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Peptide Antisera Targeted to a Conserved Sequence in Poliovirus Capsid Protein VP1 Cross-React Widely with Members of the Genus *Enterovirus*

TAPANI HOVI* AND MERJA ROIVAINEN

Enterovirus Laboratory, Department of Viral Diseases, National Public Health Institute (KTL), Helsinki, Finland

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Rabbits were immunized with synthetic peptides derived from an immunodominant region of the VP1 capsid protein of enteroviruses. This region shows a high degree of homology among all sequenced members of the genus. Peptide-induced antisera were used for immunoperoxidase staining of cell cultures infected with 41 different serotypes of enterovirus. Specific cytoplasmic staining was readily seen in all but two cases. Echovirus type 22 was previously known to differ genetically from the rest of the enteroviruses, and hence, a negative result was expected. Surprisingly, one of the tested serum samples reacted with echovirus 22-infected cells. Coxsackievirus A7-infected cells could be reliably stained with only one of the tested serum samples. For the remaining 39 serotypes, scattered infected cells resulting from 1 to 2 days of incubation with diluted inocula were easily scored as positive before the cytopathic effect became visible. The same antibodies were also used in a sandwich-type enzyme immunoassay to demonstrate poliovirus antigens in cell extracts as early as 3 h after a high-multiplicity infection. These antibodies are candidates for enterovirus group reagents, being potentially useful in both the laboratory diagnosis of enterovirus infections and research on enterovirus-host interactions.

The genus *Enterovirus* comprises about 70 human pathogenic serotypes of small RNA viruses in the family of *Picornaviridae* that are responsible for a variety of clinical symptoms (11). The best-known members of the genus, the three serotypes of poliovirus, are condemned to global eradication within the next decade as causative agents of paralytic poliomyelitis (12, 20). The other human enteroviruses, coxsackieviruses of groups A and B, echoviruses, and the four newer enteroviruses, 68 through 71, are known to cause both outbreaks and isolated cases of serious disease (3). However, the overall public health impact of the nonpoliovirus enteroviruses is poorly known because of the multitude of serotypes, the various symptoms that they cause, and cumbersome virological diagnosis.

Although identification of the serotype of an isolated virus is important for epidemiological studies, confirmation of the causative agent of a disease as an enterovirus would be the most useful part of the laboratory diagnosis in most clinical situations. Heated coxsackievirus B preparations have traditionally been used as antigen in serological group diagnosis on paired serum samples (3), but like virus isolation, this approach is too slow in providing results. The sensitivity of conventional serology in the detection of heterologous enterovirus infections is also variable and questionable, especially in children.

We recently used the peptide scanning technique (5) to study the regions of type 3 poliovirus capsid proteins that are antigenic in humans (16). One of the immunodominant regions was localized around amino acids 37 to 53 of the capsid protein VP1. This region also shows a high degree of sequence homology among all analyzed enteroviruses. Here we report the results of studies indicating that peptide antibodies targeted to this part of VP1 can be used to detect most enteroviruses replicating in cell cultures.

* Corresponding author.

MATERIALS AND METHODS

Virus strains. The virus strains used in the present study comprised reference strains of enteroviruses that were originally obtained from the Enterovirus Reference Laboratory, World Health Organization, Copenhagen, Denmark, or from the American Type Culture Collection, and made available to us through the courtesy of Mirja Stenvik of the National Public Health Institute, Helsinki, and Timo Hyypiä, University of Turku, Turku, Finland. In addition, two local isolates of type 3 poliovirus and selected unrelated virus strains were used.

Cell cultures and virus infections. The human tumor cell lines used in the present study included HeLa (Ohio), RD, and A549 cells, while Vero-KTL (fibroblastoid), A-Vero (epithelial), and GMK represented cell lines of African green monkey kidney cell origin. In addition, fibroblastoid human embryonic cells of local origin (HES cells) were used. Before inoculation, culture medium was removed and the cell monoloayers were rinsed with Hanks' balanced buffer solution supplemented with 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.4). Virus stocks were diluted in virus propagation medium (Eagle's minimal essential medium supplemented with 20 mM $MgCl_2$–20 mM HEPES [pH 7.4]–1% fetal bovine serum), and usually, a set of several 10-fold dilutions of the stock was used to infect parallel cell cultures. After 15 min at room temperature, a standard volume of virus propagation medium, according to the vial used, was added and the cultures were incubated at 36°C overnight or as indicated in the Results.

Peptide antibodies. Peptides were synthesized according to the VP1 sequence of type 3 poliovirus strain Sabin (19) with an extra C-terminal cysteine. Peptide KTL-501 (from Labsystems Research Laboratories, Helsinki, Finland) had the sequence, with single-letter codes, PALTAVETGATNPL (C), and peptide KTL-510 (from Multiple Peptide Systems Ltd., San Diego, Calif.) had the sequence KEVPALTAVET

GAT(C), and they were chemically coupled (13) to ovalbumin before immunization. Peptide KTL-511 [KEVPALT AVETGATNPLV(C); Department of Biochemistry, University of Turku] was used as the immunogen without a carrier protein. Peptide KTL-5CV, with the sequence EAIPAL TAVETGHTSQV(C), was synthesized at the Department of Biochemistry, University of Turku, according to a consensus sequence of coxsackieviruses in the same region of VP1 and was used without coupling. Rabbits were immunized with three sequential 200-μg doses of peptide by the popliteal lymph node method, including the use of Freund's adjuvant, as described previously (9). Sera collected 1 week after administration of the third dose were stored frozen as aliquots. Western blot (immunoblot) analyses carried out in parallel for peptide antisera and for antisera against purified virions showed that peptide antisera reacted specifically with VP1 in enterovirus particles.

Immunoperoxidase test. The immunoperoxidase test described by Ziegler et al. (22) was slightly modified. Medium was removed from infected cell cultures, and the cultures were rinsed once with phosphate-buffered saline (PBS) before fixing for 15 min with methanol at 4°C. In the pilot phase we also tested, as alternative fixing methods, methanol at −20°C, 80% acetone in water at 4°C, and 3.3% paraformaldehyde in PBS at room temperature and then a permeabilization step with 0.5% Triton X-100 at 4°C. In our hands, none of these fixing methods showed any advantages over the method with methanol at 4°C. After removing the methanol, the cultures were washed three times with cold PBS and were stored at 4°C if they were not immediately processed for staining. Antisera were diluted in PBS supplemented with 0.5% Tween 20 and 1% bovine serum albumin (PBS-TB) and were incubated with the fixed cultures for 1 h at 36°C in a humid atmosphere. Care was taken to use large enough volumes during all incubations to cover the entire monolayer and to prevent drying of the solutes onto the cells. Next, the cultures were washed three times with PBS containing 0.1% Tween 20 (PBS-Tw) and were supplemented with a pretested dilution of horseradish peroxidase-conjugated anti-rabbit immunoglobulin (Bio-Rad, Richmond, Calif.). After 1 h at 36°C, the cultures were again washed three times with PBS-Tw and once with 20 mM Tris-HCl (pH 7.4). The substrate for the peroxidase was prepared immediately before use by adding 30 μl of 30% hydrogen peroxide to 50 ml of 20 mM Tris-HCl (pH 7.4) and mixing it with 10 ml of 0.3% 4-chloro-1-naphthol in methanol (stored at −20°C). After adding the substrate, the cultures were inspected with a microscope after 30 to 60 min in the dark at room temperature, and when necessary, the cultures were transferred to 4°C for later, more careful evaluation.

EIA for soluble antigen with peptide antibodies. A standard sandwich schedule of enzyme immunoassay (EIA) for antigen determination was used. Microwell plates were coated (overnight at room temperature) with bovine immunoglobulin G against type 1 poliovirus (a gift from A. van Loon) and were blocked for 1 h at 36°C with 5% fetal calf serum in PBS supplemented with 0.5% Tween 20. Clarified cell extracts diluted in PBS-TB were added and incubated overnight at 4°C, and then a 1:300 dilution of antiserum to peptide KTL-510 in PBS-TB was added for 1 h at 36°C. Finally, horseradish peroxidase-conjugated rabbit immunoglobulin was added for an additional 1 h at 36°C, and its binding was quantified with the *ortho*-phenylenediamine substrate. Between each incubation, the plates were washed four times with PBS-Tw. The reaction was stopped after 30 min by adding sulfuric acid, and the A_{492}s were read. The cutoff level of absorbance was determined by subtracting the raw test values from the values obtained with a similar dilution of the preimmunization serum from the same rabbit.

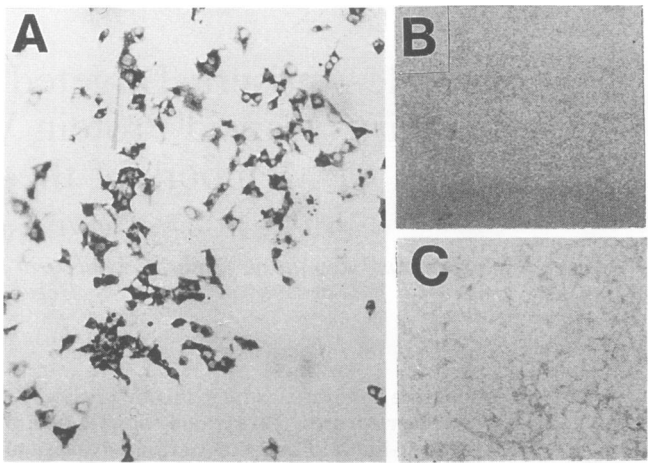

FIG. 1. Immunoperoxidase staining of poliovirus-infected cells with antipeptide antibodies. Monolayers of Vero-KTL cells were infected with poliovirus type 1 Mahoney (A and C) or were left uninfected (B). After overnight incubation, cells were fixed with cold methanol and were stained for reactivity with antibodies to peptide KTL-510 (A and B) or with a similar dilution (1:400) with the preimmune serum from the same rabbit (C). Approximate magnification, ×100.

RESULTS

Sensitive detection of poliovirus antigens with peptide antibodies. Vero-KTL cell monolayers were inoculated with different dilutions of poliovirus type 1 Mahoney or were left uninfected. After overnight incubation, the cultures were inspected with a microscope for a potential cytopathic effect and were prepared for immunoperoxidase staining. Clear-cut cytoplasmic staining was seen in virus-infected cultures, while uninfected wells showed only weak diffuse background coloring, if any reaction, with the stronger serum dilutions. With the working dilutions of 1:200 to 1:800, the uninfected cultures regularly remained completely negative (Fig. 1). Likewise, poliovirus-infected cultures incubated with similar dilutions of the preimmune sera from the rabbits showed no staining. Results comparable to those seen above for Vero cells were also obtained with GMK, HeLa, RD, HES, and A549 cells.

Positive staining was usually seen with virus inocula 1 to 2 log units smaller than the lowest CPE-positive inoculum. The intensity of staining was regularly stronger in solitary but still morphologically unaffected cells than in clusters of rounded cells representing cytopathic effect. To study the kinetics of antigen production, GMK cells were infected with a high-inoculum virus preparation and the appearance of antigen positivity was followed both by staining parallel cultures at intervals and by analyzing cell extracts by a peptide EIA. Viral antigen in cell extracts was detected as early as after 3 h of infection, and definite positivity in the immunoperoxidase test could be scored by microscopy between 6 and 8 h of infection (Fig. 2).

In other experiments, we stained poliovirus-infected cells detached from monolayers by the immunoperoxidase method by mechanically scraping them and readhering them to the microscopic slides. Recording of the results turned out

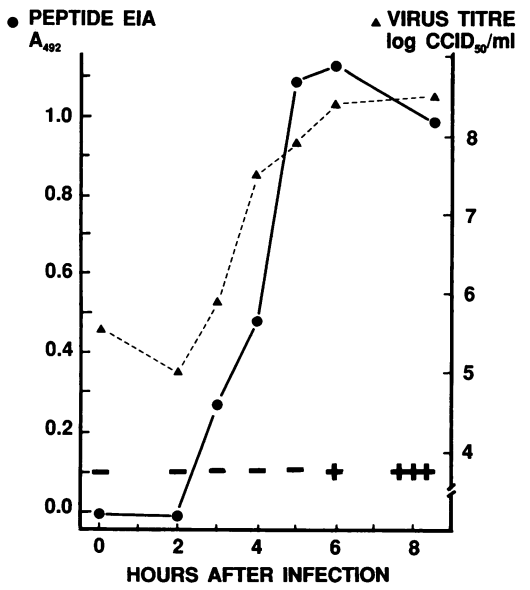

FIG. 2. Time course of appearance of reactivity with antipeptide antibodies in poliovirus-infected cells. Parallel cultures of GMK cells were infected with a high multiplicity (>50 50% cell culture infective doses per cell) of poliovirus type 1 Mahoney and were incubated at 36°C. At intervals, one of two parallel wells was fixed with methanol and stained with immunoperoxidase. Alternatively, cells were detached mechanically, frozen, and thawed, and after clarification of the extract by centrifugation, the cells were assayed for infectivity (▲) or for reactivity with peptide antiserum in a sandwich-type EIA (●). Positive staining observed by microscopy is shown by the positive or negative grading. Antiserum to peptide KTL-510 was used in both tests. $CCID_{50}$, 50% cell culture infective dose.

TABLE 1. Reactions of peptide-induced rabbit antibodies with members of the genus *Enterovirus* in immunoperoxidase-in-situ test on fixed monolayer cells

Serotype (strain)[a]	Result[b]
PV-1 (Brunhilde; Mahoney; Sabin)	+
PV-2 (Lansing; Sabin)	+
PV-3 (Fin84; Pak90; Leon; Sabin; Saukett)	+
CAV type 7	(+)[c]
CAV types 9, 13, 16, 18, 21	+
CBV types 1, 2, 3, 4, 5, 6	+
ECV types 1, 2, 3, 4, 5, 6, 7, 8, 9, 11	+
ECV types 13, 14, 15, 17, 18, 19, 21	+
ECV type 22	(+)[c]
ECV types 24, 25, 29, 30, 32	+
ENV types 69, 70, 71	+
Adenovirus types 1, 2, 7	−
HSV types 1 and 2	−
Reovirus types 1, 2, 3	−

[a] PV, poliovirus; CAV, coxsackievirus A; CBV, coxsackievirus B; ECV, echovirus; ENV, enterovirus; HSV, herpes simplex virus.
[b] Staining with antiserum to peptide KTL-510. +, positive cytoplasmic staining; −, no specific staining.
[c] Negative result with antiserum to peptide KTL-510 but positive result with antiserum to a related peptide (KTL-511).

to be much more difficult than with the monolayers stained in situ, mainly because of the variable background staining of detached uninfected cells. Medium from poliovirus-infected cultures showing an advanced CPE was regularly positive in the sandwich EIA with the peptide antisera as the source of detecting antibodies.

Wide cross-reactions within the *Enterovirus* genus. Cell cultures were inoculated with different dilutions of various enterovirus stocks and incubated at 36°C. When a CPE from the strongest inoculum was visible, cell cultures were fixed with methanol and stained with the peptide antisera and immunoperoxidase as described above. Cultures infected with different enteroviruses were regularly positively stained with the antisera (Table 1), with some interesting exceptions. Four independent strains of coxsackievirus A7 could not be stained with the otherwise most potent antiserum, derived from immunization with a poliovirus-derived peptide (KTL-510). However, brief heating of the methanol-fixed cells (15 min at 60°C in a water bath) or use of another serum sample revealed the cytoplasmic staining in coxsackievirus A7-infected cells. The latter antiserum sample was from a rabbit immunized with a slightly different but, again, poliovirus-derived peptide (KTL-511). This antiserum also detected, although only with a moderate intensity, echovirus 22-infected cells (data not shown). The best results for enterovirus 71-infected cells were obtained with yet another serum sample, which was derived from a rabbit immunized with a coxsackievirus-derived peptide (KTL-5CV). The latter serum sample was not better than those induced with the poliovirus-derived peptides in the detection of coxsackievirus-infected cells. The intensity of staining obtained with the most potent sera also varied between different, unequivocally positively reacting serotypes in a given cell line and, to some extent, between different cell types infected by a given virus, e.g., poliovirus type 1 Mahoney. In all cases, however, definite cytoplasmic staining was easily discernible in cells that were otherwise morphologically unaffected by the infection. An advanced CPE appeared to inhibit the generation of a strong staining contrast, especially in the fibroblastic HES cells.

The sensitivity of the procedure was assessed by infecting cell monolayers with different, known amounts of virus and staining the cultures after overnight incubation. About 1 PFU of all tested viruses (poliovirus type 1 Mahoney, coxsackievirus A9, coxsackievirus B3, and echovirus 9) were readily detected. If the virus was mixed with 200 μl of freshly trypsinized cells rather than inoculated onto monolayers, the detection sensitivity was between 1 and 10 PFU.

A small number of enterovirus serotypes was tested by using cells grown on microscopic slides and fluorescein isothiocyanate-conjugated antirabbit immunoglobulin rather than the immunoperoxidase conjugate for antigen detection. As expected, the dilutions of antisera used in the immunoperoxidase test described above gave strong fluorescence signals as well (data not shown).

Cultures infected with herpes simplex virus types 1 and 2, reovirus types 1, 2, and 3, and adenovirus types 1, 2, and 7 were also incubated with the peptide antisera; this was followed by incubation with the immunoperoxidase conjugate. No staining comparable to that of the enterovirus-specific cytoplasmic reaction could be seen. Clusters of adenovirus-infected cells occasionally showed some irregu-

TABLE 2. Conservation of amino acid sequence in the target region of enterovirus capsid protein VP1[a]

Virus strain	Sequence (single-letter codes)	Reference
Poliovirus		
Type 3 Sabin	KEVPALTAVETGATNPL	19
Type 1 Mahoney	—I—————	15
Type 1 Sabin	—I—————	19
Type 2 Lansing	—T—————	15
Type 2 Sabin	—I—————	19
Type 3 Finland/84	——T—————	15
Type 3 Leon	—————————	15
Coxsackievirus		
A9	AS————H–SQV	1
A21	Q————SGQA	15
B1	ESI———A——H–SQV	15
B3	EAI———A——H–SQV	15
B4	EQI———A——H–SQV	15
B5	EAI———A——H–SQV	10
Enterovirus type 70	GVI–S–N————SNT	18
Consensus	——P–L–A–ETG———	

[a] The region shown corresponds to amino acids 37 to 53 in VP1 of poliovirus type 3 Sabin (19).

lar background staining that was obtained with the preimmune sera as well. Herpesvirus-infected cells could be stained with low dilutions of both peptide antisera and preimmune sera, most likely because of the virus-induced Fc receptor, but with the working dilutions used in the present study, this was not a problem and no blocking test to avoid this nonspecific reaction was necessary.

DISCUSSION

We showed here that antibodies induced in rabbits with synthetic peptides imitating conserved sequences in the capsid protein VP1 of enteroviruses react specifically with a large number of enterovirus serotypes.

Caution must be applied when using synthetic peptide-induced antibodies in virus identification, because unexpected cross-reactions have been described with some of these types of reagents. In our case, the specificity of the reaction for enterovirus components was confirmed by showing that uninfected cells did not show the typical staining and that cultures infected with unrelated viruses were, likewise, negative in the test.

The target region of our peptide antibodies in the capsid protein VP1 was conserved among all sequenced enteroviruses (Table 2), with the exception of echovirus 22, which differed from the main enterovirus group in several other molecular features as well (7). With this in mind, our negative results for this virus with most of the sera were not unexpected. In contrast, the definite reaction obtained with some of the sera in both the immunoperoxidase test described here and Western blots (15a) is surprising and remains to be analyzed in detail by further studies. It would also be interesting to know whether the target site of our peptide antibodies coincides with that of the designated VP1 monoclonal antibody, which is reported to cross-react with a number of enterovirus serotypes (21). A further remarkable point is that the target region of our peptide antibodies in the VP1 protein of enteroviruses is also highly homologous with the corresponding region in sequenced rhinoviruses (15). Accordingly, HeLa-Ohio cells infected with several different serotypes of rhinovirus can be stained with some of the peptide antisera described here (15a).

The actual usefulness of the peptide antibodies described here in the everyday work of clinical virological laboratories must be tested in practice. Pools of individual sera may be needed to extend the detection coverage to the problematic serotypes mentioned above. Evaluation of this approach is in progress. Being technically very simple, relatively sensitive, and inexpensive, this immunoperoxidase-identification-in-situ method is likely to be superior to nucleic acid hybridization applied to virus isolates (4, 8, 17). Of course, polymerase chain reaction may detect enterovirus genomes directly in clinical specimens, even in cases in which no virus can be isolated for identification (2, 6, 14). According to our results for a few laboratory strains, the immunoperoxidase-identification-in-situ test can yield positive results in cultures inoculated with 1 PFU, resulting in a single cluster of positively staining cells. This sensitivity is likely to enable the use of this test as a rapid screening method for inoculated cultures without waiting for the appearance of a CPE. Our preliminary data support this suggestion. While immunofluorescence could be preferred to immunoperoxidase staining because of the better contrast that is usually obtained, the latter method has the advantage that it can be carried out on plastic cell culture vials and the results can be read with regular laboratory microscopes. A straightforward sandwich EIA with peptide antibodies detected poliovirus antigens in high-multiplicity-infected cell cultures a few hours earlier than the immunoperoxidase-identification-in-situ test, because the local concentration of antigen must be relatively high for a positive result in the latter test. Application of the EIA for culture confirmation might be useful in situations in which an inoculated culture already shows an advanced CPE at the first inspection.

In conclusion, peptide-induced antibodies targeted to a conserved amino acid sequence in enterovirus capsid protein VP1 appear to be promising candidates for enterovirus group reagents. Use of these antibodies in simple immunoperoxidase-in-situ or immunofluorescence tests may turn out to be a practical possibility for the rapid diagnosis that is needed for enterovirus infections, but it still requires formal documentation with clinical specimens. Our preliminary data on cross-reactions of the antibodies with a number of rhinovirus serotypes suggest that this principle may later be extended to the diagnosis of rhinovirus infections as well.

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