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Molecular Basis for Serological Cross-Reactivity between Enteroviruses

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The recognition sites for human antibodies which are cross-reactive between different types of enteroviruses were determined and characterized. Serum samples obtained from 58 patients with culture-confirmed enteroviral infections were analyzed in enzyme immunoassays against two sets of overlapping synthetic peptides covering residues 31 to 96 of poliovirus 1 VP1 (Mahoney strain) and residues 31 to 148 of coxsackievirus B1 VP1 (positions based on alignment with poliovirus 1 VP1, Mahoney strain). A major antigenic region eliciting cross-reactive antibodies could be located to residues 37 to 51 of VP1. Furthermore, a single peptide covering residues 42 to 55 almost completely inhibited the binding of human antibodies to heat-inactivated enteroviruses, indicating that residues 42 to 55 of VP1 contain a major region eliciting cross-reactive antibodies. By using peptide analogs in which each residue within positions 42 to 55 of VP1 was sequentially substituted by Ala or Gly, we were able to determine the most essential residues for human antibody binding in 38 of the convalescent-phase patient serum samples. In a majority of the serum samples, the most essential residues for antibody binding were found to be Pro-42, Ala-43, Leu-44, Thr-45, Ala-46, Glu-48, Thr-49, and Gly-50. All of these residues are conserved, according to known enterovirus sequences, with the divergent echovirus 22 excepted. In conclusion, we could demonstrate that the essential residues for binding of cross-reactive antibodies are well conserved within the enterovirus family. These findings provide a molecular basis for the observed antibody cross-reactivity within the enterovirus group.

Enteroviruses are small, nonenveloped, icosahedral viruses containing a positive-sense, single-stranded RNA genome of about 7,400 nucleotides. Sixty copies of each structural protein, namely, VP1, VP2, VP3, and VP4, constitute the viral capsid. The enterovirus family includes poliovirus (3 serotypes), coxsackievirus A (23 serotypes), coxsackievirus B (6 serotypes), echovirus (31 serotypes), and the human enteroviruses 68 to 71 (4).

Four antigenic sites of polioviral proteins eliciting neutralizing antibodies, which have been referred to as 1, 2, 3A, and 3B, were identified by using neutralization escape mutants (14, 16, 26). The locations of the mutated amino acid residues indicate that the majority of these are located within prominent structural features of the virus surface (5, 16). However, several additional antigenic regions were identified in VP1, VP2, and VP3 of poliovirus type 3 by using peptides linked to polyethylene rods and five human serum samples (18).

We have recently described synthetic peptides from the amino-terminal part of VP1, selected by various prediction algorithms, that bind group-common immunoglobulin G (IgG) antibodies to enterovirus (1, 22). The major cross-reactive peptide covered residues 42 to 55 of VP1. In a recent work, similar peptides have been utilized for the production of antisera which are cross-reactive in the enterovirus group (7).

In the present study, we further map the amino-terminal part of VP1 and characterize the human immune recognition and cross-reactivity of it by using overlapping synthetic peptides and a large panel of human serum samples.

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MATERIALS AND METHODS

Human sera. A serum panel consisting of acute- and convalescent-phase serum samples from 58 patients with culture-confirmed enterovirus infections was used. The enteroviral strains were typed by monospecific antisera in a complement fixation assay (4). The patients were infected with any 1 of the 10 following enteroviral serotypes: coxsackievirus A9 (seven patients), coxsackievirus B3 (three patients), coxsackievirus B4 (five patients), coxsackievirus B5 (six patients), echovirus 4 (four patients), echovirus 6 (six patients), echovirus 9 (six patients), echovirus 11 (seven patients), echovirus 18 (six patients), and echovirus 30 (eight patients). As negative controls, a pool of sera from children aged 1 to 2 years was used. The pool was nonreactive in two different enterovirus IgG enzyme-linked immunosorbent assays (ELISAs), one using heat-treated enterovirus antigens (23) and the other using synthetic peptides as antigens (22).

Protein sequences and alignment. All sequences were obtained from GenBank (release 74) by using the program DNAsis (Hitachi Incorporated), and alignment was performed by using the program MultAlin 4.0 (Cherwell Scientific Publishing Ltd). Sequences from the following enteroviruses were used: poliovirus 1 strain Mahoney (12), poliovirus 1 strain Sabin (15), poliovirus 2 (17), poliovirus 3 strain Leon (24), coxsackievirus B1 (10), coxsackievirus B3 (13), coxsackievirus B4 (11), coxsackievirus A9 (2), coxsackievirus A21 (8), coxsackievirus A24 (25), echovirus 22 (9), and enterovirus 70 (19). Throughout this work, all numbering of residues refers to alignment with poliovirus 1 strain Mahoney (12).

The one-letter amino acid code is used in sequences, and the three-letter code is used otherwise.

Peptide synthesis. The peptides were synthesized, cleaved, and deprotected by a "tea-bag" method using 9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids as described pre-

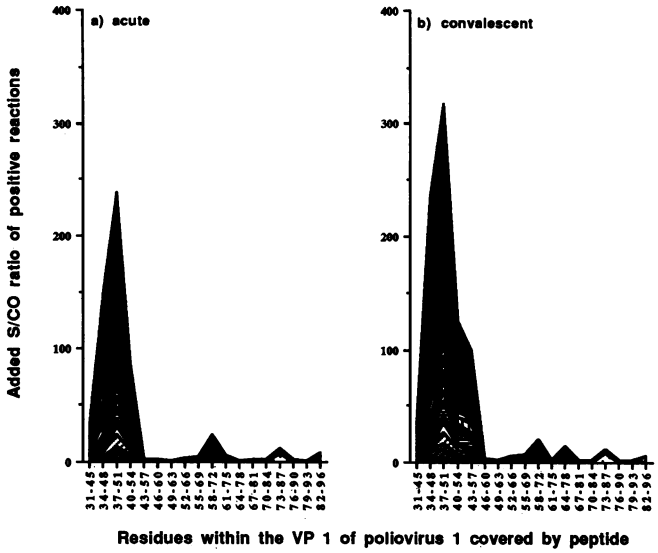

FIG. 1. Reactivity to peptides covering residues 31 to 96 of poliovirus 1 VP1 in acute (a)- and convalescent (b)- phase sera. Reactivity is given as the added-signal-to-cutoff (S/CO) ratio of serum samples giving positive reactions.

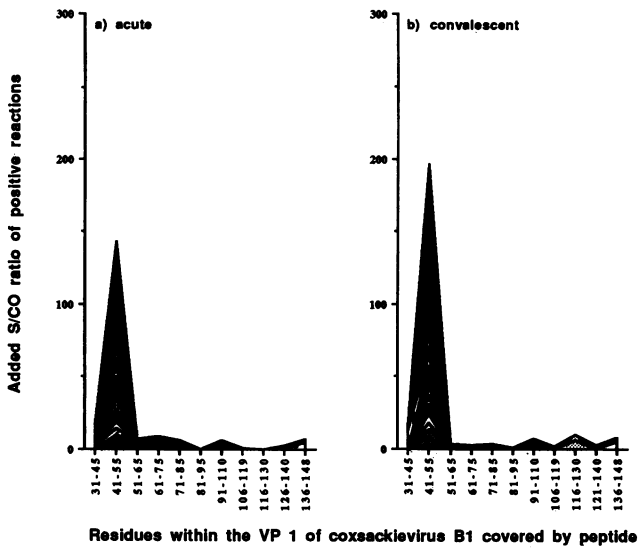

FIG. 2. Reactivity to peptides covering residues 31 to 148 of coxsackievirus B1 in acute (a)- and convalescent (b)- phase sera. All peptides are 15 amino acids long, but the numbering of the residues is based on alignment with poliovirus 1 strain Mahoney. Reactivity is given as the added-signal-to-cutoff (S/CO) ratio of serum samples giving positive reactions.

viously (6, 21). Peptides containing 15 residues, with a 12-amino-acid overlap, were synthesized to cover residues 31 to 96 of poliovirus type 1 Mahoney strain. An additional set of peptides, with a five-amino acid overlap, was synthesized to cover residues 31 to 148 of coxsackievirus type B1. Also, one peptide covering residues 39 to 56 of echovirus 22 was synthesized.

A set of 14 substitution peptide analogs covering residues 42 to 55 of poliovirus type 1 was produced to characterize the human antibody binding. Each residue was sequentially substituted by alanine or glycine as described in a previously published protocol (20).

All peptides were analyzed by high-performance liquid chromatography using a Pep-S 5-μm reverse-phase column (Pharmacia, Uppsala, Sweden). All substitution peptide analogs were found to have the same overall characteristics (mean retention time, 15.3 ± 0.83 min) and purity (50 ± 17%). Also, the shift in retention time of the major peak always correlated to the theoretical change in overall polarity of a substitution peptide, which further implies that the main peaks corresponded to the correct sequence. Since all peptides had similar purities and the same overall characteristics, the peptides were used in ELISAs without prior purification.

Indirect IgG ELISA using peptide antigens. The peptides were coated onto polystyrene microtiter plates (Maxisorp; Nunc AS, Roskilde, Denmark) at a concentration of 1 μg of peptide material in 0.05 M sodium carbonate buffer (pH 9.6) per well. The dilutions were based upon the purities of the substitution peptide analogs and were corrected to correspond to the amount of the original peptide (a purity of 64% for the peptide without substitution corresponds to 0.64 μg per well). Plates were sealed and stored at room temperature for one night and then at +4°C until use (minimum, 2 days).

Phosphate-buffered saline (PBS; pH 7.4) with 0.05% Tween 20 was the wash buffer used. To prevent nonspecific binding, diluent buffer for serum and conjugate consisting of PBS (pH 7.4) with 1% bovine serum albumin (fraction V; Boehringer GmbH, Mannheim, Germany), 2% milk powder (Semper AB, Stockholm, Sweden), 0.05% Tween 20, and 0.02% NaN_3 was used.

After the plates were washed four times, 100 μl of serum diluted 1/100 was added. When testing the overlapping peptides covering VP1 and the echovirus 22-derived peptide, single determinations were made. When characterizing the antibody binding of the substitution peptide analogs, duplicate determinations were made. The plates were then incubated overnight at ambient temperature.

When testing the overlapping peptides covering VP1, the negative control pool was added to seven wells on each plate.

After the plates were washed, 100 μl of alkaline phosphatase-conjugated goat anti-human IgG (A-3150; Sigma Chemical Co., St. Louis, Mo.) diluted 1/1,500 was added. After the plates were incubated for 90 min at 37°C and washed; the substrate *p*-nitrophenyl-phosphate (5 mg; Sigma 104 phosphatase substrate tablets) diluted in 5 ml of 1 M diethanolamine buffer (pH 9.8) was added, and the plates were incubated for 30 min at room temperature. The reaction was stopped by the addition of 50 μl of 3 M NaOH, and the A_{405}s were read.

To give the assays a high specificity in the mapping test, the cutoff was defined as the mean absorbance value for the negative control plus seven times the standard deviation. A signal-to-cutoff ratio was calculated for each serum sample.

For characterization of the antibody binding, the cutoff was defined as 50% of the absorbance value obtained with the peptide with the original sequence. Thus, a substitution peptide analog which yielded an absorbance of less than 50% of that of the original peptide was considered to have lost a residue essential for antibody binding.

Indirect IgG ELISA with heated antigens. The use of echovirus 9, echovirus 30, and coxsackievirus B5 antigens in a cross-reactive IgG ELISA has been described previously (23). In brief, the antigens were prepared by differential centrifugation, heated at 56°C, and applied to plates as described above.

The incubation times, buffers, conjugate, and substrate used were those described above. For each serum sample, a dilution that gave an A_{405} value of around 1.0 was chosen to ensure that the tested serum samples all gave values within the linear phase of the absorbance curves. In inhibition assays, 50 μl of

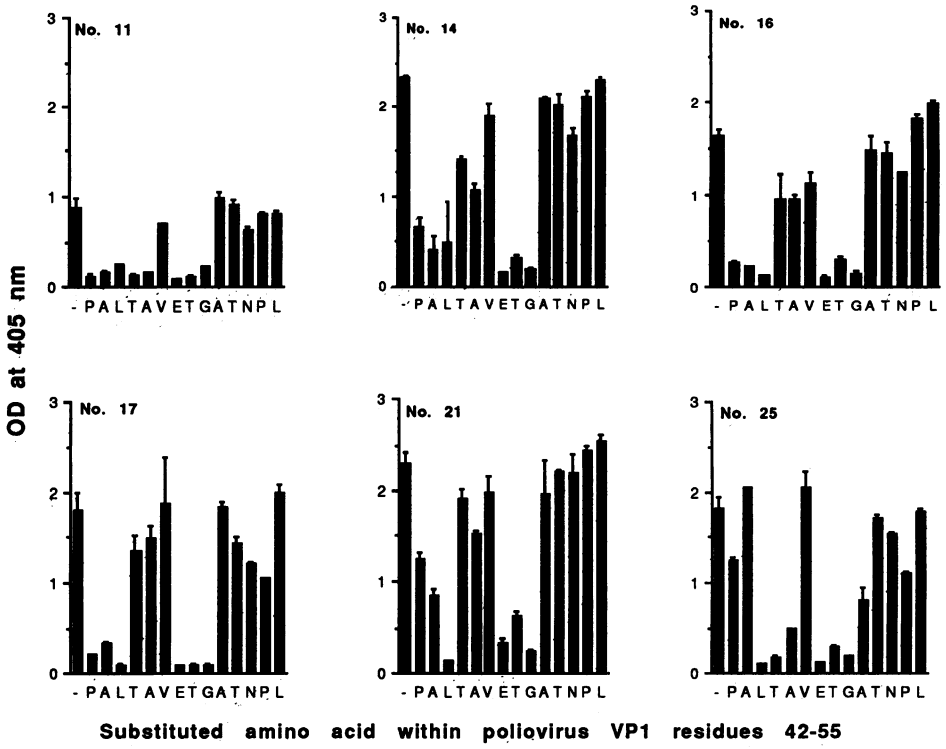

FIG. 3. Reactivity pattern for 6 (of 54) serum samples when tested against substitution peptide analogs. Black bars indicate the mean A_{405}s. The difference in absorbances between the duplicates is indicated by the thin bars on top of the thick bars. Numbering refers to the serum identification shown in Fig. 4. OD, optical density.

the peptide PALTAVETGATNPL (residues 42 to 55) serially diluted 10-fold from 200 to 0.02 μg/ml was added to 50 μl of serum diluted 1/50, and the mixture was incubated.

Specific inhibition was determined to take place when the absorbance of the reaction mixture with the heat-treated virions was reduced by more than 50% by the inhibiting peptide.

RESULTS

Mapping of antigenic regions within poliovirus 1 and coxsackievirus B1 VP1. Figure 1 shows the results from testing 44 paired acute- and convalescent-phase serum samples with the 18 overlapping peptides covering residues 31 to 96 of poliovirus 1. Figure 2 illustrates the results from testing the same serum samples with the 11 overlapping peptides covering residues 31 to 148 of coxsackievirus B1. With both sets of peptides, a major antigenic region was found to reside within residues 37 to 51. The cutoff values for all of the overlapping peptides ranged within an optical density at 405 nm of 0.108 to 0.504. The optical density at 405 nm of the acute-phase serum samples giving positive reactions with the peptides covering residues 37 to 51 of poliovirus VP1 and residues 41 to 55 of coxsackievirus B1 VP1 ranged from 0.289 to 2.540 and from 0.305 to 2.045, respectively. The optical density at 405 nm of the convalescent-phase serum samples giving positive reactions with the peptides covering residues 37 to 51 of poliovirus VP1 and residues 41 to 55 of coxsackievirus B1 VP1 ranged from 0.252 to 2.719 and from 0.297 to 2.428, respectively.

Antibody binding to the echovirus 22 peptide covering residues 39 to 56. The reactivity of forty-four paired acute- and convalescent-phase serum samples to a peptide covering the

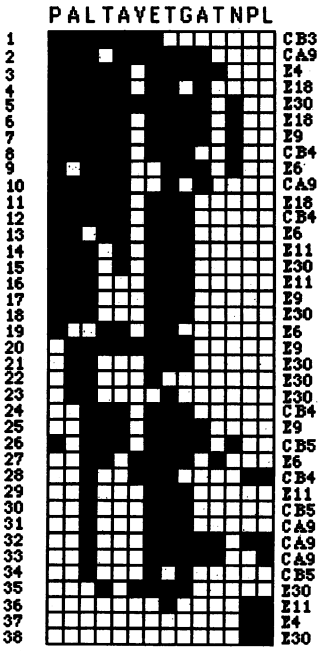

FIG. 4. Determination of essential residues for antibody binding to residues 42 to 55 of poliovirus VP1 by substitution peptide analogs. The black boxes indicate residues that cannot be substituted without loss of reactivity to the peptide for each of the 38 serum samples. Open boxes indicate residues which can be substituted without loss of reactivity. Serum identification numbers are given to the left, and the corresponding isolated virus types are given to the right.

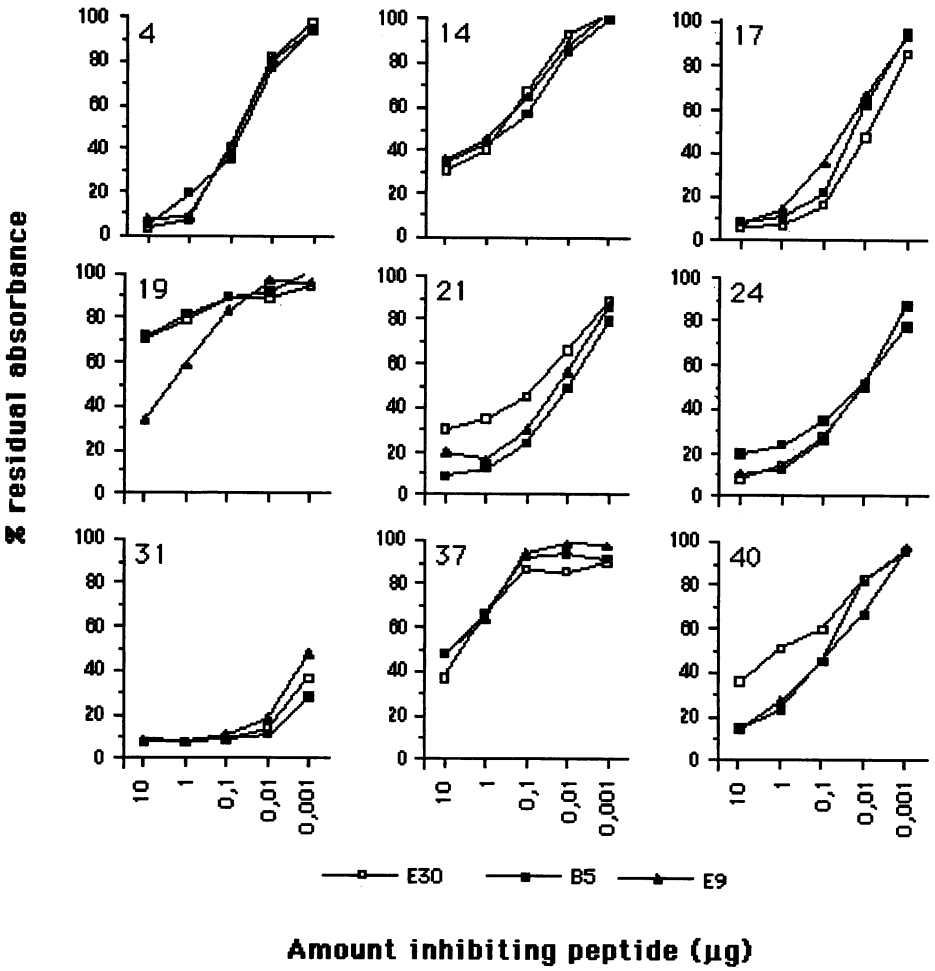

FIG. 5. Inhibition of IgG antibody binding to heated enterovirus antigens by peptides covering residues 42 to 55 of poliovirus 1 VP1. The percentages of residual absorbance obtained with three different antigens in relation to the amounts of inhibiting peptide are given for nine different serum samples. Serum identification numbers 1 to 38 are identical in Fig. 4 and Fig. 5. In this figure, the serum identification numbers are shown in the upper left corners of the panels.

same region on echovirus 22 as the indicated antigenic region on poliovirus 1 and coxsackievirus B1 was tested (data not shown). For 43 paired serum samples, no reactivity was seen. Only two serum samples from one patient, who was infected by echovirus 30, were reactive.

Characterization of antibody binding to poliovirus 1 VP1 residues 42 to 55. To determine the essential residues present for human antibody recognition of the antigenic region at residues 42 to 55 of VP1, 54 convalescent-phase serum samples were assayed with the set of substitution peptide analogs. Each residue within positions 42 to 55 was sequentially substituted by Ala or Gly. In Fig. 3, the results from testing 6 of the 54 serum samples are shown to illustrate the effect of substitution of each residue.

The recognition pattern for 38 of the 54 analyzed serum samples could be clearly defined. The results are given in Fig. 4. In more than 50% of the 38 serum samples with a defined reactivity pattern, the residues Pro-42 (20 of 38), Ala-43 (21 of 38), Leu-44 (32 of 38), Thr-45 (19 of 38), Ala-46 (20 of 38), Glu-48 (32 of 38), Thr-49 (33 of 38), and Gly-50 (28 of 38) were found to be essential for antibody binding. No differences were seen in the recognition patterns irrespective of the enterovirus serotype that had infected the patient.

Inhibition of antibody binding to heat-treated enterovirus antigens by peptide. In 9 of 11 tested convalescent-phase serum samples, the presence of the peptide PALTAVETGAT NPL (residues 42 to 55) reduced the absorbance obtained with heat-treated enterovirus antigens by more than 50% (Fig. 5). With the addition of 10 µg of inhibiting peptide, less than 40% of residual binding was seen in all nine serum samples, and for seven serum samples, the residual binding was less than 20% binding to one or more antigen (Fig. 5).

Variability of VP1 residues 42 to 55 within the enterovirus family. The sequence PALTAVETG is a highly conserved sequence within the enterovirus family, except for echovirus 22 (Table 1). Residues Pro-42, Leu-44, Ala-46, Glu-48, Thr-49, and Gly-50 are completely conserved among enteroviruses.

DISCUSSION

By using a large number of human serum samples from patients with a defined enteroviral serology and overlapping synthetic peptides, we have identified and characterized one major antigenic region within enteroviral VP1 which in humans elicits antibodies cross-reactive within the enterovirus family. On the basis of our mapping data, the major antigenic

TABLE 1. Alignment of an immunodominant antigenic region cross-reactive within different types of enterovirus serotypes

Enterovirus	Sequence[a]
Poliovirus 1 (Mahoney)	P A L T A V E T G A T N P L
Poliovirus 1 (Sabin)	- - - - - - - - - - - - -
Poliovirus 2W2	- - - - - - - - - - - - -
Poliovirus 3L	- - - - - - - - - - - - -
Coxsackievirus A9	- - - - - - - - - H - S Q V
Coxsackievirus A21	- - - - - - - - - - S G Q A
Coxsackievirus A24	- - - - - - - - - V S G Q A
Coxsackievirus B1	- - - - - A - - - H - S Q V
Coxsackievirus B3	- - - - - A - - - H - S Q V
Coxsackievirus B4	- - - - - - - - - H - S Q V
Enterovirus 70	- S - N - - - - - - - S N T
Echovirus 22	G L T S - Q D D - P L G Q E
Consensus sequence	P A L T A * E T G * * * * *
Most essential residues for antibody binding	P A L T A * E T G * * * * *

[a] –, amino acid homology; *, variable or nonessential position.

region is located around residues 37 to 51, with the sequence HSKEIPALTAVETGA (poliovirus 1, Mahoney strain), which is in good correlation with previous findings (18). We were also able to determine the most essential amino acid residues responsible for antibody binding within the region of residues 42 to 55. By using substitution peptide analogs, we found that the majority of the essential residues for antibody recognition, i.e., Pro-42, Ala-43, Leu-44, Thr-45, Ala-46, Glu-48, Thr-49, and Gly-50, are located at positions which are all highly conserved among the enteroviral serotypes. This finding provides an explanation at the molecular level for the observed serological cross-reactivity within the enterovirus family. This explanation also seems plausible for the majority of echoviruses represented in the study, although sequence data are not yet available. The lack of reactivity to peptides covering the corresponding region of echovirus 22 was clear. The sequence of this region is highly divergent in this virus. Echovirus 22 is known to differ from the main enterovirus group in other molecular (9) and clinical (3, 9) aspects. Our findings further support the idea that echovirus 22 should not be considered an enterovirus.

The assumption that the identified region forms the dominant cross-reactive antigenic site is further corroborated by the fact that IgG antibody binding to heat-treated enteroviral antigens is inhibited by the peptide with the sequence PAL TAVETGATNPL. However, inhibition was not complete and not recorded for all sera. This discrepancy may be explained by the different conformations of the linear peptide and the antigenic site of the viral capsid. It also raises the question of whether there are additional antigenic sites. In a previous study of human sera using the peptide scanning technique (18), antigenic regions were also found in VP2 and VP3. It is, however, not known whether they represent antigenic regions eliciting cross-reactive antibodies.

Further studies using large panels of human sera must be performed to explore the possibility of additional cross-reactive antigenic sites within VP1, VP2, VP3, and VP4.

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