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Detection of Antibodies and Antigens of Human Parvovirus B19 by Enzyme-Linked Immunosorbent Assay

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Acute-phase serum from a patient with aplastic crisis provided sufficient human parvovirus B19 to make a monoclonal antibody against B19 and to develop antigen and immunoglobulin M (IgM) and IgG antibody detection enzyme-linked immunosorbent assays (ELISAs). The indirect capture antibody method was used for all three assays. Antigen was detected in 8 of 29 sera drawn within 2 days of onset of illness from patients with aplastic crisis. These sera had high titers of virus by electron microscopy and DNA hybridization and had no detectable B19 antibody. Antigen was not detected in serum specimens that had low titers of B19 DNA and had B19 antibody. With the IgM ELISA, we detected B19 IgM in over 85% of clinical cases of aplastic crisis and fifth disease and less than 2% of controls. The prevalence of B19 IgG antibodies increased with age. Approximately 2% of children <5 years of age and 49% of adults >20 years of age had B19 IgG antibodies. The B19 antibody ELISAs are sensitive and specific tests to detect B19 infections.

Human parvovirus B19 was identified and characterized in 1975 by Cossart et al. while they were evaluating assays for hepatitis B virus in serum (11). Initially, studies to identify the clinical and epidemiological characteristics of B19 infections used counterimmunoelectrophoresis tests. Counter immunoelectrophoresis and later, a more sensitive radioimmunoassay (RIA), showed that B19 infection was associated with aplastic crisis in patients with hemolytic anemias in 1981 (31), with fifth disease in 1983 (6), with stillbirths in 1984 (8, 20), and with arthritis in 1985 (30, 33). The antibody assays require B19 antigen (P), and since B19 has not been grown in tissue culture or in laboratory animals, sera from humans remain the sole source of this antigen (7). Recently, the development of DNA hybridization assays (5, 9) has improved our ability to detect the virus, but immunoglobulin G (IgG) and IgM antibody assays remain the most sensitive way to detect B19 infection (5).

An epidemiological investigation of simultaneous outbreaks of aplastic crisis and fifth disease in Ohio in the spring of 1984 (30a; T. L. Chorba, P. Coccia, R. C. Holman, P. Tattersall, L. J. Anderson, J. Sudman, N. S. Young, E. Kurczynski, U. M. Saarinen, D. N. Lawrence, J. M. Jason, and B. Evatt, J. Infect. Dis., in press) provided the antigen to develop serological asays for B19 infections. Virus obtained from serum collected from patients acutely ill with aplastic crisis was used to make a monoclonal antibody (MAb) against B19. This MAb then was used to develop B19 antibody and antigen detection enzyme-linked immunosorbent assays (ELISAs) patterned after the previously described RIA for B19 antibody and antigen (10).

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MATERIALS AND METHODS

B19 MAb. Virus was purified from 2 ml of serum specimen S-36 (from a 14-year-old female with aplastic crisis, Cleveland, Ohio, 1984) that had a titer of greater than 10^8 B19-like particles per ml by electron microscopy and 40 μg of B19 DNA per ml by hybridization (12, 30a). The serum was centrifuged twice at 30,000 rpm in an SW41 rotor for 20 h at 5°C through 10% glycerol in TE buffer (0.05 M Tris plus 0.05 mM EDTA at pH 8.7) into 60% metrizamide (Nyegaard and Co. AS, Oslo, Norway) in TE buffer. The virus in the metrizamide was dialyzed against phosphate-buffered saline (PBS) and stored at −70°C. Female 8- to 12-week-old BALB/c mice were immunized intraperitoneally with 0.2 ml of a 1:1 mixture of a 1:10 dilution of purified B19 and incomplete Freund adjuvant on day 0 and with 0.2 ml of a 1:10 dilution of purified B19 alone on days 30, 31, and 32. On day 33, the spleen was harvested, and the spleen cells were fused with SP2/0-Ag14 myeloma cells by an adaptation of the procedure described by Oi and Herzenberg (24). The hybridomas were screened by an adaptation of the antigen detection ELISA described below, and the positive hybridoma was cloned twice by limiting dilutions and then injected into pristane-primed BALB/c mice to make ascites fluid. The MAb (162-2B) was characterized as an IgM antibody by ELISA with subclass-specific antiserum (Litton Bionetics, Kensington, Md.) as the capture antibody and peroxidase-conjugated goat anti-mouse IgG (heavy and light chains) antibody (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) as the detector antibody. MAb 162-2B reacted against five strains of B19 from Ohio, one strain each from Michigan, Connecticut, and Mississippi, and two previously characterized strains, BrI and BrII, from England in the antigen detection ELISA (10). The MAb reacted to a titer of 1:10,000 against one of the strains from Cleveland in an adaptation of the antigen detection ELISA.

IgG and IgM ELISAs. A seven-step indirect capture antibody ELISA was used to detect IgG and IgM antibodies to B19. (i) Flat-bottomed 96-well Immulon-2 microtiter plates

(Dynatech Laboratories, Inc., Alexandria, Va.) were coated with 75 μl of goat anti-human IgG or IgM antibody (Tago, Inc., Burlingame, Calif.) per well diluted 1:1,000 in 0.01 M carbonate buffer (pH 9.6) and incubated overnight at 4°C. (ii) After the plate was washed, 250 μl of PBS plus 0.5% gelatin (PBS-G) and 2% normal goat serum (PBS-G-NGS) was added and was incubated for 30 min at 35°C. (iii) After washing, 75 μl of the human serum specimen diluted in PBS-G plus 0.15% Tween 20 (PBS-GT; Sigma Chemical Co., St. Louis, Mo.) was added and incubated at 35°C for 1.5 h. (iv) After washing, P, either serum specimen S-36 at a 1:1,000 dilution or S-744 (from a 12-year-old male with aplastic crisis, Cleveland, Ohio, 1984) at a 1:750 dilution, or control antigen (N) at a 1:1,000 or 1:750 dilution in PBS-GT plus 0.02% sodium azide was added and was incubated overnight at room temperature. (v) After washing, 75 μl of MAb 162-2B, diluted 1:1,000 in PBS-GT, was added and was incubated for 1.5 h at 35°C. (vi) After washing, 75 μl of peroxidase-conjugated, goat anti-mouse IgM antibody (Kirkegaard and Perry Laboratories, Inc.) diluted 1:3,333 in PBS-GT (for the IgG test) or in PBS-GT-NGS (for the IgM test) was added and was incubated for 1 h at 35°C. (vii) After washing, a substrate (0.4 mg of *o*-phenylenediamine per ml and 0.015% hydrogen peroxide in 0.15 M citrate phosphate buffer [pH 5.5]) was added. The reaction was stopped with 3.5 M HCl after 30 to 45 min, and A_{490} was measured (MR 580 Microelisa Auto Reader; Dynatech Laboratories, Inc., Alexandria Va.).

To screen for the presence of IgG and IgM antibodies, each serum specimen was tested at a 1:100 dilution in PBS-GT against both P and N (serum which tested negatively by ELISA for B19 IgM and IgG antibodies and for P). The P/N value was calculated from the geometric mean of P, two wells with the specimen reacted against P, and the geometric mean of N, two wells with the specimen reacted against N. The dilution of the respective reagents was the one that, in checkerboard titrations, gave the highest P/N value and conserved that reagent.

Antigen detection ELISA. The antigen detection ELISA is identical to the B19 IgM antibody ELISA except that in step (iii) a known high-titered, positive serum specimen replaced the unknown serum and in step (iv) the unknown antigen replaced P and N. Two negative controls were used. One was an unknown antigen reacted against an IgM MAb against respiratory syncytial virus 62-9E. The other was an unknown antigen reacted against diluent instead of against the high-titered positive serum. The first control usually gave the higher absorbance readings. Serum was tested at a 1:10 dilution in PBS-GT. The P/N value was caculated from the geometric mean of P (two wells with the specimen reacted against 162-2B) and the geometric mean of N (two wells with the specimen reacted against 62-9E or against diluent).

Clinical specimens. Serum specimens from 61 patients with fifth disease, 43 patients with aplastic crisis and their 107 controls, and 102 patients with a variety of other illnesses and their 37 controls were tested in this study. The patients with fifth disease were physician diagnosed and were part of outbreaks in Kentucky, Mississippi, and Ohio, and the patients with aplastic crisis were physician diagnosed and were part of outbreaks in Connecticut, Illinois, Michigan, and Ohio. The onset of illness for patients with fifth disease was taken as day 1 of the rash, and onset of illness for patients with aplastic crisis was taken as the date of diagnosis. The aplastic crisis controls were randomly selected from patients with chronic hemolytic anemias who had no history of aplastic crisis, and the fifth disease controls were either well students from the same school or patients without evidence of fifth disease who saw the same pediatrician as did the case patients. The "other illness" controls were age- and sex-matched patients without the respective illness.

TABLE 1. B19 antibody response by patients with aplastic crisis or fifth disease

Antibody	% Positive results in no. of days after onset of illness (no. tested):					
	0–2	3–7	8–28	29–56	57–112	>112
IgM	78 (32)	93 (30)	93 (29)	74 (31)	74 (23)	67 (6)
IgG	42 (36)	90 (30)	100 (30)	94 (31)	91 (23)	100 (6)

Analysis of results. To calculate the cutoffs for positive and negative sera, we considered the results a mixture of two populations, one of negative results and the other of positive results. This analysis was based on the log of the P/N values for a subset of the serum specimens we tested. This subset of 280 sera included sera from persons with fifth disease and aplastic crisis and from well persons with illnesses unrelated to B19 infection. The mean and standard deviation of the two populations were obtained by using a computer program to determine maximum-likelihood solutions (18). This program was based on one previously published by Agha and Ibrahim (1). It was also possible to clinically identify populations of sera that should be negative (from well persons) or positive (from ill persons) for IgM but not for IgG. P/N values 3 standard deviations above the mean of the well population were considered positive, and those below the mean of the well population were considered negative. Since the background for the IgG ELISA was often very low, we also required that the specific absorbance (P − N) be >2 standard deviations above the mean of P − N.

RESULTS

IgM ELISA. For the IgM test, 90% of the time the background absorbance (N) values were less than 0.125, and 90% of the ratios of the higher value to the lower value for duplicate wells were less than 1.15. The coefficient of variation in P/N for 23 replicate tests on four different days for one positive serum specimen was less than 15% for within-day variation and was less than 25% for day-to-day variation. The natural log of P/N values produced a well-defined curve corresponding to the negative results and a scattering of points corresponding to the positive results (Fig. 1). The mean P/N for the negative curve was 1.12 and 3 standard deviations above the mean was 1.42. For the well population, the mean P/N was 1.13 and 3 standard deviations above the mean was 1.47. We chose a P/N of 1.5 as our cutoff value.

IgM antibodies were acquired earlier than IgG antibodies. Most patients had IgM antibodies within a few days after onset of illness (Table 1), but the titer of IgM antibodies and the rate of positivity began to fall by month 2 after onset of illness (Table 1 and Fig. 2). Among clinical cases of aplastic crisis, 38/43 (88%) were positive at least once for B19 IgM, and among controls 1/34 (3%) were positive. Among clinical cases of fifth disease, 51/61 (84%) were positive at least once for B19 IgM antibodies and among controls 1/73 (1%) were positive. A total of two of the five aplastic crisis patients with sera negative for IgM antibodies had these sera drawn within 2 days of onset of illness, two patients had sera drawn between 2 days and 2 months after onset of illness, and one

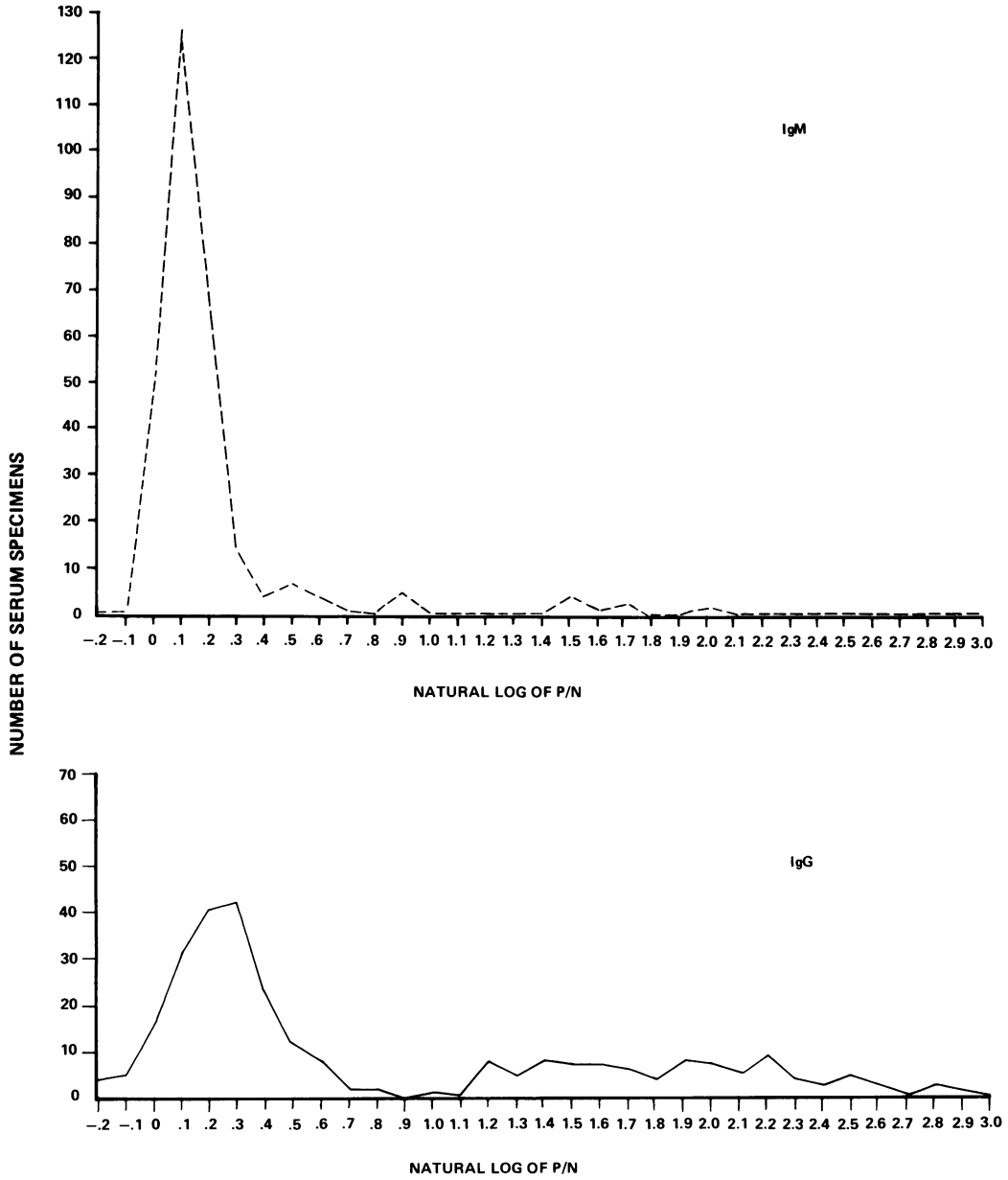

FIG. 1. B19 antibodies in 280 serum specimens. P/N was calculated from the geometric mean absorbance for the serum specimen reacted against P divided by the geometric mean absorbance of the serum specimen reacted against N. The natural log of the cutoff value for the IgM test is 0.41, and the natural log of the cutoff value for the IgG test is 0.74. The 280 serum specimens are a subset of the specimens tested and include sera from persons with B19-related illnesses and other illnesses.

patient had sera drawn 4 months after onset of illness. A total of four of the 10 fifth disease patients with sera negative for IgM antibody had these sera drawn within 2 days after onset of illness, four patients had sera drawn between 3 days and 2 months after onset of illness, one patient had sera drawn 3 months after onset of illness, and one patient had sera drawn at an unspecified time after onset of illness. Less than 1% of serum specimens from patients with other illnesses or their controls were positive for B19 IgM antibodies (Table 2). In a comparison study of 25 sera, the results for the IgM ELISA were similar to those for the previously described IgM RIA (10) (Table 3).

IgG ELISA. For the IgG test, 90% of the time the background absorbance (N) was <0.045 and 90% of the ratios of the larger value to the smaller value for duplicate wells were less than 1.5. The coefficient of variation in P/N values for 28 replicate tests over 4 days for one positive serum specimen was <10% for within-day variation and was <25% for day-to-day variation. As with IgM, the distribution of P/N values defined two overlapping curves, a sharp curve corresponding to negative results and a broad curve corresponding to the positive results (Fig. 1). The mean P/N value for the negative curve was 1.27, and 3 standard deviations above the mean was 2.13. Two standard deviations above the mean of the absolute absorbance, P − N, for the negative curve was 0.030. We chose a P/N value of >2.1 and an absolute absorbance of >0.030 for our cutoff values. As with the IgM detected by ELISA, the results for the IgG ELISA

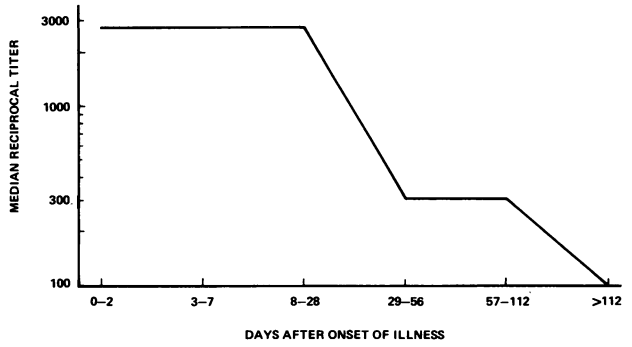

FIG. 2. IgM antibody response to B19 infection. Titer is the median titer of from 6 to 20 serum specimens for the respective time periods. Titer was determined in serial threefold dilutions in the IgM ELISA. The endpoint was taken as the last dilution with a P/N value of >1.5.

were similar to those for a previously described IgG RIA (10) (Table 3). IgG antibodies were usually acquired during week 1 of illness (Table 1), and once present, presisted in any follow-up serum specimens. Among controls and patients with diseases not related to B19 infection, the prevalence of B19 IgG antibodies and, therefore, evidence of past infection increased with age (Table 4).

Antigen detection ELISA. The mean background absorbance of individual specimens varied from 0.064 to 0.241 in the antigen detection test. We tested 42 serum specimens that had been previously tested for B19 DNA by hybridization (30a). A total of 26 sera were negative for B19 DNA, 10 sera had medium or low titers of B19 DNA, and 6 sera had high titers of B19 DNA. The mean P/N value for the 26 sera negative for B19 DNA was 1.63, and 3 standard deviations above the mean was 2.75. Thus, we considered a serum specimen positive if the P/N value was greater than 2.8 for both of the controls. The 10 sera with a medium or low titer of B19 DNA also had B19 antibodies and were negative for P when tested by ELISA. The six sera with high titers of B19 DNA were drawn from patients within 2 days of onset of aplastic crisis, had no B19 antibody and were positive for P. To date, we have tested 29 sera drawn within 2 days of onset of aplastic crisis, and 8 sera were positive for P.

DISCUSSION

The results of the B19 IgG and IgM antibody ELISAs are consistent with other reports that convincingly show that B19 infection is the cause of aplastic crisis in patients with hemolytic anemias and fifth disease (3, 6, 7, 14, 15, 17, 19, 22, 25, 26, 28, 29, 32). We were able to detect B19 IgM antibodies and to demonstrate recent B19 infection in over 85% of cases. B19 IgM antibody was found in <2% of controls and in <1% of patients with other illnesses. Some of the case sera that were negative for IgM antibodies may have been drawn too early or too late after onset of illness for IgM to be detected. It is also possible that these patients had B19 infection and did not devlop an IgM antibody response or that their illness was unrelated to B19 infection. Some of the control sera positive for IgM antibodies may have come from persons with clinically inapparent B19 infection. The B19-IgM antibodies developed and declined rapidly. Over 90% of cases had IgM antibody by the end of 1 week of illness, and the titer and rate of positivity began to drop after 1 month of illness. Despite the drop in IgM antibodies, four of the six sera drawn 4 or more months after onset of illness were still positive, though at a low titer. This pattern of antibody response is similar to that previously described with the B19 IgM RIA (4, 6, 10, 21, 25). These data suggest that the B19 IgM ELISA is similar to RIA in sensitivity and specificity testing for B19 infection, though the ELISA may be less sensitive than the RIA for B19 antibody detection. The test is most sensitive when the serum specimen is drawn between 3 days and 1 month after onset of illness. B19 IgG antibodies developed slower than IgM antibodies but were present by the end of 1 week of illness in 90% of patients. The IgG antibodies persisted for at least 4 months in the epidemic sera and persisted for many years in the age-specific antibody prevalence study. The prevalence of IgG antibodies by age is consistent with other reports that show low rates among preschool children (2, 16) and rates of 30 to 60% among adults (2, 10, 11, 13, 23, 25, 27). Some of the differences in reported rates of IgG positivity represent differences in the sensitivity of the test. The highest rate of IgG positivity (61%) was by RIA, while only 43% of the same sera were positive by counterimmunoelectrophoresis (10).

The antigen detection ELISA was not as sensitive as the DNA hybridization assay. The lower sensitivity probably was caused in part by B19 antibody in the patient's serum-blocking antigenic sites on the virus, as suggested for the detection of P by RIA (5).

The B19 IgG and IgM ELISA appear to be sensitive and specific for detecting B19 infections. With this ELISA, we were able to use P at a high enough dilution to conserve our limited supply without compromising the sensitivity of the test. The principal advantages of the ELISA over the RIA

TABLE 3. Comparison of RIA and ELISA for B19 antibodies in 25 serum specimens

RIA result	No. of specimens with ELISA result			
	IgM		IgG	
	Positive	Negative	Positive	Negative
Positive	19	1	18	1
Negative	0	5	0	6

TABLE 2. B19 IgM antibodies among patients with other illnesses

Illness	No. positive/no. tested	
	Cases	Controls
Kawasaki disease	0/26	0/12
Thyroiditis	0/17	0/25
Transient erythroblastopenia or aplastic anemia	0/10	
Other viral infections[a]	1/43[b]	
Hemolytic uremic syndrome	0/6[b]	

[a] Includes 19 patients with enterovirus infections, 12 patients with adenovirus infections, 6 patients with measles, and 6 patients with rubella.
[b] Acute- and convalescent-phase serum pairs.

TABLE 4. B19 IgG antibody by age group[a]

Age group (yr)	% Positive tests (no. tested)
<5	2 (51)
5–9	21 (32)
10–19	36 (49)
20–39	49 (173)

[a] Sera were obtained from controls or patients with non-B19-related diseases from different locations in the United States.

are that it is more easily automated and that it bypasses the use of radioactive reagents with their attendant hazard, cost, and disposal problems.

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