Quantitative Analysis of Neutralizing Immune Responses to Human Parvovirus B19 Using a Novel Reverse Transcriptase–Polymerase Chain Reaction–Based Assay

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Infection with human parvovirus B19 causes fifth disease, acute and chronic red cell aplasia, fetal hydrops, arthropathy, and other disorders. Antiviral antibodies limit B19 infection in vivo; however, the identification of serologic markers of protection has been hampered by the lack of a quantitative assay for parvovirus neutralization. A novel in vitro test for parvovirus neutralization has been developed using reverse transcriptase–polymerase chain reaction to detect viral transcripts in a B19-permissive cell line. Parvovirus neutralizing activity was measured in sera from naturally infected individuals, and common features of sera with high neutralizing capacity were identified as protection correlates. Sera that suppressed B19 replication in vitro demonstrated IgG reactivity with capsid proteins VP1 and VP2, but no linear relationship between antibody titer and neutralizing capacity was observed. Sera from experimental animals and human volunteers immunized with a virus-like particle vaccine candidate exhibited B19 neutralizing titers equal to or greater than those observed in natural infections.

Human parvovirus B19, first discovered in asymptomatic plasma donors [1], has been identified as the causative agent of a variety of clinical disorders. In normal children, B19 infection leads to an exanthematous rash called fifth disease [2]. In adults, particularly women, B19 infection may result in persistent arthropathy in ~50% of patients [3]. Because parvovirus B19 replicates in erythroid progenitor cells, infection can lead to serious complications, including transient aplastic crisis in persons with underlying hemolysis or chronic red cell aplasia in immunodeficient patients [4, 5]. Fetal infection can result in hydrops fetalis, spontaneous abortion early in pregnancy, and congenital anemia [6].

Multiple studies have highlighted the role of antiviral antibodies in B19 viral clearance [7–11]. Pooled human immunoglobulin containing B19-specific IgG is effective therapy for persistent B19 infection [10], suggesting that humoral immunity is predominant in limiting parvovirus infection. However, the lack of quantitative surrogate assays for virus neutralization has hampered the study of neutralizing sera and the identification of markers of protection. The only assay commonly used measures the ability of sera to protect colony-forming erythroid cells (CFU-E) from parvovirus killing in vitro [12]. Several problems with the hematopoietic progenitor cell assay limit its usefulness. Target CFU-E cells for viral infection are obtained from primary bone marrow cultures, which are inconvenient to obtain and contain cells of various differentiation states and lineages, introducing assay-to-assay variability. In addition, neutralization is typically seen at high concentrations of sera, which have highly variable composition and contain cytokines that can affect the growth of erythroid colonies for these reasons. Direct, quantitative comparisons of virus neutralization are difficult to perform by CFU-E analysis. In addition, the week-long incubation period for each CFU-E test makes screening of large numbers of sera cumbersome.

The purpose of this study was to develop a more sensitive, quantitative assay for parvovirus neutralizing capacities of immune sera obtained from individuals infected with B19. We also sought to determine the potential utility of this assay for measuring neutralizing titers in sera after immunization with a B19 vaccine candidate. We developed a new in vitro parvovirus neutralization test using a novel reverse transcriptase–polymerase chain reaction (RT-PCR)–based system.

Materials and Methods

Recombinant B19 antigens. B19 parvovirus empty capsid particles consisting of ~25% minor capsid protein VP1/75% major capsid protein VP2 were produced as previously described [13],

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Human experimentation guidelines of the US Department of Health and Human Services were followed in the conduct of the clinical research described. Informed consent was obtained from all human volunteers. Nonhuman primate research and immunization protocols were reviewed, approved, and performed according to experimentation guidelines of the NIH Animal Care and Use Committee.

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using a recombinant baculovirus system. CroVP1 was expressed in *Escherichia coli* and purified as previously described [14].

Antisera. Clinical specimens and patient diagnoses were obtained from subjects who were screened for clinical studies at the National Institutes of Health or were provided by Larry Anderson (CDC, Atlanta) and Bernard Cohen (Public Health Laboratory Service, London). Sera were also obtained from healthy volunteers immunized with B19 virus-like particles (VLPs) in studies conducted by MedImmune (unpublished data). Sera were complement-inactivated at 54°C for 30 min before storage at −20°C.

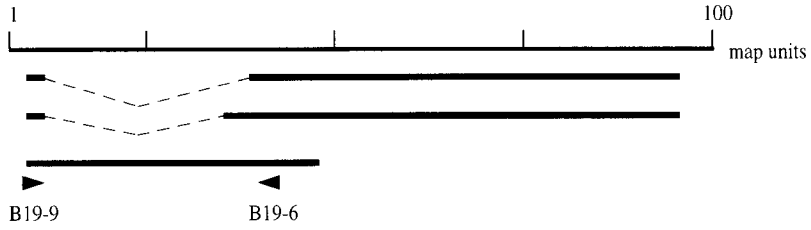
Animal studies and antisera. High-titer anti-B19 capsid antisera were generated by immunizing BALB/c mice and guinea pigs [15] three times with VLPs formulated with aluminum hydroxide (alum) or experimental adjuvants described below. Rhesus monkeys were maintained at the National Heart, Lung and Blood Institute (NIH) nonhuman primate research facility.

Adjuvants. Surfactant-stabilized emulsion adjuvant MF-59 was provided by Dan Granoff (Chiron, Emeryville, CA). MF-59 is an oil-in-water formulation that contains surfactants (Tween 20 and Span 85) along with a metabolizable oil (squalene) emulsified under high-pressure conditions [16]. Nonionic block copolymer adjuvant CRL-2690 was provided by Mark Newman (Vaxcel, Norcross, GA). CRL-2690 is a micellar adjuvant consisting of 12-kDa polyoxypropylene core components copolymerized with 10% polyoxyethylene [17].

ELISA. Anticapsid and anti-croVP1 antibody levels in sera were measured by ELISA. Immulon II plates (Dynex, Chantilly, VA) were coated with 100 μL of PBS buffer containing 100 ng/μL recombinant antigen overnight at 4°C. Plates were washed four times in PBS containing 0.1% Tween 20 (PBS-T), then incubated 60 min at 37°C with blocking buffer (PBS containing 5% nonfat milk and 0.1% Tween 20). After four washes in PBS-T, serum diluted in blocking buffer (100 μL total volume) was added directly to the wells and incubated 60 min at 37°C. The plates were washed in PBS-T, then incubated for 60 min with peroxidase-conjugated secondary antibody (goat anti–mouse IgG, 1:5000; Jackson Immunoresearch, West Grove, PA; goat anti–human IgG1, IgG2, IgG3, IgG4, or all IgG, 1:500; Zymed, South San Francisco) at 37°C. The plates were washed with PBS-T and developed with 100 μL/well peroxidase substrate (Kirkegaard & Perry, Gaithersburg, MD) for 15 min. Absorbance was read at 405 nm using an ELISA plate reader (Dynex). End-point titer was designated as the reciprocal of the highest serial dilution yielding an optical density reading equal to the reading of the control serum control wells plus 3× SD. All serum dilutions were performed in duplicate.

RT-PCR–based neutralization. Megakaryocytoblastic UT-7 cells adapted to growth in erythropoietin (Epo) were provided by N. Komatsu [18, 19]. UT-7/Epo cells were maintained in RPMI containing 10% fetal calf serum and 2 U/mL recombinant Epo (R&D Systems, Minneapolis). For each neutralization reaction, 3×10^5 UT-7/Epo cells were seeded in 1 well of a 24-well plate (Falcon/VWR, South Plainfield, NJ). Neutralizing serum was added at a dilution of 1:200 to 1:20,000, and viremic serum was simultaneously added at a final dilution of 1:25,000. Infected cultures were incubated at 37°C for 36 h. After 36 h, the cells were harvested, and total cellular RNA was collected using an RNAgents kit (Promega, Madison, WI) according to the manufacturer's instructions. Parvovirus infection of cultured UT-7/Epo cells was demonstrated by RT-PCR to detect spliced parvovirus mRNA species [20]. As a control for reverse transcription and amplification, cellular β-actin mRNA was simultaneously detected, using primers designed to overlap splice donor/acceptor sites. The location of primers used in these studies, their nucleotide sequences, and the final expected size of PCR amplimers are listed in figure 1.

Total cellular RNA (1 μg/reaction) was diluted in 10 μL (total volume) of nuclease-free distilled H_2O, incubated at 65°C for 5 min



B19-9 primer
5' GTTTTTTGTGAGCTAACTAACA 3'
nt 384-405

B19-6 primer
5' CAAAGGTGTGTAGAAGGCTT 3'
nt 2950-2972

Expected PCR Product Sizes: 262 bp; 125 bp; 2538 bp

Actin-3 primer
5' GATGACCCAGATCATGTTTG 3'
nt 1578-1587 / 2029-2039

Actin-4 primer
5' GGAGCAATGATCTTGATCTTC 3'
nt 2735-2744 / 2857-2867

Expected PCR Product Size: 635 bp

Figure 1. RT-PCR primers for detection of B19 and β-actin transcripts. Schematic depicts splice junction spanned by primers B19-9 and B19-6 and its location on genetic map of B19 parvovirus. Two alternatively spliced transcripts observed in infected UT-7/Epo cells and unspliced transcript are demonstrated. Nucleotide sequences and expected sizes of parvovirus and β-actin RT-PCR products are listed.

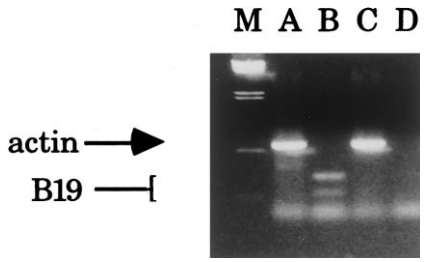


Figure 2. In vitro neutralization of B19 parvovirus detected by RT-PCR. In vitro neutralization reactions were carried out with high-titer guinea pig serum raised against irrelevant antigen (lanes A and B) or against B19 recombinant VLP (lanes C and D). Lanes A and C, amplification using actin-specific primers; lanes B and D, amplification using B19-specific primers. Lack of detection of B19 RT-PCR products in lane D demonstrates neutralization of input virus in presence of high-titered anticapsid antibodies. Lane M, molecular mass standards.

to denature the RNA, and stored on ice. Transcription of cDNA was primed using the B19-6 primer and ACT-4 primers in a reaction mix containing 1 μL of a 2.5 μM solution of each primer, 2 μL of a 25 mM stock of dNTPs, 5 μL of 5× RT buffer, 2 μL of 0.1 M dithiothreitol, 0.5 μL of RNase inhibitor, and 0.5 μL of murine leukemia virus RT (Promega) in a 25-μL reaction volume. Each reaction was incubated for 60 min at 37°C, then heat-inactivated at 65°C for 5 min. An equal volume (5 μL) from each RT reaction was amplified in a 50-μL reaction mix containing 2 μL of a 25 μM stock of each primer, 2 μL of a 2.5 mM dNTP stock, 10 μL of 5× PCR buffer, 10 μL of 25 mM $MgCl_2$, and 0.5 μL of Taq polymerase (Perkin-Elmer, Foster City, CA). Thirty-five cycles of PCR were carried out: 94°C for 30 s, 58°C for 30 s, and 72°C for 2 min. A final extension step at 72°C for 10 min was performed before analysis of product amplimers by 2% agarose/ethidium bromide gel electrophoresis. Neutralization was reported as the highest dilution of serum that yielded no virus-specific RT-PCR products. All RT-PCR reactions were performed in duplicate.

Results

In vitro parvovirus neutralization assay. An in vitro, quantitative assay for B19 parvovirus neutralization was developed using RT-PCR to amplify virus-specific RNA from a megakaryocytoblastic leukemic cell line (UT-7/Epo [18, 19]), adapted to growth in erythropoietin and semipermissive for parvovirus replication. Virus-specific transcripts were detectable as early as 12 h after infection. Total RNA was collected, quantitated, and reverse transcribed, and a standard PCR was used to amplify alternatively spliced viral products (262- and 125-bp, figure 1) from UT-7/Epo cells exposed to B19 parvovirus in the presence of a dilution of neutralizing serum for 36 h. The cellular transcript control, 635-bp and β-actin–specific, was also amplified using primers spanning splice junctions. PCR products were analyzed by 2% agarose/ethidium bromide electrophoresis.

Actin- and B19-specific products from representative control neutralization reactions are shown in figure 2. Experimental guinea pig serum raised against irrelevant antigen was used in reaction 1 (lanes A and B), while guinea pig serum with a high anticapsid end-point titer [15] was used in reaction 2 (lanes C and D). Virus-specific products of the predicted sizes were observed in lane B, demonstrating that virus neutralization had not occurred in the presence of negative control serum. The identity of the 262- and 125-bp products was confirmed by gel purification of the amplicons and DNA sequencing (data not shown). Virus-specific PCR products were absent in lane D, indicating that input virus had been neutralized in the presence of B19-specific antiserum. Reverse transcription was successful in both reactions, based on comparable actin products in lanes A and C.

To establish the reliability of the RT-PCR neutralization test, the neutralizing capacity of a panel of high-titer anticapsid sera was examined. Representative results for 6 control sera are shown in figure 3. Immune sera raised in mice against irrelevant antigen did not neutralize parvovirus, as evidenced by virus-specific products observed in reaction 2. Sera with high anti-B19 titer, from animals vaccinated with B19 VLPs (reactions 1, 3, and 4) and immune volunteers (reactions 5 and 6), neutralized input virus. These results confirmed that sera with anti-B19 reactivity from different sources consistently inhibit viral replication. End-point neutralization titers were established by serial dilution of test serum (figure 4), permitting quantitative comparisons of clinical and experimental antiparvovirus neutralization responses.

Clinical correlates of parvovirus neutralization. To determine the spectrum of endogenous parvovirus neutralizing activity in sera from individuals potentially at risk of B19 disease, sera from subjects with various hematologic and oncologic dis-

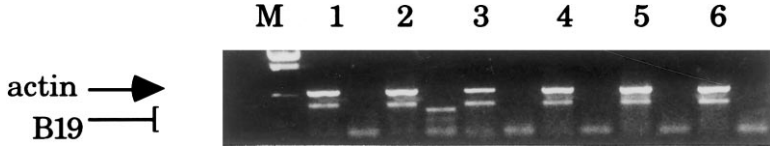


Figure 3. High-titered anti-B19 sera from various sources neutralize parvovirus in vitro. In vitro neutralization reactions were carried out using high-titered mouse serum raised against various formulations of B19 VLP (reactions 1, 3, and 4), high-titered mouse serum raised against irrelevant antigen (reaction 2), or serum from human donors with high anticapsid serum reactivity as determined by ELISA (reactions 5 and 6). RT-PCR products using actin-specific primers (left lane of each reaction) are comparable in all groups. RT-PCR products using B19-specific primers (right lane of each reaction) are not detected in presence of anti-B19 antibodies, indicating that input parvovirus was neutralized. No neutralization, as measured by detection of viral transcripts, occurs in presence of irrelevant immune serum. Lane M, molecular mass standards.

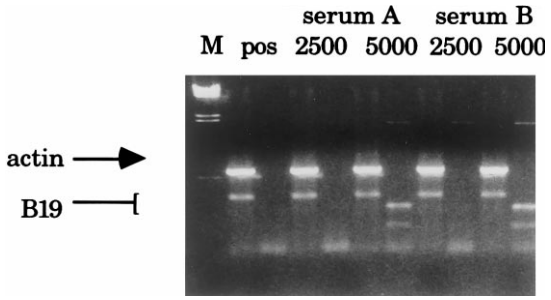


Figure 4. End-point determination of in vitro neutralizing capacity. In vitro neutralization reactions were carried out using positive control serum (pos; high-titered anti-B19 serum raised in mouse) or serum from human donors A and B with high anticapsid reactivity as determined by ELISA. Sera A and B were diluted to 1:2500 and 1:5000 for this assay; positive control serum dilution was 1:200. RT-PCR products using actin-specific primers (left lane of each reaction) and parvovirus-specific primers (right lane of each reaction) are shown. End point of B19 neutralizing capacity is defined as last serum dilution at which virus-specific RT-PCR products are not detected. Lane M, molecular mass standards.

orders were tested in this RT-PCR neutralization assay. Sera from 2 healthy donors were used as controls; 1 of these had the highest anticapsid response as determined by ELISA. The ability of dilute sera to neutralize parvovirus in vitro is shown in table 1. Neutralizing capacity is reported as the highest dilution of serum that prevents detection of parvovirus-specific products by RT-PCR. To evaluate whether neutralization titers correlated with other serologic responses, end-point titers against whole recombinant capsid and the minor capsid protein VP1 were measured by ELISA. The IgG subclasses of sera reactive with VP1 were also examined and compared with neutralization to assess whether a particular subclass was associated with functional antiparvovirus responses.

Of 25 clinical samples studied, 12 neutralized parvovirus in vitro. All neutralizing sera contained antibodies reactive with both recombinant capsid and VP1, consistent with the proposed role of antiparvovirus antibodies in viral clearance. End-point capsid and VP1 reactivity was highly variable. End-point neutralizing titers of these sera also varied, ranging from 200 to 5000. No linear correlation between neutralizing capacity and ELISA titer against capsid ($r = .44$) or against VP1 ($r = .44$) was observed. Sera with relatively high anticapsid (>6250) and anti-VP1 (>250) end points showed the highest in vitro neutralization. On the other hand, several clinical sera with moderate to high anticapsid titer demonstrated low or no neutralizing activity (e.g., 7340, 7345). We hypothesize that these sera did not recognize important neutralizing epitopes on the B19 virion. No relationship between the specific IgG subclass elicited against VP1 and neutralizing capacity was observed.

Table 1. Analysis of anti–parvovirus B19 response in clinical sera.

Serum no.	End-point titer				Neutralization in vitro by RT-PCR	Clinical profile[a]
	Anticapsid	Anti-VP1 (intact)	Anti-VP1 (denatured)	Anti-VP1 subclass		
6904	31,250	1250	250	G1>G4	2500	A
6966	1250	1250	250	G1>G3=G4	2500	A
7025	6250	250	250	G1>G3=G4	1000	NA
7273	6250	250	250	G1>G4	1000	A
6887	6250	250	50	G1	2500	A, B
062	31,250	1250	1250	G1>G4	5000	N
6850	6250	250	250	G1>G4	500	A
7353	1250	250	50	G1	500	D
CS-1	1250	250	250	G1>G4	500	N
7124	6250	250	250	G1>G3	200	A
7138	1250	1250	1250	G4>G1	200	A
CS-3	250	50	<50	G1	200	F
7081	250	250	50	G1>G3=G4	<200	C
7001	1250	250	<50	G1>G3	<200	A
7340	6250	250	50	G1	<200	A
7017	250	<50	<50	ND	<200	A
7072	50	<50	<50	ND	<200	A
7345	6250	50	50	G1=G3=G4	<200	A
CS-2	1250	<50	<50	ND	<200	E
CS-4	1250	<50	<50	ND	<200	G, H
CS-5	50	50	50	G1	<200	H
CS-6	50	50	50	G1	<200	G
CS-7	<50	<50	<50	ND	<200	I
CS-8	<50	<50	<50	ND	<200	J
CS-9	<50	<50	<50	ND	<200	E

NOTE. ND, none detected. Anticapsid and anti-VP1 end-point titers were evaluated by ELISA as described in Materials and Methods.

[a] A, exposed to parvovirus B19 during pregnancy; B, neonatal death; C, marrow myelodysplasia with B19 exposure, aplastic crisis; D, polyarthralgia with aplastic anemia; E, anemia; F, marrow myelodysplasia; G, parvovirus infection; H, chronic lymphocytic leukemia; I, sarcoidosis; J, liver transplant; N, normal clinical profile; NA, not available.

Table 2. Analysis of anti–parvovirus B19 response in paired clinical sera.

Serum no.	End-point titer				Neutralization in vitro by RT-PCR	Clinical profile[a]
	Anticapsid	Anti-VP1 (intact)	Anti-VP1 (denatured)	Anti-VP1 subclass		
4052	<50	50	50	G1=G3	<200	L
	250	50	50	G1=G3	1000	
436	<50	50	<50	G1	<200	S
	250	1250	50	G1=G3=G4	1000	
2763	<50	50	<50	ND	<200	R
	50	250	<50	ND	500	
0443	<50	<50	<50	ND	<200	A
	50	250	<50	ND	500	
2469	50	<50	<50	ND	200	R
	1250	<50	<50	ND	200	
2426[b]	250	250	250	G4>G1=G3	1000	R
	1250	250	250	G1>G3=G4	2500	
6726	1250	250	250	G1=G4>G3	200	P
	1250	250	250	G1=G3=G4	5000	
8102[b]	6250	250	250	G1=G3	200	R, A
	6250	250	250	G1=G3>G4	1000	

NOTE. ND, none detected. Anticapsid and anti-VP1 end-point titers were evaluated by ELISA as described in Materials and Methods.

[a] L, laboratory infection; S, stillbirth, R, rash; A, arthropathy/arthralgia; P, polyhydraminos.

[b] Positive for B19-specific IgM by RIA.

We extended our analysis of clinical sera to include 8 pair-matched samples from individual donors presumed from clinical history to be infected with B19 (table 2). Serum samples were obtained during the first clinical visit for parvovirus-attributed symptoms, and second samples were collected later during the convalescent period, 2 weeks to 6 months afterward. The detection of anti-B19 IgM responses in only 2 subjects probably reflects delay in serum acquisition after parvovirus infection. In general, convalescent-phase sera demonstrated higher neutralization titers, suggesting maturation of the functional antiparvovirus immune response through the convalescent period, even in the absence of enhanced anticapsid or anti-VP1 specific responses measured by ELISA (e.g., 6726, 8102). In addition, changes in anti-VP1 isotype were detected over time in some volunteers, suggesting that affinity maturation may have coincided in these individuals with improved virus neutralization. The overall magnitude of neutralizing titers in convalescent sera were comparable in range to those of immune subjects selected randomly (table 1).

Since the passive administration of immune globulin is used therapeutically to arrest acute parvovirus infection in some patients [10], we measured the in vitro neutralization characteristics of several commercial lots of immune globulin (table 3). Commercial immunoglobulin lots were adjusted to a midrange of normal serum IgG levels (1000 mg/dL). All lots of immune globulin examined neutralized B19. End points observed (250–1000) were similar to titers found in random donors.

Recombinant B19 capsid elicits neutralizing antibodies. Recombinant capsid proteins have been used to elicit an anti-B19 humoral response as the basis of a candidate vaccine for humans [21]. We examined immune sera generated in mice using the RT-PCR–based neutralization assay (table 4) and found that sera from mice vaccinated with B19 capsid in alum adjuvant neutralized parvovirus in vitro, similar to reported results using the CFU-E neutralization assay [21, 22]. Next, we examined sera from 3 human volunteers immunized three times with B19 VLP formulated in alum (Alhydrogel; Reheis, Berkeley Heights, NJ) (table 4). Of the 3 post–second boost sera from human volunteers, only 1 exhibited moderate humoral reactivity with capsid and VP1 by ELISA and also exhibited a modest in vitro neutralization titer.

Since alterations in formulation, particularly the use of adjuvants, can have dramatic effects on serum antibody titers and functional immune responses, we sought to determine if alternative adjuvants could improve anti-B19 responses. Preliminary studies were carried out in mice immunized with VLPs formulated in either a surfactant-based emulsion or a block copolymer adjuvant. These adjuvants had a pronounced effect on antiparvovirus responses measured by ELISA and by RT-PCR neutralization (table 4). A trend was observed between high capsid/high VP1 end-point titers by ELISA and in vitro neutralization, although no linear correlation was seen.

Table 3. In vitro parvovirus B19 neutralization with commercial immune globulin.

Immune globulin product	In vitro neutralization titer	
	Product lot A	Product lot B
Bayer[a] standard IVIG	1000	200
Sandoz[b] standard IVIG	200	200
Respigam[c] immune globulin	1000	500
Cytogam[c] immune globulin	200	500

NOTE. Intravenous immune globulin (IVIG) products were diluted to 1000 mg/dL before evaluation by RT-PCR–based neutralization assay. Two separate lots of each commercial product were evaluated.

[a] Bayer, Elkhart, IN.

[b] Sandoz Pharmaceuticals, East Hanover, NJ.

[c] Medimmune, Gaithersburg, MD.

Table 4. Anti-parvovirus B19 response in paired sera from human volunteers vaccinated with B19 virus-like proteins (VLPs) in alum and in sera from mice vaccinated with various VLP formulations.

Serum description	End-point titer		Neutralization, in vitro by RT-PCR
	Anticapsid	Anti-VP1	
Volunteer 1			
Day 0	>50	250	<200
2nd boost	250	250	<200
Volunteer 2			
Day 0	>50	50	<200
2nd boost	250	50	<200
Volunteer 3			
Day 0	>50	50	<200
2nd boost	1250	1250	<200
Murine sera[a]			
Alum/VLP	62,500	12,500	500
Emulsion/VLP	312,500	62,500	1000
Copolymer/VLP	100,000	12,500	2500

NOTE. Human volunteers received 3 intramuscular vaccinations of 100 μg of VLPs in alum adjuvant. Anticapsid and anti-VP1 end-point titers were determined by ELISA as described in Materials and Methods.

[a] Pooled ($n = 5$); BALB/c mice received intramuscular vaccinations of VLPs formulated with experimental adjuvant as listed.

To investigate further the relationship between adjuvant and anti-VLP response, a dose-escalating immunization study of VLP formulated with three different adjuvants (alum, block copolymer, or surfactant-based emulsion) was performed in rhesus monkeys (table 5). Sera from rhesus monkeys immunized with VLP in emulsion or copolymer adjuvant demonstrated markedly enhanced antiparvovirus responses, with detectable in vitro neutralizing capacity after a single vaccination. Similar to results in human studies, antiparvovirus responses elicited by a single vaccination with alum-formulated VLPs were absent or just above background values. After two booster immunizations, sera from all 12 rhesus monkeys neutralized parvovirus in vitro, with ~2- to 5-fold enhancement in copolymer and emulsion adjuvant recipients compared with alum recipients. End-point anticapsid ELISA titers after two booster immunizations ranged from 2500 (VLP in alum adjuvant) to >3,000,000 (VLP in emulsion adjuvant).

CFU-E analysis of selected rhesus sera after tertiary immunization was performed to confirm our RT-PCR neutralization results (figure 5). Sera with strong neutralizing activity by RT-PCR also protected erythroid progenitor cells in CFU-E. Rhesus sera with the lowest neutralizing scores by RT-PCR (alum adjuvant recipients) were found to be marginally protective or not protective by CFU-E, which may indicate the relative insensitivity of the colony-forming assay in detection of parvovirus-neutralizing activity.

Conclusions. The resolution of active infection and prevention of natural parvovirus B19 infection is dependent upon the production of antibodies directed to either one or both of the two capsid proteins VP1 and VP2. Passive administration of immune globulins containing anti-B19 reactivity to patients with active infection has been shown to abrogate viral replication and disease [7–11]. Developing an assay that quantifies this functional antiviral activity could have potential clinical value: to characterize surrogates for protection against infection, to identify individuals who may be at risk of infection (particularly those undergoing chemotherapy or with immune deficiencies), to assist in standardizing immune globulins for functional anti-B19 responses, and to guide vaccine design by setting standards of immune protection that should be achieved by active vaccination. The results described here begin to address many of these issues.

The RT-PCR assay developed was found to be a sensitive and quantitative method for B19 neutralization. Furthermore, larger numbers of samples could be evaluated simultaneously using this assay, compared with the conventional method using donor bone marrow cells. It is certainly feasible that other cell lines, besides UT-7, that are permissive for B19 infection [22, 23] might be useful in standardizing neutralization responses. Additional studies are warranted to establish reference standards for both viral replication and antibody activity, possibly with a neutralizing monoclonal antibody. The RT-PCR assay appears to be considerably more sensitive than the CFU-E assay in detecting functional neutralizing activity, since serum

Table 5. Antiparvovirus responses in rhesus monkeys immunized with B19 virus-like particles.

Adjuvant, animal no.	Dose (μg)	3 weeks after primary		3 weeks after secondary		3 weeks after tertiary	
		Anticapsid	Neutralization	Anticapsid	Neutralization	Anticapsid	Neutralization
Alum							
800	3	<50	<200	500	<200	25,000	1000
940	3	500	<200	2500	<200	5000	1000
705	30	<50	<200	500	<200	25,000	1000
9SM	30	2500	<200	2500	200	5000	2500
Copolymer							
406	3	500	1000	12,500	5000	25,000	5000
717	3	500	1000	2500	2500	25,000	5000
831	30	2500	1000	62,500	2500	125,000	10,000
84315	30	2500	<1000	62,500	5000	125,000	5000
Emulsion							
851	3	62,500	1000	1,562,500	5000	>3,125,000	10,000
859	3	62,500	5000	1,562,500	5000	>3,125,000	10,000
828	30	312,500	1000	1,562,500	5000	>3,125,000	10,000
1255	30	2500	<1000	312,500	5000	>3,125,000	5000

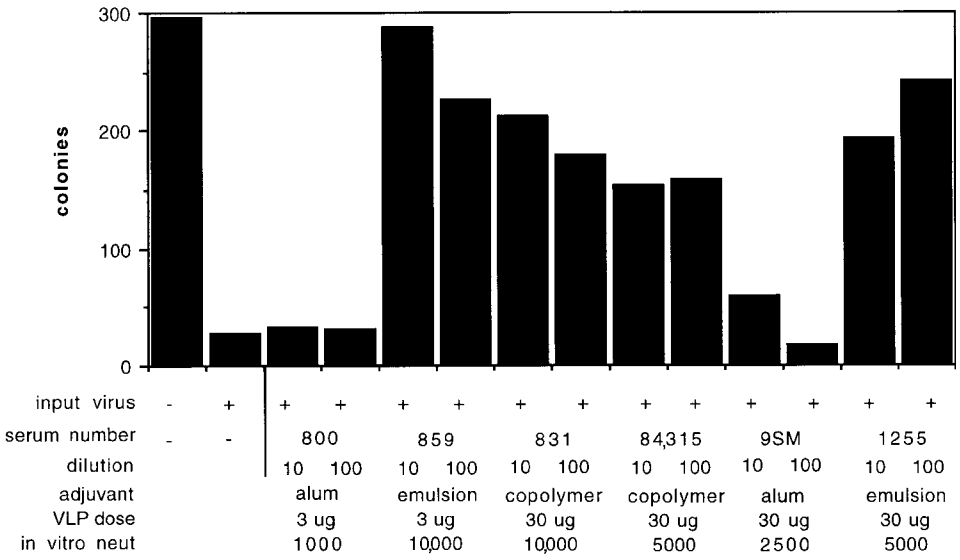


Figure 5. CFU-E analysis of immune serum generated in rhesus monkeys. Erythroid progenitor cells obtained from primary bone marrow were incubated with B19 parvovirus and dilution of rhesus immune serum. Surviving erythroid cells were cultured in semisolid medium, and colonies from duplicate plates were counted. Colonies reported are sum of duplicate plates. VLP, virus-like particle; neut, neutralizing titer.

samples that failed to demonstrate any neutralizing responses in the latter had low neutralizing activity in this assay. Whether such titers are sufficient for in vivo protection will still need to be discerned, although titers achieved were well within the range of activity measured in randomly selected lots of immune globulin.

In vitro neutralizing titers, as expected, were identified only in subjects with measurable anticapsid antibodies. While particular individual sera with high binding titers to capsid components contained strong neutralizing activity, neither anti-VP1 specific titers nor anticapsid responses correlated directly with neutralizing titers. This suggests that varying responses are elicited in infected subjects to epitopes defining neutralizing domains. While previous studies with VLP vaccines have suggested that increasing the content of VP1 was important in enhancing neutralizing responses [15, 21], functional anti-B19 activity after natural infection may provoke responses to both capsid proteins [24–28]. Also, it is unclear if the neutralizing responses in polyclonal sera are directed primarily to epitopes that are conformationally dependent or linear, since neutralizing monoclonal antibodies recognizing VP1 and VP2 have been identified that bind to both native and denatured VLP structures [29]. It will be of interest to define these neutralizing epitopes with regard to the required structure for optimal vaccine development.

The survey of neutralizing responses in sera from subjects suspected and observed to develop B19-associated disorders established a range for neutralizing responses required for recovery from acute infections. The maximum titer observed in these subjects was 1:5000, and most subjects had titers of ⩽1:1000. The titers observed in the immune globulin serum derived from large plasma pools of >1000 individuals were in a similar range, quite consistent with the titers of individual donors. These results establish the boundaries of titers that would be expected to yield effective responses after vaccination, given the effectiveness of random immune globulin pools in abrogating B19 replication. These titer ranges may also be of value in identifying individuals at high risk of developing B19 infections, particularly subjects with immune deficiency, those undergoing chemotherapy, or pregnant women. It was quite interesting, for example, that individuals with neutralizing titers of ⩽1:200 developed documented B19 infections (table 2), although 1 subject with a baseline neutralizing value of 1:1000 also developed a rash-like illness. The limitation of these data is that primary serum samples were obtained after clinical illness was observed in most cases, and initial sampling may not represent accurate preexposure neutralizing levels.

The studies we report also demonstrated that two adjuvants other than alum markedly improved the induction of functional neutralizing responses to VLPs composed of the two B19 capsid proteins. While significant increases in neutralization were observed with both the blocked copolymer and the emulsion (MF59), in mice, the magnitude of the enhancement was even more pronounced in rhesus monkeys.

Several valuable observations emerged from this experiment. Anticapsid and neutralizing titers were low in the vaccine formulated in alum, comparable to what was observed in a clinical trial of seronegative volunteers immunized with a similar formulation. In fact, neutralizing activity in the clinical study was seen in a single volunteer and only after three immunizations;

three doses of vaccine with alum adjuvant were required in rhesus monkeys to achieve consistent neutralizing responses. In contrast, neutralizing responses were observed in the majority of animals immunized with VLPs formulated in either of the experimental adjuvants after a single inoculation and were as high as any titer observed in naturally infected subjects after a booster vaccination. Furthermore, after a tertiary immunization, neutralizing titers in many of samples from the vaccinated monkeys exceeded any serum sample examined to date from humans previously infected with parvovirus B19. This would suggest that the formulation of either adjuvant with the B19 VLPs might induce effective functional responses in humans.

Of interest is the finding that despite the quantitative differences in the anticapsid responses induced by the two experimental adjuvants, neutralizing titers were quite similar with the two vaccine doses tested. It is possible that at doses <3 μg, differences in functional responses might be observed. In addition, the results suggest that MF59 might enhance responses to both neutralizing and nonneutralizing domains more effectively than the copolymer. Whether similar effects will be observed in human volunteers remains to be determined in future trials.

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