Evaluation of an Enterovirus Group-Specific Anti-VP1 Monoclonal Antibody, 5-D8/1, in Comparison with Neutralization and PCR for Rapid Identification of Enteroviruses in Cell Culture

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We evaluated the usefulness of a commercially available monoclonal antibody (MAb) directed against a group-specific epitope of the capsid protein VP1 of enteroviruses for the rapid identification of these viruses in cell culture. The MAb was assayed in an indirect immunofluorescence test with cultured cells infected by various serotypes of enterovirus; all 39 serotypes tested, including echoviruses 22 and 23, which are considered atypical enteroviruses, were reactive. The MAb was also tested with 61 strains recovered from clinical specimens inoculated into cell cultures in comparison with seroneutralization with intersecting pools of hyperimmune sera and PCR with primers from the 5′ untranslated region of enteroviruses. There was total agreement between the results obtained with the MAb and those obtained by PCR, even for those strains of enteroviruses which were found to be untypeable with polyclonal antisera. These data demonstrate the usefulness of the MAb for rapid identification of enteroviruses in cell culture.

The genus *Enterovirus*, which belongs to the family *Picornaviridae*, consists of positive-stranded RNA viruses and is subdivided into five groups: poliovirus (PV), coxsackievirus A (CA), coxsackievirus B (CB), echovirus (EV), and numbered enteroviruses. There are 68 serotypes which have been associated with infections in humans ranging from asymptomatic to fatal, including respiratory, cardiac, hepatic, and gastrointestinal diseases, meningitis, and poliomyelitis (for a review, see reference 13).

Distinguishing enterovirus-related infections from those due to bacteria or other viruses is important for prognostic, therapeutic, and epidemiologic purposes. Viruses are currently detected by isolation in cell culture followed by neutralization typing with antiserum pools. These methods are laborious and time-consuming. In addition, some factors may impair the interpretation of neutralization tests: many enteroviruses replicate at low titers in cell culture, unstable genomes enable neutralizing-escape mutants to occur at high rates, and the isolate may be a mixture of viral types (14). Recently, the application of PCR to the direct detection of enterovirus genomes in clinical specimens has offered exciting perspectives for the rapid diagnosis of enteroviruses (3, 6, 8, 9, 11, 15, 16, 18, 20, 25). The 5′ untranslated region (5′ UTR) is highly conserved among human enteroviruses, allowing the detection of almost all of the serotypes with a single pair of primers (2, 5, 19, 25). However, these techniques still need to be standardized before the criteria for routine diagnosis will be fulfilled.

In 1987, Yousef et al. described a mouse monoclonal antibody (MAb), designated 5-D8/1, which was able to recognize an enterovirus group-specific (EGS) epitope that is located on the capsid protein VP1 and is common to many serotypes of enteroviruses (23, 24). This MAb is now commercially available (code no. M 7064; DAKO S.A., Trappe, France). The present study was designed to delineate the diagnostic usefulness of this MAb for the rapid identification of enteroviruses in primary-culture cell lysates by indirect immunofluorescence (IIF). The results were compared with those obtained by seroneutralization and PCR with the same specimens.

MATERIALS AND METHODS

Viruses and cell cultures. The enterovirus strains used in this study consisted of reference strains and of clinical isolates from our laboratory, kept frozen at −20°C. Isolates of rhinovirus, herpes simplex virus type 1, and adenovirus were used as controls. Human embryonic lung fibroblasts (Hel line; BioWittakers, Fontenay-sous-Bois, France) and KB cells were used for culture propagation of viruses.

Culture and identification of enteroviruses by seroneutralization. Culture of enteroviruses was performed according to standard procedures as described previously (17). When a cytopathic effect was compatible with the presence of an enterovirus, the strain was passaged onto fresh cells. The culture supernatant was titrated on 96-well microplates seeded with susceptible cells. Ten to 1,000 50% tissue culture infectious doses were used for neutralization testing with intersecting pools of hyperimmune sera (Statens Seruminstitut, Copenhagen, Denmark) according to the Lim–Benyesh-Melnick scheme (10, 14); in some cases, the identification was achieved by neutralization with a specific monovalent polyclonal antiserum.

Identification of viruses by immunofluorescence. Cells from primary-culture lysates were harvested when the cytopathic effect was at least 50%. They were seeded onto slides, dried in an incubator at 37°C, and fixed in cold acetone at 4°C. Mouse MAbs directed against viruses were obtained commercially. The EGS MAb 5-D8/1 was supplied in liquid form as tissue culture supernatant; the concentration in mouse immunoglobulin G was 110 μg/mL. The working dilution was 1:200 in phosphate-buffered saline (PBS), pH 7.2. MAbs directed against adenovirus (Sanofi Diagnostics Pasteur, Marne-la-Coquette, France) and herpes simplex virus type 1 (Syva-Mérieux, Dardilly, France) were used according to the manufacturers' instructions. The last MAb was conjugated to fluorescein, while the two others were used in an IIF test with a fluorescein-conjugated anti-mouse immunoglobulin (Sanofi Diagnostics Pasteur) diluted 1:10 in PBS as the second antibody. Incubations were performed at 37°C for 30 min in a moist chamber. After being washed for 5 min in PBS, the slides were mounted in buffered glycerin and read under UV light with a Leitz epifluorescence microscope at a magnification of ×400. The reading was done in a blinded fashion.

PCR assay for enterovirus identification. The viral RNA from culture supernatants was extracted as described by Johnston et al. (8). Four hundred microliters of sample was treated with 200 μg of proteinase K (Boehringer Mannheim,

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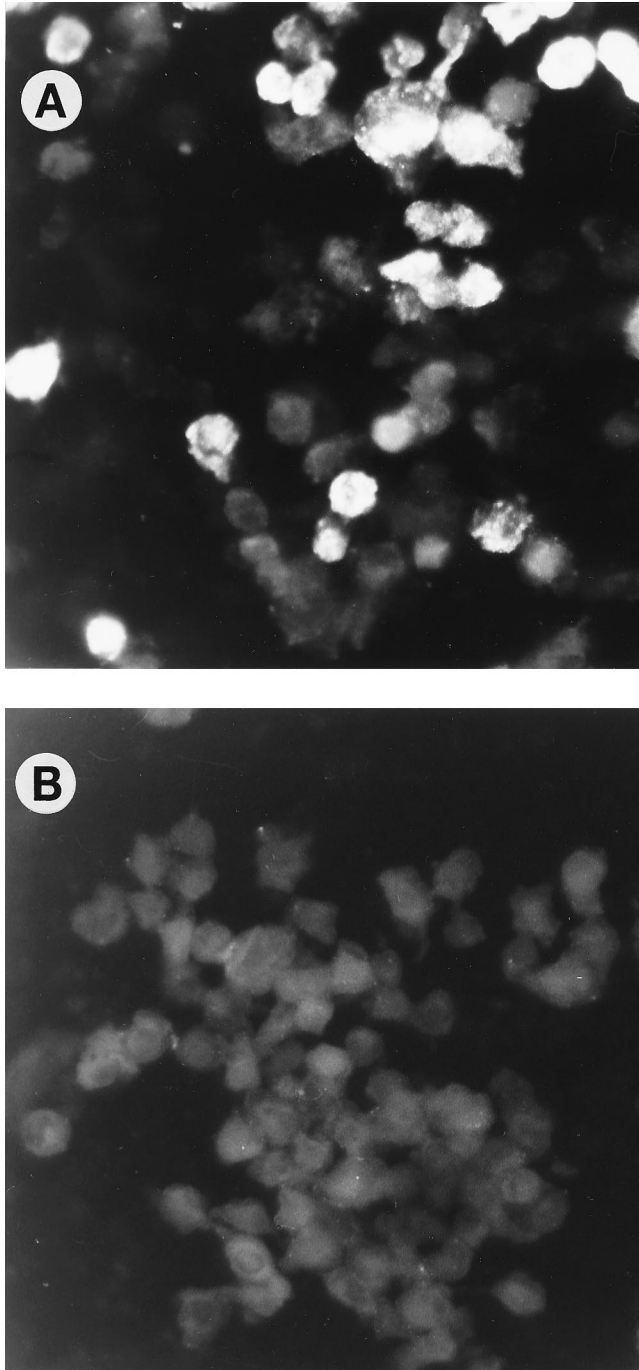
FIG. 1. Photomicrographs of KB cells infected by PV3 (Sabin strain) (A) or uninfected (B) and tested by IIF with the EGS MAb 5-D8/1 as described in Materials and Methods (magnification, ×400).

TABLE 1. Strains of enteroviruses tested with the EGS MAb (5-D8/1) by the IIF test[a]

Enterovirus serotype	Origin of strain[b]	Cell line
PV1	Sabin	KB
PV2	Sabin	KB
PV3	Sabin	KB
CB1	89-5145	KB
CB2	Pretorius	KB
CB3	T.vDee	KB
CB4	Tilo	KB
CB5	Dekking	KB
CB6	Schmitt	KB
EV1	Farouk	Hel
EV2	Cornelis	Hel
EV3	88-11044	Hel
EV4	86-8934	Hel
EV5	84-7632	Hel
EV6	84-4359	Hel
EV7	85-4680	Hel
EV8[c]	Bryson	Hel
EV9	86-8934	Hel
EV11	87-3289	Hel
EV12	Travis 2-85	Hel
EV13	4-11-1D	Hel
EV15	Ch 96-51	Hel
EV17	89-9796	Hel
EV18	85-6268	Hel
EV19	Burke	Hel
EV20	88-8012	Hel
EV21	87-7617	Hel
EV22	Harris	Hel
EV23	Williamson	Hel
EV24	84-4617	Hel
EV25	89-2627	Hel
EV26	Coronel	Hel
EV27	Bacon	Hel
EV29	83-3259	Hel
EV30	87-7468	Hel
EV33	82-3851	Hel
CA7	Russian AB IV	Hel
CA9	81-3363	Hel
CA16	8-610	Hel
CA21	88-11568	Hel

[a] All strains were positive by the IIF test.
[b] Except for the origin of EV13, numbers correspond to clinical isolates; EV13 and the other strains are reference strains.
[c] EV8 is a variant of EV1.

Meylan, France) for 1 h at 56°C and then subjected to a single phenol-chloroform-isoamyl alcohol (25:24:1) extraction. After addition of 40 μl of 3 M sodium acetate (pH 5.5) to the supernatant, RNA was precipitated for 1 h at −80°C with glacial ethanol. The RNA pellet was washed in 70% ethanol and resuspended in 30 μl of DEPC-treated water containing 3 μl of RNase inhibitor (RNAsin; Sigma, Saint-Quentin-Fallavier, France). Oligonucleotide panenteroviral primers were purchased from Genset (Paris, France). They were derived from the conserved sequences in the 5′ UTR of the enteroviral genome and were previously described by Zoll et al. (25). The sequences were 5′ATTGTCACCATAAGCAGCCA3′ for primer A and 5′TCCTCCGGCCCCTGAATGCG3′ for primer B. The amplification resulted in a 155-bp product. For reverse transcription,

a 20-μl reaction mixture containing 50 mM Tris HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 1 mM dithiothreitol (Gibco-BRL Life Technologies, Eragny, France), 0.2 mM (each) deoxynucleoside triphosphates (dNTP) (Boehringer Mannheim), 40 pmol of primer B, 100 IU of murine Moloney leukemia virus reverse transcriptase (Gibco-BRL), and 10 μl of template RNA extracted from the culture supernatant was prepared. After incubation at 42°C for 45 min, 2 μl of the previous reaction mixture was added to 48 μl of PCR mixture for the amplification assay. The PCR mixture contained 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM (each) dNTP, 80 pmol of each primer, and 1.25 IU of *Taq* polymerase (ATGC Biotechnology, Noisy-le-Grand, France). After the mixture was overlaid with mineral oil, the tubes were placed in a thermal cycler (Thermocis; Cis Bio International, Gif-sur-Yvette, France); RNA-cDNA hybrids were denatured at 94°C for 5 min. The amplification was performed in 30 cycles, each consisting of denaturation for 0.5 min at 94°C, primer annealing for 1 min at 42°C, and elongation for 2 min at 72°C. The last extension step was prolonged for 10 min. Thirteen-microliter aliquots of DNA samples from the PCR were run on a horizontal, submerged 2% agarose gel in Tris acetate buffer at 90 V for 45 min and stained with ethidium bromide. Only samples with clearly visible bands of the correct size for the primer pair were considered to be positive by gel electrophoresis. To prevent PCR contamination within the laboratory, the following precautions were taken: the preparation of PCR reagents, the extraction of viral RNA from cell lysates, and the amplification step were conducted in three different rooms; tips equipped with filters were used for pipetting reagents

TABLE 2. Testing of cell lysates from primary cultures of 61 clinical specimens with the EGS MAb (5-D8/1) by IIF assay

Group	Result of:		Interpretation	No. positive in IIF assay with 5-D8/1 MAb/no. tested
	Enterovirus-specific neutralization test	Enterovirus-specific PCR		
1	+	+	Typeable enterovirus strains[a]	16/16
2	−	+	Untypeable enterovirus strains[b]	25/25
3	NT[c]	−	Virus belonging to another family[d]	0/9
4	NT	−	Unidentified virus[e]	0/11

[a] Strains were identified as follows: one PV1, one PV2, one PV3, four CB2, one CB3, 3 CB5, one CA9, one EV11, two EV20, and one EV30.

[b] Untypeability may be due to very low titers in cell culture, to variant strains, or to simultaneous infection with several serotypes.

[c] NT, not tested.

[d] Ten strains of adenovirus and one strain of herpes simplex virus type 1.

[e] The strains of this group gave a cytopathic effect in cell culture (which was passaged several times) but could not be identified by routine testing.

introduced into the PCR tubes; and negative controls consisting of water and of DNA extracts from noninfected cells were tested in parallel in each experiment to exclude carryover.

RESULTS

Evaluation of the reactivity of the EGS MAb by IIF. An IIF test was used to evaluate the reactivity of the EGS MAb. As shown in Fig. 1, the MAb showed a bright cytoplasmic fluorescence in entovirus-infected cells but not in uninfected cells. Table 1 lists the enterovirus strains which were tested with the EGS MAb and found to be positive by the IIF test. The remaining serotypes either were not available in the laboratory or could not be tested in cell culture. The nonenterovirus strains (rhinovirus, adenovirus, and herpes simplex virus type 1) which were tested with the EGS MAb were all negative by IIF, as were noninfected cells (data not shown).

Ability of the EGS MAb to identify enteroviruses in cell culture in comparison to neutralization and PCR. Cell lysates from primary cultures of 61 clinical isolates classified in four groups (Table 2) were tested by IIF with the EGS MAb. The sensitivity of this assay was 100%, since all of the strains identified as enteroviruses by PCR were positive by IIF, even when the determination of the serotype was not possible by seroneutralization (group 2 in Table 2). The specificity of the assay was excellent, since no false-positive results occurred with nonenterovirus strains (groups 3 and 4 in Table 2) (although the number and the diversity of such strains were rather limited).

DISCUSSION

The initial study of Yousef et al. (23) had evaluated the reactivity of the EGS MAb against a limited number of enterovirus serotypes (CB1 through CB6, PV1, PV3, CA7, CA9, EV11, and EV22). The results of the present study confirm the broad reactivity of this MAb, since all tested serotypes of enteroviruses were found to be positive (Table 1). By contrast, no reactivity was seen with nonenterovirus strains either belonging (rhinovirus and hepatitis A virus) or not belonging to the *Picornaviridae* family (reference 23 and this study). However, only a limited number of strains was used to assess the specificity of the assay; additional testing would be useful before definitive conclusions about the specificity of the MAb are made.

It is worthwhile to note that EV22 and EV23 were recognized unambiguously by the EGS MAb. These two serotypes have been shown to differ from the other enteroviruses in important features, including cytopathic effect (22), receptor properties (12, 21), lack of hybridization with enterovirus-specific probes from the 5′ UTR (4, 25), and the presence of only three capsid proteins (21). These findings led some authors to propose a new classification of these two serotypes outside the *Enterovirus* genus (7, 21). However, the group-specific epitope located on VP1 and recognized by the 5-D8/1 antibody is common to all of the serotypes of enteroviruses, including EV22 and EV23, as shown by Yousef et al. (23) and ourselves (this study). Similar results were obtained by Coller et al. (4), who studied the proliferative cellular response to a group-specific antigen on VP1: spleen lymphocytes from mice primed with CB3 were shown to exhibit an anamnestic response after challenge with other serotypes of enteroviruses, including EV22, but not after challenge with other members of the *Picornaviridae*. These findings suggest that the VP1 group-specific antigen is a more conserved region than the 5′ UTR in the phylogenetic evolution of the family *Picornaviridae*.

Total agreement was found between the results obtained with the EGS MAb and those obtained by PCR for the identification of enteroviruses, especially for those strains that could not be typed by seroneutralization. For identification of the latter strains at the serotype level, a restriction enzyme digest of PCR products from the 5′ UTR may be assayed, as recently described (9).

Overall, our results indicate that use of the EGS MAb permits rapid confirmation of the presence of an enterovirus in cell culture. The test is inexpensive, specific, and easy to perform, even in laboratories not specialized in enteroviral diagnosis. Further studies would be needed to delineate whether the MAb could detect enteroviruses directly in clinical specimens exhibiting high viral loads (nasopharyngeal aspirates, for instance) or after short cultures, as recently reported for rhinoviruses (1).

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