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Counterimmunoelectrophoresis Test for Immunoglobulin M Antibodies to Group B Coxsackievirus

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A counterimmunoelectrophoresis test was developed for immunoglobulin M (IgM) antibodies to group B coxsackievirus (CB) types 1 through 5. The IgM precipitin line could be identified and differentiated from the IgG line by treating sera with 2-mercaptoethanol. Antigen purity was demonstrated by single precipitin lines occurring only to the homologous antigen when tested with type-specific hyperimmune rabbit sera. Serum pairs from 19 of 22 patients with documented CB type 1, 3, 4, and 5 infections were positive for IgM antibody to the infecting serotype, whereas 2 of 7 pairs from CB type 2 patients were positive. Heterologous IgM antibodies were present in sera from 14 of 29 CB patients. Of the 14 patients with heterologous IgM antibodies, 12 also had ≥4-fold rises in whole serum neutralizing antibody to heterologous serotypes. Only three control sera from 72 patients with coxsackievirus group A, echovirus, or other viral infections had IgM antibody to CB serotypes.

A variety of means have been reported for the detection of antiviral immunoglobulin M (IgM) antibodies in human sera as an indication of a current or recent infection. A simple and reliable procedure for demonstration of IgM antibodies to group B coxsackieviruses (CB) is a microslide Ouchterlony gel diffusion test developed by Schmidt and colleagues (4-7). Two distinct precipitin lines were observed with this immunodiffusion (ID) test. One line was group specific and was composed of IgG antibodies and noninfectious viral particles. The other line was type specific and consisted of IgM antibodies and intact infectious viral particles. A critical requirement for highly concentrated antigen preparations and a failure to detect CB type 2 antibodies were the major limitations of this technique.

The counterimmunoelectrophoresis (CIE) test has potentially greater sensitivity than the ID test because of the unilinear as opposed to radial migration of reactants. Furthermore, in most instances the test can be completed in 1 h. A CIE test has been used to type CB isolates with hyperimmune animal antisera (2). This report describes a rapid and easily performed CIE test for IgM antibodies to CB types 1 through 5 in human sera and discusses the sensitivity and type specificity of the technique.

MATERIALS AND METHODS

Serum specimens. Paired acute- and convalescent-phase sera from 101 patients, age 6 days to 57 years, were obtained from the Wisconsin State Laboratory of Hygiene, Virus Section. All patients had illnesses that were associated with isolation of viruses from the throat, stools, or cerebrospinal fluid and a ≥4-fold serum antibody titer rise to the isolate. The test group was comprised of sera from 29 patients with CB type 1 through 5 isolates. The control group consisted of paired sera that were negative for CB type 1 through 5 whole serum neutralizing antibody rises. This group included sera from 22 patients with group A coxsackievirus or echovirus isolates and 50 patients with non-enteroviral isolates.

Virus preparation and concentration. Strains of CB types 1 through 5 used in the CIE test were isolated in our laboratory from patient specimens during 1976 and 1977. Terminal dilutions of virus were passed three times in cell cultures and tested for purity and type specificity by neutralization with rabbit hyperimmune antisera (Microbiological Associates, Bethesda, Md.). Type specificity also was evaluated by testing each antigen with rabbit antisera in the CIE test. Virus suspensions were prepared by collecting the maintenance medium from tubes of primary rhesus monkey kidney cells or flasks of HEp-2 cell cultures that showed 100% infection. Cell debris was removed by centrifugation at 1,500 rpm for 20 min. The suspension was further clarified by centrifugation at 15,000 rpm (15,000 × *g*) for 30 min in a Spinco model L ultracentrifuge. A 50-fold concentration was achieved by centrifugation at 40,000 rpm (106,500 × *g*) for 1 h and resuspension of pellets in phosphate-buffered saline, pH 7.2. The titer of the viral concentrate was determined with rhesus monkey kidney or HEp-2 cell cultures by the tube 50% tissue culture infective dose method (3). Antigens were stored at 5°C for up to 1 month.

CIE. An agar plate was prepared by covering a

projector slide cover glass (8.3 by 10.2 cm; Eastman Kodak, Rochester, N.Y.) with 15 ml of a boiled solution of 1% Seakem agarose (Marine Colloids, Rockland, Maine) in high-resolution buffer [tris-(hydroxymethyl)aminomethane-barbital-sodium barbital buffer, pH 8.8; Gelman Instrument Co., Ann Arbor, Mich.] with a 0.075 ionic strength. The plate was stored overnight at 5°C in a humid atmosphere to permit maturation of the agar. Pairs of wells, 3 mm in diameter and 6 mm center to center, were cut in the agar. Three rows, each consisting of 12 pairs of wells, were arranged on the plate.

Wells were filled with 5 μl of reactant. Undiluted antigens were located on the cathode side, and whole sera were placed on the anode side. A single plate was placed in an electrophoresis chamber (Gelman) and connected by paper wicks to each reservoir containing 400 ml of high-resolution buffer with a 0.075 ionic strength. The power source was set for constant voltage at 200 V, and the current (initially 32 mA) was applied for 1 h. The plate was held for 1 h in a humid environment and then examined with a Precision Viewer (Hyland, Costa Mesa, Calif.). Finally, the plate was immersed in a 0.85% NaCl solution, held overnight at 5°C, and then reexamined.

Microneutralization test. Sera were inactivated (56°C, 30 min), and 50 μl of twofold dilutions (1:8 to 1: 8,192) in growth medium were prepared in duplicate in 96-well tissue culture microtiter plates (Linbro Scientific, New Haven, Conn.) by using an automated microdilutor (Medimixer; Linbro). A 50-μl volume of growth medium containing 32 to 320 50% tissue culture infective doses of virus was added to each well. A single row of serum dilutions containing no virus was prepared for each serum. A virus back titration (100 to 0.01 50% tissue culture infective doses per 50 μl) was done in quadruplicate. Four wells containing only growth medium were included with each plate. The plates were gently agitated and then incubated at 37°C for 1 h in a 5% CO_2 atmosphere. A suspension of LLC-MK_2 cells (one 75-cm^2 flask to five microtiter plates) was prepared in growth medium, and 0.15 ml was added to each well. The plates were gently agitated, reincubated for 6 days, and examined microscopically for a viral cytopathic effect.

Mercaptoethanol treatment. Nine volumes of whole serum were mixed with one volume of a 1.25 M solution of 2-mercaptoethanol (Sigma Chemical, St. Louis, Mo.) in phosphate buffered-saline, pH 7.2. Controls consisted of mixtures of 9 volumes of serum and 1 volume of phosphate-buffered saline. Mixtures were incubated for 2 h at 37°C. Treated sera were tested immediately, eliminating the necessity of removing the 2-mercaptoethanol by dialysis.

RESULTS

Virus concentration. The use of virus strains obtained from recent patient isolates was critical to formation of precipitin lines in the CIE test. Initial experiments not described in this paper revealed that concentrates of prototype strains that were prepared in an identical manner and had comparable virus titers either failed to form visible precipitin lines or only sporadically formed them.

An average of about 50% of the total virus present in the original suspensions was recovered in the viral concentrates when the clarified suspensions were centrifuged at 40,000 rpm for 1 h. Approximately 2% of the total virus present originally was detected in the supernatant fluid. Centrifugation at 40,000 rpm for 1 h resulted in higher recoveries than did centrifugation at 30,000 rpm for 6 h ($\leq$10%) or at 30,000 rpm for 16 h (10 to 20%).

Eighteen viral concentrates prepared in rhesus monkey kidney cells during the study had virus titers that ranged between $10^{8.0}$ and $10^{9.75}$ 50% tissue culture infective doses per ml. Concentrates with lower titers performed comparably to those with higher titers. Titers of antigens prepared in HEp-2 cells were similar to those prepared in rhesus monkey kidney cultures and performed equally well.

CIE test parameters. More satisfactory results were achieved with a continuous buffer system than with a discontinuous one in which the ionic strength of the buffer in the agar was lower than that in the reservoirs. A continuous system with a 0.075 ionic strength buffer was superior to one with a 0.05 buffer. A potential of 200 V was high enough to permit strong lines to form but low enough to avoid excessive heat production in the agar. The current had to be applied for a minimum of 50 min to ensure optimum line formation; hence, a time of 1 h was selected.

Lines that formed with the majority of patient sera were distinct, and IgM-positive reactions usually were easily distinguished from negative reactions (Fig. 1). When weak lines formed, overnight storage in a normal saline bath increased the density of the lines and provided more contrast between the lines and the agar. On occasion, precipitin lines which were not evident immediately after electrophoresis became visible after overnight saline immersion. Consequently, this procedure was adopted routinely.

Sera from most CB patients were tested on at least three different occasions. Homologous IgM precipitin lines were reproducible, whereas heterologous lines were not as consistently reproduced.

Tests with rabbit antisera. Each CB antigen formed a distinct single line with its homologous rabbit hyperimmune antiserum and did not form a line with any of the heterologous antisera.

Mercaptoethanol treatment. Treatment of sera with 0.1 M 2-mercaptoethanol in many cases reduced the intensity but did not prevent formation of the line that was closer to the

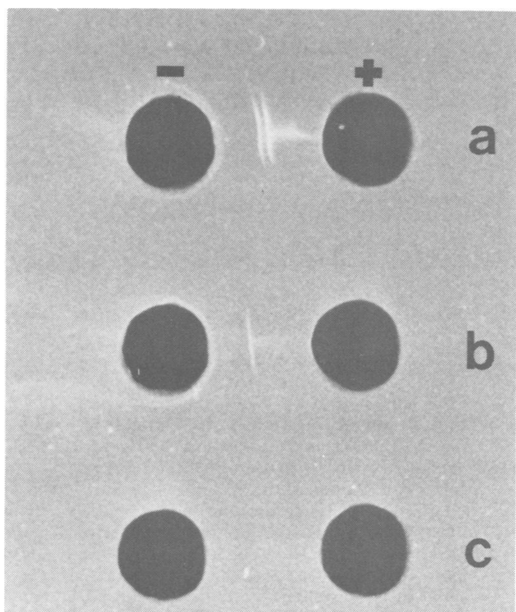

FIG. 1. *Formation of precipitin lines by CIE. −, Cathode; +, anode. (a) IgG-IgM positive; (b) IgG positive; (c) IgG-IgM negative.*

antiserum well. After treatment with 0.125 M mercaptoethanol, the proximal line was eliminated in 8 of 10 instances and was barely discernable in the other 2 treated sera compared with results with untreated samples diluted similarly with phosphate-buffered saline. The proximal line, therefore, represents the IgM antibody-antigen complex since it is well known that 2-mercaptoethanol selectively degrades the IgM antibody molecule. Formation of the distal line was unaffected by the treatments and represents IgG activity.

IgM in human sera. Serum pairs from 20 of 22 patients (91%) with CB type 1, 3, 4, and 5 infections were positive for IgM antibody, and 19 patients had IgM to the infecting serotype (Table 1). Only three of seven paired sera from CB type 2-infected patients had detectable IgM antibody, and in one of the three pairs there was no homologous IgM. In 14 of 21 instances where IgM to the infecting serotype was demonstrated, the homologous IgM occurred only in the convalescent sample.

Of the 29 serum pairs from CB patients, 14 had IgM to heterologous serotypes (Table 2). Activity to a single serotype was encountered in six serum pairs, and seven pairs were associated with two types. Of the 14 patients with heterologous IgM antibodies, 12 also had ≥4-fold rises in whole serum neutralizing antibody to heterologous serotypes. Multiple rises also occurred in five of nine patients who had IgM antibody to a single serotype.

Of the 72 control patients, 3 had CB IgM (Table 2). Two of the patients had echovirus type 6 infections, and the third was an influenza virus type A patient. Both the acute and convalescent sera of all three control pairs were IgM positive, and each involved only a single CB serotype. Two of the IgM diagnoses were associated with a high sustained neutralizing antibody titer to the reacting serotype (≥1:1,024).

TABLE 1. *Detection of CB type 1 through 5 IgM antibodies by CIE in sera from patients with CB infections*

CB infecting serotype	No. of patients	No. of patients with IgM to infecting serotypes[a]		
		Ho only	Ho + Ht[b]	Ht but no Ho
1	5	2	2	0
2	7	0	2	1
3	6	3	3	0
4	7	3	3	0
5	4	1	2	1

[a] Ho, Homologous antibody; Ht, heterologous antibody.

TABLE 2. *Heterologous IgM antibody to CB serotypes in human sera detected by CIE*

Infecting serotype	No. of patients	No. of patients with heterologous antibody to:		
		One type	Two types	Three types
CB type 1	5	1	1	0
CB type 2	7	1	2	0
CB type 3	6	0	3	0
CB type 4	7	2	0	1
CB type 5	4	2	1	0
Other enteroviruses[a]	22	2	0	0
Other viruses[b]	50	1	0	0

[a] Coxsackievirus group A type 5; echovirus types 3, 4, 5, 6, 7, 11, and 22.

[b] Influenza virus types A and B; parainfluenza virus types 1, 2, and 3; adenovirus types 2, 3, and 21; Herpes simplex virus types 1 and 2; cytomegalovirus.

DISCUSSION

The data presented above indicate that demonstration of type-specific IgM by CIE is a rapid and reliable technique for serological diagnosis of CB infections. Homologous IgM was demonstrated in 19 of 22 patients (86%) with CB type 1, 3, 4, and 5 infections, and an additional patient had heterologous but no homologous IgM. In

contrast, only three of seven patients with CB type 2 infections had IgM antibody, and one of these was only a heterologous reaction. This limited success with CB type 2 mirrors the experience of Schmidt and colleagues (4-7), who had good success with their ID test for CB type 1, 3, 4, and 5 infections but failed to diagnose any CB type 2 infections, even when the antigen was concentrated 750-fold. The reason why neither immune precipitin test works satisfactorily for this serotype is not clear.

Heterologous reactions were common in our CB-infected patients, as determined by both CIE and neutralizing antibody testing. Schmidt and colleagues also observed heterologous IgM reactions with the ID test in 20 of 96 patients with CB type 1 through 5 isolates (5) and in 14 of 70 patients with myopericarditis or pleurodynia (7). The explanation of why heterologous reactions occur with these viruses is far from clear. One approach to this question may be more extensive purification of the CIE antigens.

Finding CB IgM in control patient sera was not surprising. Schmidt et al. (7) found CB IgM in 8% of 259 control patients.

The greatest immediate benefit of the ID and CIE tests can be derived from their application to diagnosing CB-related myopericarditis. Although the CB have been most frequently associated with this disease, viral isolation attempts and/or demonstration of significant neutralizing antibody rises in these patients seldom yield diagnoses (1, 7). A plausible explanation for this anomaly is that the onset of cardiac symptoms may occur late in the infection. Schmidt et al. (7) found that 27% of 259 patients with myopericarditis or pleurodynia had CB IgM antibodies by the ID test. We have tested sera from several hundred myopericarditis patients to date by CIE and obtained nearly identical results (unpublished data).

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