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Subclass Restriction of Human Enterovirus Antibodies

EINAR G. TORFASON,[1]† CHARLES B. REIMER,[2] AND HARRY L. KEYSERLING[1]*

Department of Pediatrics, Emory University School of Medicine, Atlanta, Georgia 30303,[1] and Division of Host Factors, Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia 30333[2]

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We studied antibodies to enteroviruses in four groups of serum specimens: those from healthy adults, cord blood specimens, serum specimens known to contain immunoglobulin M (IgM) to coxsackie B (CB) viruses by radioimmunoassay, and serum specimens from children with symptomatic enteroviral infections. Enzyme-linked immunosorbent assays (ELISAs) were developed to detect the IgG class- and subclass (IgG1, IgG2, IgG3, and IgG4)-specific responses to CB3. The CB3 virus ELISA was not type specific. There was very poor correlation between CB3 virus neutralizing titer and IgG anti-CB3 virus ELISA results, indicating that antibodies to heterologous picornaviruses cross-react with CB3 virus in the assay. All serum specimens tested except one were IgG positive for CB3 virus. All 32 cord serum specimens were positive for IgG1 and IgG3. No enterovirus-specific IgG2 or IgG4 was detected in any serum specimen tested. Most serum specimens from the IgM-positive group, healthy adults, and children with enterovirus infections were positive for IgG1 and IgG3. Class and subclass antibody titers remained constant over time. IgG antibodies to enteroviruses appear to be restricted to the IgG1 and IgG3 subclasses. This pattern is similar to results obtained by other investigators evaluating IgG subclass antibodies to protein antigens.

The coxsackie B (CB) viruses are members of the genus *Enterovirus* of the *Picornaviridae* family. Enteroviruses are small, nonenveloped RNA viruses with no detectable lipids or carbohydrates. They may produce rashes, pharyngitis, upper respiratory infections, conjunctivitis, parotitis, pericarditis, myocarditis, hepatitis, pancreatitis, orchitis, pleurodynia, meningitis, and encephalitis. Although many infections are asymptomatic or self-limited, fulminant hepatitis, myocarditis, or encephalitis may be fatal. Neonates are more susceptible to severe disease than older children and adults are. Increasing evidence links CB viruses with juvenile-onset, insulin-dependent (type I) diabetes mellitus (1, 6, 8).

There are four subclasses of immunoglobulin G (IgG) (IgG1, IgG2, IgG3, and IgG4) in humans (4). All the IgG subclasses are transported across the placenta during pregnancy (3, 13). There seem to be at least three subclass patterns of IgG responses to different antigens (15). Responses to most viruses studied are predominantly IgG1, with IgG3 as the second most abundant subclass (9, 18, 20). A second pattern, seen after prolonged immunization to proteins, involves IgG1 and IgG4. A third pattern, apparently as a response to polysaccharide antigens, involves IgG1 and IgG2. Children are less likely to develop IgG2 or IgG4 responses than are adults. For a review of subclass responses, see Rautonen et al. (15) and Rubin et al. (17).

To our knowledge, the immunoglobulin subclass distribution of antibodies to the nonpolio enteroviruses has not been reported. The purpose of this study was to examine the subclass restriction pattern of a CB virus. This information may be useful for diagnostic tests and vaccine development.

In the present study, we examined serum specimens from four groups: healthy adults, cord blood, serum known to contain IgM reacting in type-specific radioimmunoassays (RIA) with CB1 to CB5 virus antigens, and a few serum specimens from infants and children with symptomatic enterovirus infections documented by virus isolation. Conventional neutralizing antibodies to CB1 to CB6 virus were determined in 34 serum specimens from the healthy adults to compare results with those of enzyme-linked immunosorbent assays (ELISAs). Assays were developed for antibody classes and subclasses by using an ELISA system with CB3 virus antigen and monoclonal antibodies to the human immunoglobulin subclasses.

MATERIALS AND METHODS

Cells and viruses. FL (amnion, human) and Vero (kidney, African green monkey) cells were obtained from the American Type Culture Collection, Rockville, Md. Cells were propagated in Eagle minimum essential medium with Earle salts, supplemented with MEM nonessential amino acids. Growth medium contained 10% newborn calf serum (NCS), and maintenance media contained 2% NCS.

Prototypic strains of CB1 to CB6 virus were used in the neutralization titer tests.

Virus purification. The purification procedure was a minor modification of techniques described previously (7, 19). Briefly, clarified medium and clarified cell lysate from virus-infected monolayers were combined, and the virus particles were concentrated and purified by two cycles of ultracentrifugation, the first being done with a cushion of CsCl and the second being an isopycnic ultracentrifugation in CsCl.

Serum specimens. The Infectious Diseases Division, Department of Pediatrics, Emory University, Atlanta, Ga., provided 60 serum specimens from 38 laboratory staff, collected between 1979 and 1986 for herpes simplex virus serology. The Grady Memorial Hospital, Atlanta, Ga., provided 32 cord blood specimens, collected in spring 1986 for sickle cell anemia screening. The State Laboratory of Public Health, Raleigh, N.C., provided seven specimens collected in 1985 from five children with proven enterovirus infection (CB2 virus, echovirus type 11, and polio virus type 2). Of these, one pair of specimens was from a 6-year-old child with a CB2 virus infection; the other children ranged in age from newborn to 6 months. The Department of Medical Virology, University of Uppsala, Uppsala, Sweden, provided 20 serum

* Corresponding author.
† Present address: Department of Microbiology, Virus Laboratory, University of Iceland, Reykjavik, Iceland.

specimens from 14 individuals with suspected enterovirus infections. These all tested IgM positive against at least one CB virus type in a direct, reverse RIA (7, 19). Finally, by using estimated IgG subclass concentrations of an intramuscular immune serum globulin preparation (Gamastan; Cutter Biological, Berkeley, Calif.) and a purified preparation of human IgG (Jackson ImmunoResearch Laboratories, Avondale, Pa.), we attempted to estimate the limiting concentration sensitivity of the assays.

Mouse monoclonal antibodies. Mouse monoclonal antibodies (present as lyophilized ascitic fluids) to each subclass of human IgG were obtained from the Centers for Disease Control, Atlanta, Ga. Validation of the specificity of the anti-human IgG isotype reagents has been described previously (16). The mouse anti-human IgG1 (HP6001) was Fc specific; the anti-human IgG2 (HP6014) was Fab specific; the anti-human IgG3 (HP6047) was specific for the hinge; and the anti-human IgG4 (HP6024, HP6025) was Fc specific. The monoclonal antibodies were titrated for maximum immunochemical sensitivity consistent with specificity (low background) in ELISA plates coated with serial dilutions of purified IgG and the immune serum globulin preparation (see above), as well as control wells coated with bovine serum and lysate of Vero cells. Monoclonal antibodies HP6001 and HP6014 were diluted 1:2,000, and HP6047, HP6024, and HP6025 were diluted 1:1,000.

Class-specific ELISA. Polystyrene 96-well plates (Immulon II; Dynatech Laboratories, Inc., Alexandria, Va.) were coated at 4°C overnight with CB3 virus antigen and a control antigen preparation consisting of clarified lysate of uninfected cells, diluted 1:100 in carbonate buffer (pH 9.4 to 9.6). After the coating and between all subsequent incubations, the plates were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20. The specimens were serially diluted fourfold in PBS containing 1% bovine serum albumin (BSA), 0.05% Tween 20, and 10% NCS starting at a 1:32 dilution. Two wells each of control antigen and virus antigen each received 100 μl of the appropriate serum dilution and were incubated at 37°C for 2 h. After being washed, the plates were incubated at 37°C for 45 min with 100 μl of affinity-purified biotinylated $F(ab')_2$ fraction of goat anti-human IgG Fc (Jackson), diluted 1:2,000 in PBS containing 1% BSA, 0.05% Tween 20, and 0.1% NCS. Then 100 μl of streptavidin-horseradish peroxidase conjugate (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) diluted 1:2,000 in PBS containing 1% BSA and 0.05% Tween 20 was added. The plates were incubated at 37°C for 15 min. The enzyme reaction took place at room temperature in citrate buffer (pH 6.0) containing 0.4 mg of *o*-phenylenediamine and 0.3 μl of 30% H_2O_2 per ml (100 μl per well). After 10 to 15 min in the dark, the reaction was terminated by the addition of 50 μl of 4 N H_2SO_4 per well. The color reaction was measured at 492 nm in a Titertek Multiskan spectrophotometer. The titer of a serum specimen was defined as the highest dilution at which the mean of the CB3 antigen wells was still 0.2 optical density units higher than the mean of the control antigen wells.

Subclass-specific ELISA. The plates, the coating, the washing procedure, and the incubation with the human serum were all identical to those used for the class-specific assay described above. After the serum incubation, mouse monoclonal antibody in PBS containing 1% BSA, 0.05% Tween 20, and 10% NCS was added. The plates were incubated with the monoclonal antibodies at 37°C for 2 h, washed, and incubated at 37°C for 45 min with the conjugate, Biotin-SP-AffiniPure $F(ab')_2$ fragment sheep anti-mouse IgG (heavy plus light chains) (Jackson) diluted 1:2,000 in PBS containing 1% BSA, 0.05% Tween 20, and 0.1% NCS. Incubation with streptavidin-horseradish peroxidase, enzymatic reaction, reaction termination, reading of the plates, and determination of the serum titer were all identical to those used for the class-specific assays described above.

Confirmation of anti-human antibody activity. Wells coated with immune serum globulin or purified IgG (instead of virus antigen) provided a method to demonstrate the biologic activity of the polyclonal and monoclonal reagents.

Neutralization tests. Neutralization titer tests for CB1 to CB6 virus were performed on serum specimens from 34 laboratory personnel. The serum specimen was first screened at a 1:10 dilution with 100 50% tissue culture infective doses ($TCID_{50}$) of the virus. If wells showed signs of partial or complete neutralization, the specimen was titrated in twofold dilutions, starting at 1:10. The procedure was reported previously (19).

TABLE 1. Neutralization titers against CB1 to CB6 viruses in serum specimens from 34 laboratory personnel

Virus	No. (%) negative[a]	No. (%) positive	Titer range
CB1	23 (67.6)	11 (32.4)	10–1,280
CB2	15 (44.1)	19 (55.9)	10–640
CB3	21 (61.8)	13 (38.2)	10–320
CB4	6 (17.6)	28 (82.4)	10–1,280
CB5	16 (47.1)	18 (52.9)	10–320
CB6	28 (82.4)	6 (17.6)	10–80

[a] Measured at 1:10 dilution.

RESULTS

CB3 virus ELISA antigen. The infectivity titer of the crude harvest from two 850-cm^2 roller bottles and one 150-cm^2 flask used to purify CB3 virus antigen was 10^8 $TCID_{50}$/ml; after the first ultracentrifugation it was $10^{8.5}$ $TCID_{50}$; and after the second it was 10^{10} $TCID_{50}$. The final protein concentration was approximately 400 μg/ml (Bio-Rad Protein Assay; Bio-Rad Laboratories, Richmond, Calif.). The CB3 virus antigen preparation gave approximately 10 ml of antigen stock. This provided enough reagent to coat approximately 200 ELISA plates.

Neutralization antibody. In the specimens from the 34 laboratory personnel, the prevalence of neutralizing antibody varied from 17.6% for CB6 to 82.4% for CB4 virus (Table 1). Titers ranged from 10 to 1,280 for CB1, 10 to 640 for CB2, 10 to 320 for CB3, 10 to 1,280 for CB4, 10 to 320 for CB5, and 10 to 80 for CB6 virus. Specimens from two individuals had no neutralizing antibody against any of the CB viruses.

ELISA antibody. The results of the ELISA are summarized in Table 2. Although CB3 virus antigen was used as the prototypic enterovirus antigen, all the specimens except one were positive. (CB3 virus has antigens common to many other picornaviruses. This ELISA would detect antibodies against a broad spectrum of picornavirus epitopes.) All 21 specimens negative in the CB3 virus neutralization titer test were positive in the CB3 virus antigen IgG ELISA (comparison not shown). Titers for serial specimens from laboratory personnel remained unchanged for several years.

(i) IgG. All the cord serum specimens, all the serum specimens from laboratory personnel, all the RIA IgM-positive serum specimens, and five of six specimens from children with enterovirus infections proven by virus isola-

tion (enterovirus group) were IgG positive (Table 2). The titers ranged from 128 to 16,384 in the cord serum group. For specimens from the laboratory personnel, the titers ranged from 512 to 8,192. One serum specimen from the enterovirus group was negative, one pair had a titer of 128, one had a titer 512, one had a titer of 2,048, and one had a titer of 65,536 or more. The titers of the RIA IgM-positive specimens ranged from 128 to 4,096.

(ii) IgG1. All 32 cord serum specimens were IgG1 positive. In the laboratory personnel group, 54 of 57 (94.7%) specimens were positive. Two of the three negative specimens in that group were taken from the same individual. All the specimens in the RIA-positive group were IgG1 positive. In the enterovirus group, five of six serum samples were positive, and the negative specimen was also negative in all the other assays. The titers ranged from 64 to 8,192.

(iii) IgG2. No IgG2 reacting with the CB3 virus antigen was found in any serum specimen.

(iv) IgG3. All 32 cord serum specimens were positive for IgG3. In the laboratory personnel group, 53 of 59 (89.8%) specimens were positive, and the six negative specimens came from three individuals (92% of individuals were positive). In the RIA IgM-positive group, 11 of 20 (55%) specimens were positive. In the enterovirus group, two paired specimens were positive and five specimens were negative. The IgG3 titers ranged from 32 to 4,096.

(v) IgG4. No IgG4 reacting with the CB3 antigen was detected in any of the specimens.

Confirmation of anti-human antibody activity. The estimated minimum concentration of IgG class or subclass detected was calculated on the basis of expected normal values of IgG subclasses present in human serum (4). Results when wells were coated with purified IgG and Gamastan were 0.05 and 0.02 μg/ml, respectively, for IgG, 1.0 and 0.6 μg/ml for IgG1, 0.1 and 0.04 μg/ml for IgG2, 0.4 and 0.01 μg/ml for IgG3, and 0.3 and 0.1 μg/ml for IgG4. The discrepancy in results obtained with purified IgG and Gamastan may reflect selective losses or enrichment of subclasses during purification, particularly denaturation of the labile, sulfhydryl-containing hinge region (2). We have no data indicating how much of each immunoglobulin subclass actually bound to the solid phase, nor of the molecular orientation of its antigenic epitopes. These results provide evidence of biologic activity rather than a direct quantitative assessment of immunochemical sensitivity.

DISCUSSION

We report the detection of the enterovirus-specific IgG class and subclasses by using purified CB3 virus as the enterovirus antigen. In several studies, immunoassays involving the use of enterovirus antigen for coating the solid phase have given group-specific (broadly reactive) rather than type-specific results, even when the antigen consists of purified virions (5, 10, 12, 14, 19). This was also the case in the present study.

TABLE 2. Class- and subclass-specific IgG ELISA results with CB3 virus antigen

Specimen group	No. positive specimens/no. assayed for:				
	Total IgG	IgG1	IgG2	IgG3	IgG4
Cord serum	32/32	32/32	0/32	32/32	0/32
Laboratory personnel	59/59	54/57[a]	0/59	53/59[b]	0/59
IgM-positive (RIA) patients	20/20	20/20	0/20	11/20	0/20
Enterovirus patients	5/6	5/6	0/7	2/7[c]	0/7

[a] Two of the three negative specimens came from the same individual.
[b] The six negative serum specimens came from three individuals.
[c] Both positive specimens came from the same individual.

TABLE 3. Distribution of IgG1 and IgG3 titers against CB3 virus antigen

IgG1 titer	No.[a] with following IgG3 titer:								
	<32	32	64	128	256	512	1,024	2,048	4,096
<32	1	1				2			
32	4	3	2						
64	3	7	7	2					
128	10	7	2	6	1	3			
256	1	3	3	20	6				
512				1	8	1			
1,024				1	2	3			
2,048						1	2		
4,096								1	
8,192									1

[a] Of the 119 specimens in the study, 4 were not available for both IgG1 and IgG3 assays. The total number recorded in the table is therefore 115.

In our class-specific assay, we detected IgG in all specimens except one. The negative specimen was from a 4.5-month-old child with a stool isolate positive for poliovirus type 2. One would expect decreased levels of passively acquired transplacental antibody in an infant of this age.

In the subclass assays, all but four specimens (97%) were IgG1 positive. IgG3 was detected in 98 of 118 (83%) specimens. All of the cord serum specimens were positive for IgG1 and IgG3, possibly indicating that the lack of competition from IgM and IgA and the concentration effect with respect to the maternal serum (13) may unmask weak IgG3 responses in samples from adults that are otherwise below the detection limit in our assays. The IgG1 and IgG3 titers generally remained the same when serial specimens from the same individual, spanning up to 6 years, were tested. Table 3 gives the IgG1 and IgG3 titers for 115 serum specimens. The ratio of IgG1 to IgG3 is generally 2:1 to 4:1, indicating a fixed mode of IgG antibody response to CB3 virus antigen. The high prevalence of IgG1 and IgG3 indicates that the assay is group specific rather than CB3 virus specific. No IgG2 or IgG4 responses were detected. The sensitivity of the IgG4 assay is perhaps low for a subclass that is present in such minute amounts as IgG4, but the IgG2 assay appears to be one of our most sensitive assays. The pattern of IgG1 and IgG3 responses and the absence of IgG2 and IgG4 are in good agreement with the general assumption that IgG1 or IgG3 responses or both are to be expected against protein antigens.

In the present study we settled for available techniques. We are aware of certain limitations, such as immunoglobulin class-subclass competition, but we consider that the information about the IgG subclass restriction pattern of the human antibody response against enteroviruses is important. A reverse type of assay might solve the sensitivity problem and give more virus type-specific assays. For this purpose, monoclonal antibody with proven high affinity for the class-subclass in question may be suitable if used as a solid-phase capture antibody. The loss of activity, frequently encountered (11, 16) when monoclonal antibody is used for coating, requires that better coating techniques be developed before monoclonal antibodies can be routinely used for selective capture of human antibodies of a given isotype, particularly if they are present at very low concentrations, such as IgG4, IgA1, IgA2, IgD, or IgE in normal serum.

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