nucleotide sequences of the coding region for the structural protein of B19 virus (34). Twelve peptides were selected for synthesis from hydrophilic regions of the protein (20). The 12 synthetic peptides were tentatively named S-1 to S-12, from the amino-terminal to the carboxy-terminal end of VP2. Their location and sequences are provided in Fig. 1 and Table 1. As a control peptide, FLNTEPSQLPPTAPPLLPHSNLDHI, tentatively named Env, corresponding to amino acids 179 to 199 from the amino-terminal end of the *env* gene product of human T-lymphotropic virus type I (HTLV-I), was synthesized (16). The peptide synthesis was performed manually with a stepwise solid-phase procedure (22), using *tert*-butoxycarbonyl (t-Boc) aminoacyl-4-(oxymethyl)-pam resin as the solid support. Protected amino acids were obtained from the Protein Institute Inc. (Osaka, Japan). The α amino acid groups were protected with t-Boc, and the side chain-protecting groups were as follows: *O*-benzyl for Thr, Ser, Asp, Glu, and Tyr; *N*-tosyl for Arg and His; *N*-2-chlorobenzoxycarbonyl for Lys; *S*-4-methylbenzyl for Cys; and *N*-formyl for Trp. The peptides were cleaved from the resin with liquid hydrogen fluoride and lyophilized. After being dissolved in 5% acetic acid, they were eluted with the same solution to remove by-products of low molecular weight. The purity of individual peptides was examined by reverse-phase high-performance liquid chromatography with an octadecyl (C_{18})-silicated column. The majority of peptides were isolated as a single peak and were used for ELISA without further purification.

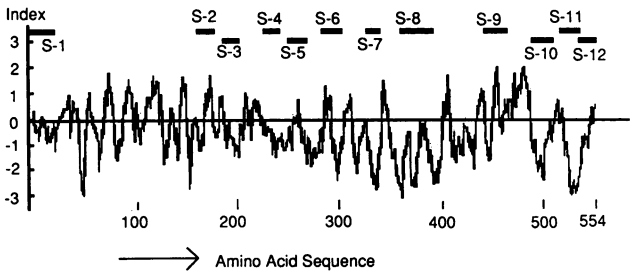

FIG. 1. Hydropathy curve for the polypeptide corresponding to VP2 of human parvovirus B19 (20). The location of each synthetic peptide (named S-1 to S-12) is shown (see Table 1).

ELISA. The wells of a 96-well microtiter plate (Costar, Cambridge, Mass.) were coated with 500 ng of each synthetic peptide dissolved in 0.01 M $NaHCO_3$ buffer (pH 9.55) at 4°C and left overnight. Unreacted sites on the solid phase were blocked with 20% goat serum in phosphate-buffered saline (PBS), pH 7.2, at room temperature for 3 h. Then, hybridoma culture supernatant or human sera was added to the coated microtiter wells and incubated at 37°C for 1 h after six washings with PBS containing 0.05% Tween 20. The plates were further incubated with goat antibodies to mouse or human IgG conjugated with horseradish peroxidase (MBL, Nagoya, Japan) at 37°C for 30 min. The plates were washed six times and developed with 150 μl of 0.1% H_2O_2 and 0.04% *ortho*-phenylenediamine in 0.1 M sodium citrate buffer (pH 5.5). The reaction was stopped by the addition of 50 μl of 2.5 M H_2SO_4, and color development was measured in a plate reader (SLT-Labinstruments) at 492 nm. In a competition ELISA for confirmation, monoclonal antibody preincubated with 5 μg of synthetic peptides or PBS at 37°C for 30 min was added to microtiter wells coated with the peptide. An antibody showing more than a 30% decrease in absorbance in the competition ELISA was judged to be positive.

TABLE 1. Amino acid sequences of synthetic peptides

Peptide	Amino acids[a]	Peptide sequence[b]
S-1	1–23	MTSVNSAEASTGAGGGGSNSVK
S-2	157–176	YKYPYYLGQGQDTLAPELPI
S-3	185–209	AYLTVGDYNTQGISGDSKKLASEES
S-4	223–242	GTGGTASMSYKFPPVPPENL
S-5	253–272	YNPLYGSRLGVPDTLGGDPK
S-6	288–311	FMPGPLVNSYSTKEGDSSNTGAG
S-7	328–344	LRPGPVSQPYHHWDTDK
S-8	359–382	YGNAEDKEYQQGVGRFPNEKEQ
S-9	449–468	LHQPPPQIFLKILPQSGPIG
S-10	491–515	TFKLGPRKATGRWNPQPGVYPPHAA
S-11	520–544	YVLYDPTATDAKQHHRHGYEKPEEL
S-12	536–554	HGYEKPEELWTAKSRVHPL

[a] The numbers represent amino acid numbers in VP2.
[b] Each amino acid is represented in the single-letter code.

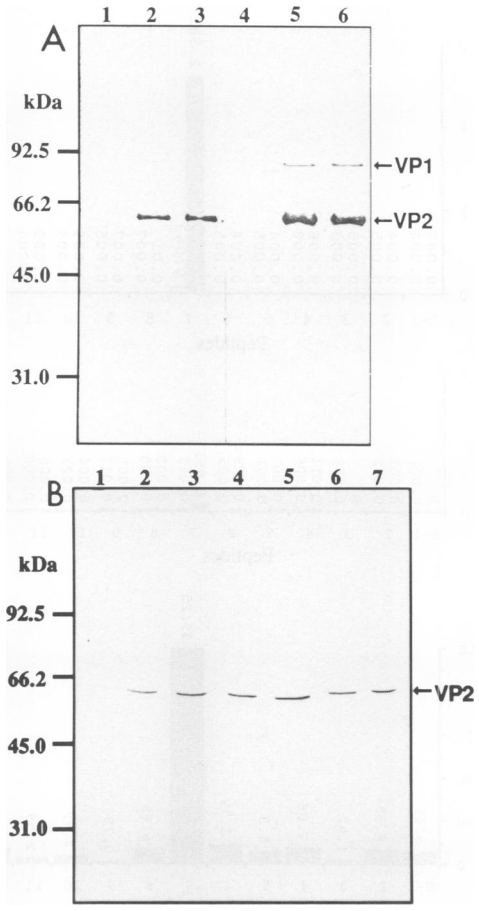

FIG. 2. (A) Antibody specificity of monoclonal antibody BE11 detected by immunoblotting. Either 0.5 μl (lanes 1, 2, and 3) or 3.0 μl (lanes 4, 5, and 6) of purified virus solution was loaded on SDS-PAGE gels and blotted onto nitrocellulose sheets. The sheet was treated with paired human preimmune (lanes 1 and 4) and convalescent-phase (lanes 2 and 5) sera or BE11 cultured supernatant (lanes 3 and 6). (B) Reactivity of BE11 with various isolates of B19 virus by immunoblotting. Lanes: B19 virus-free plasma (lane 1) and independently collected plasma containing B19 virus (lanes 2 to 7).

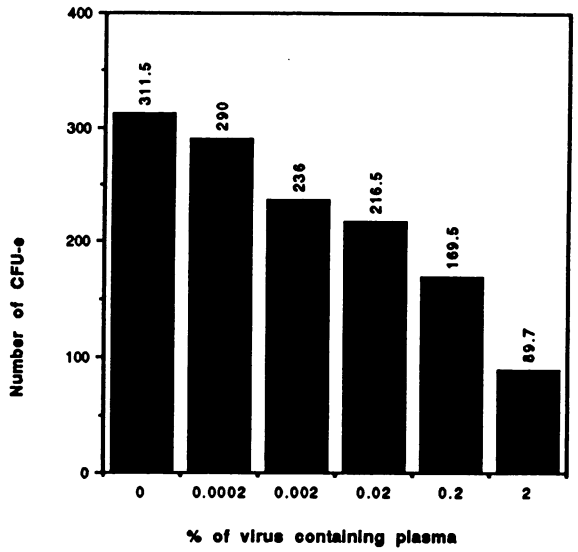

FIG. 3. Cytotoxicity of B19 virus on CFU-e. Bone marrow cells (10^5) were preincubated with serially diluted plasma containing the B19 virus and cultured in a plasma clot in the presence of erythropoietin.

RESULTS

To produce monoclonal antibodies against human parvovirus B19 proteins, we fused the splenocytes from an immunized mouse with myeloma cells to generate hybridomas. Fourteen days after hybridization, the cultured supernatant of each well of three microtiter plates was screened by a dot blot ELISA. Only one hybridoma which secreted antibody against B19 virus was identified by immunoblotting and then was cloned twice. Using rabbit antibodies specific for various subclasses of mouse immunoglobulins, we found that the monoclonal antibody, named BE11, was IgG1(κ).

The antibody specificity of BE11 was examined by immunoblotting. When a small amount of purified virus was used in immunoblotting, a 58-kDa protein corresponding to VP2 of B19 virus reacted with the serum of a patient with erythema infectiosum collected 20 days after B19 virus viremia was diagnosed but not with the serum of the same patient collected 3 months before the infection. The monoclonal antibody reacted with the 58-kDa protein (Fig. 2A). When greater quantities of virus were loaded in immunoblotting, the BE11 also reacted with an 83-kDa protein corresponding to VP1 that was found in lesser amounts in the virion than VP2.

The reactivity of BE11 with six isolates of the B19 virus was examined. Three of them were from asymptomatic blood donors collected in different years and from different populations. Two of them were from erythema infectiosum patients from different hospital, and one was from a patient with hereditary spherocytosis during an aplastic crisis. Virus samples collected in the same hospital and in the same year were excluded from the examination. When whole plasma containing B19 virus was applied in immunoblotting, BE11 reacted with a viral antigen corresponding to VP2 in six isolates of B19 virus but did not react with virus-free plasma (Fig. 2B).

The neutralizing activity of BE11 was examined for protective effect against injury of late erythroid progenitor cells (CFU-e) in the bone marrow by the B19 virus. First, the cytotoxic effect of the virus used in this experiment against CFU-e was confirmed by adding serially diluted plasma containing the virus to the CFU-e assay system as shown in Fig. 3. A dose-dependent decrease in the CFU-e count was observed. When the virus-containing plasma was added at a final concentration of 2% to the bone marrow culture, the CFU-e count showed a decrease of 71.2% versus the culture to which virus-free plasma was added. Then, the B19 virus-containing plasma was preincubated with BE11 for 2 h at 4°C, and the mixture was then added to the CFU-e assay system. As a control experiment, paired sera which were collected from a patient before and after infection with B19 virus were mixed with the virus-containing plasma and added to the assay system. RF465, a monoclonal antibody against p19 of the HTLV-I *gag* gene product was used instead of BE11 as a control ascites fluid (32). As shown in Fig. 4, the cytotoxicity to CFU-e of the virus was prevented by convalescent-phase serum which had anti-B19 antibody, but was not prevented by a serum collected before infection. By pretreatment of the virus with BE11, CFU-e was protected from injury by the virus, and 91% of CFU-e were recovered. The control ascites fluid RF465 was unable to

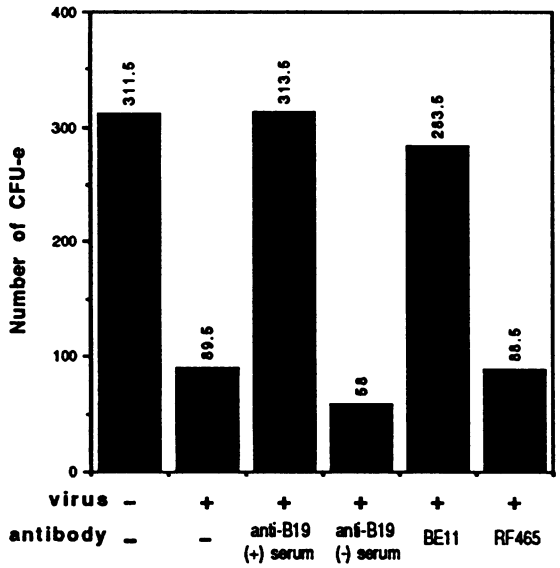

FIG. 4. Protective effect of anti-B19-positive human serum and BE11 from CFU-e injury by B19 virus. B19-containing plasma at a final concentration of 2% was preincubated with human serum or BE11 ascites fluid at 37°C for 30 min, further incubated with bone marrow cells at 4°C for 2 h, and cultured in a plasma clot in the presence of erythropoietin at 37°C for 7 days.

protect CFU-e. Then, serially diluted BE11 was preincubated with virus-containing plasma and added to the CFU-e assay. Ascites fluid of BE11 showed a neutralizing effect at up to a 1,000-fold dilution. BE11 itself did not affect the CFU-e count in the absence of the virus (Fig. 5).

The epitope of the structural protein of the virus which reacted with BE11 was examined by ELISA with synthetic peptides suggested by the expected secondary structure (4) and hydropathy analysis (20) of the amino acid sequence of the coding region for the structural protein of the B19 virus. One of 12 peptides synthesized, LRPGPVSQPYHHWD

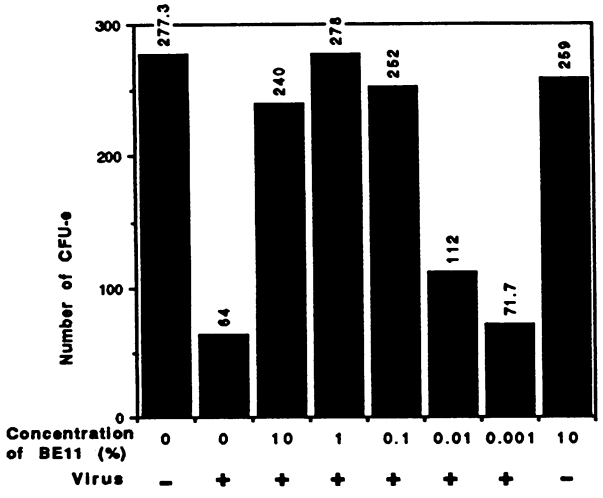

FIG. 5. Protective effect of BE11 against CFU-e injury by B19 virus. B19-containing plasma at a final concentration of 2% was preincubated with serially diluted BE11 ascites fluid at 37°C for 30 min, further incubated with bone marrow cells at 4°C for 2 h, and cultured.

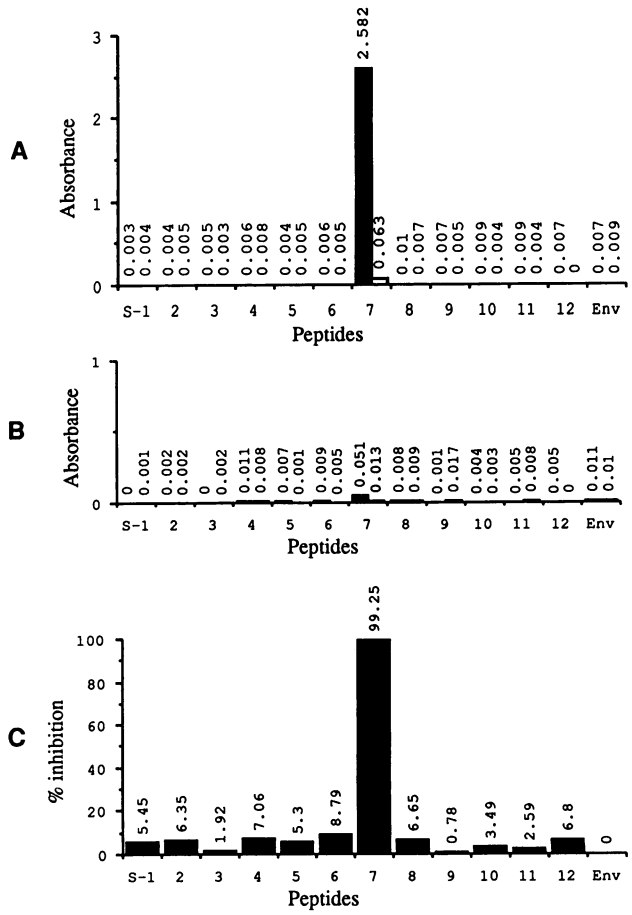

FIG. 6. Reactivity of BE11 with synthetic peptides. The binding capacity of BE11 (A) and RF465 (B) with each peptide was examined by ELISA (solid bars) and competition ELISA (open bars). Competition ELISA was done by preincubating BE11 with the same peptide that was used in the ELISA. Each value was obtained by measuring the A_{492}. (C) Inhibition rate in the reaction of BE11 with S-7 peptide by preincubating BE11 with each peptide is shown.

TDK, named S-7, corresponding both to amino acids 328 to 344 from the amino terminus of VP2 and to amino acids 555 to 571 from the amino terminus of VP1, reacted with BE11 in the ELISA (Fig. 6A). The control ascites fluid RF465 did not show significant binding with any peptide (Fig. 6B).

The specific binding of BE11 was examined by competition ELISA. Each peptide was preincubated with BE11, added to the S-7-coated microtiter well, and then measured for the binding capacity of BE11. The reaction of BE11 with S-7 was specifically inhibited by the S-7 peptide (Fig. 6C). In addition, the binding capacity of each peptide with anti-B19 positive and negative human sera used in this study was measured. The S-7 peptide reacted with the seropositive serum, but no peptide reacted with seronegative serum (Fig. 7).

DISCUSSION

Monoclonal antibodies against the B19 virus were used in a diagnostic assay system for detection of the IgM antibody with high sensitivity (6). However, there has been no previous report on a monoclonal antibody having neutralization activity. In this study, a new monoclonal antibody against

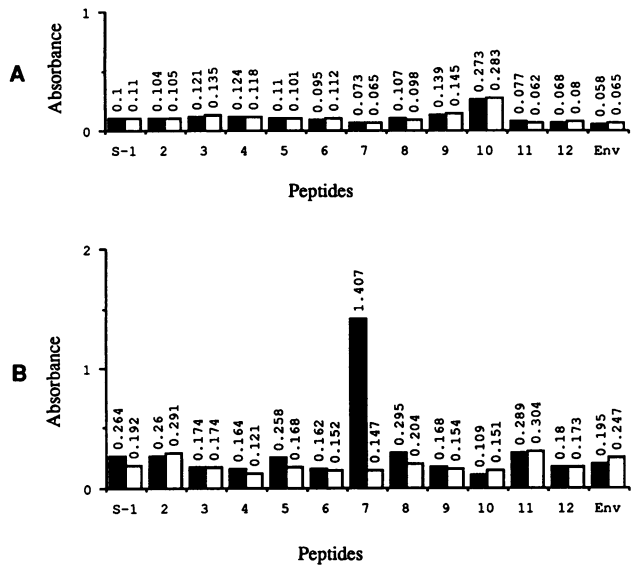

FIG. 7. Reactivity of human sera with synthetic peptides. Reaction of preimmune (A) and convalescent-phase (B) human serum with each peptide was measured by ELISA (solid bars) and competition ELISA (open bars).

the B19 virus could be produced by the hybridoma technique. Unfortunately, only one B19-specific monoclonal antibody could be isolated. The reason for this might be that in the first screening of the hybridomas, clones that were positive by dot blot ELISA but negative by immunoblotting were excluded from further cloning to make it easier for the following analysis. It was thought, however, that some conformation-recognizing antibodies might be missed in this step. The monoclonal antibody termed BE11 reacted with both VP1 and VP2 of the B19 virus capsid protein by immunoblotting. This suggested that the antigenic determinant of BE11 was common to VP1 and VP2, because the amino acid sequence of the 58-kDa major capsid protein VP2 is completely contained within the 83-kDa minor capsid sequence VP1 (27). In order to test whether the monoclonal antibody causes aggregation of the B19 virion, we examined lattice formation of the virion by BE11 in an agarose gel by the Ouchterlony double-diffusion technique and counterimmunoelectrophoresis in various antigen-antibody ratios. No immunoprecipitation could be observed by either method (data not shown). It is suggested that BE11 might have no ability to agglutinate the virion, as is the case for many other monoclonal antibodies.

Genetic variability of human parvovirus B19 was observed by restriction enzyme analysis (23). For vaccination, it is desirable that epitopes included in the protein used as a vaccine be highly conserved beyond the strain of virus used. The epitope defined by BE11 was conserved at least in the six isolates of the virus collected from different places and in different years, though there is no evidence that each isolate belonged to a different strain. But the amino acid sequences of the S-7 region, which BE11 reacted to, originated from the nucleotide sequence of the B19 virus isolated in the United States (34). These observations suggested that the BE11 defined epitope existed in at least six isolates in Japan and one in the United States.

It is known that the B19 virus has a cytotoxic effect on hematopoietic colony formation in vitro. CFU-e is more sensitive to the virus than BFU-e and CFU-c in bone marrow cell culture. The damage to CFU-e was strongly inhibited by the serum obtained in the convalescent phase of B19 virus-related illness (25, 39). We used this phenomenon in measurement of the neutralizing activity of the monoclonal antibody against the B19 virus. In the CFU-e assay in a bone marrow culture challenged with an appropriate amount of the virus, BE11 was shown to be able to protect the CFU-e from injury by the B19 virus as well as from human serum collected in the convalescent phase of a patient with erythema infectiosum. These findings suggested that the CFU-e escaped viral infection by BE11 by means of neutralization rather than aggregation of the virus.

Moreover, the region including the antigenic determinant against the monoclonal antibody was identified by using site-specific synthetic peptides deduced from the amino acid sequence of VP2 of the B19 virus reported by Shade et al. (34). Among the 12 peptides, BE11 reacted with only 1 peptide, S-7, which was mapped to a site corresponding to amino acids 328 to 344 from the amino terminus of VP2. The convalescent serum also reacted to only one peptide, S-7. As the serum is thought to contain polyclonal antibodies, there might be another neutralizing antibody which recognizes conformational or sequential structure other than that of the 12 peptides tested. However, it is suggested that the S-7 site is potentially immunogenic for humans. From the observation that the monoclonal antibody against the epitope within the S-7 site had the ability to neutralize B19 virus, it is suggested that both VP1 and VP2 may bind cellular receptors for the virus and that the region including the S-7 site of capsid protein is on the surface of the virus. Adhesion to the cellular membrane at a specific receptor was probably inhibited by combining of the antibody with the S-7 site in the capsid protein.

The S-7 peptide and BE11 are considered useful tools for analysis of the cellular receptor. The formation of neutralizing antibodies is a consistent feature of acute infection in animals by members of the family *Parvoviridae* (35). In humans, the production of neutralizing antibodies to capsid protein was shown to play a major role in limiting parvovirus infection in a CFU-e assay (17). Prior to preparing a component vaccine for the B19 virus, it is necessary to confirm the immunogenicity of the protein used as a vaccine for humans. Further epitope mapping of the virus capsid protein with human sera will be necessary. An immunogenic polypeptide including the S-7 site of the virus can be a candidate for the production of a vaccine for human parvovirus B19.

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