JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1993, p. 911–916
0095-1137/93/040911-06$02.00/0

Vol. 31, No. 4

Identification of Group-Common Linear Epitopes in Structural and Nonstructural Proteins of Enteroviruses by Using Synthetic Peptides

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Received 8 September 1992/Accepted 21 January 1993

Synthetic peptides were employed in enzyme-linked immunosorbent assays to identify group-common linear epitopes in the structural and nonstructural proteins of enteroviruses. Nine linear epitopes were recognized by using sera from patients with heterotypic immunoglobulin G antibody responses to enterovirus infections. The most-reactive peptides were derived from conserved regions of the amino-terminal part of VP1, whereas peptides representing sequences from other conserved regions of VP1, as well as VP2, VP3, and VP4, and from a nonstructural region showed no or poor reactivity. These findings may be useful in the development of serological tests for the diagnosis of infections caused by a broad range of enteroviruses.

Enteroviruses include 69 serotypes: polioviruses, coxsackieviruses, echoviruses, and enterovirus types 68 to 72 (35). Infections due to enteroviruses range in severity from the asymptomatic to aseptic meningitis, encephalitis, paralysis, pneumonia, and myocarditis (24). The enteroviruses are small (24 to 30 nm), nonenveloped, single-stranded RNA viruses, made up of 60 copies of four proteins, VP1 to VP4 (35).

Knowledge of the primary structure and genetic organization of enteroviruses has increased recently (31, 35, 40), and it has been shown that conserved sequences can be found in structural and nonstructural proteins among enteroviruses. Considerable antigenic homology between individual serotypes has been shown by serological cross-reactivity in assays such as the enzyme-linked immunosorbent assay (ELISA) (36, 42), the radioimmunoassay (28, 41), complement fixation (15), the hemadsorption technique (13), gel double diffusion (6, 37, 38), and the immunoblotting technique (4, 25, 32, 33). Enterovirus group-common determinants are exposed in defective, heated, or disrupted virions (7, 8, 20, 22, 23, 29). It has been shown by the immunoblotting technique that cross-reactive immunoglobulin G (IgG) antibodies reacted only with epitopes of capsid protein VP1, which is not present on the surface of intact virus particles (25, 33). According to some authors (4, 25), cross-reactive enterovirus IgM antibodies reacted exclusively with VP1, although reactions with VP2 and VP3 have subsequently been demonstrated elsewhere (33).

Information about the amino acid sequences and the exact location of group-common epitopes is limited (34). Since heterotypic antibody responses are observed in patients with enterovirus infections, there is considerable interest in obtaining a broadly reactive antigen that can detect antibodies against a wide range of enterovirus serotypes. Identification of the group-common epitopes eliciting cross-reactive antibodies has potential value for serodiagnosis of enterovirus infections.

Synthetic peptides have been used to study antigen-antibody interactions (11) and to map immunogenic domains or linear epitopes on virus proteins (14, 18, 26). They have also been found to be specific and sensitive for the detection of antibodies when used as antigens in different diagnostic tests (2, 18, 39).

In the present report, we describe the use of synthetic peptides for the identification of group-common linear epitopes in the structural and nonstructural proteins of enteroviruses. Two panels of serum samples, one from patients showing a rise in the level of heterologous enteroviral IgG antibodies and another from patients with heterotypic enteroviral IgM responses, were used to characterize the reactivity of different peptides by ELISA.

MATERIALS AND METHODS

Patients and sera. To evaluate the IgG reactivities of the synthetic peptides, we tested acute- and convalescent-phase sera from 11 patients with aseptic meningitis due to the following enteroviruses: echovirus 6 (1 patient [n = 1]), echovirus 24 (n = 1), echovirus 30 (n = 7), coxsackievirus B4 (n = 1), and coxsackievirus B5 (n = 1) (confirmed by isolation from cerebrospinal fluid or feces). Acute-phase sera were obtained within 7 days, and convalescent-phase sera were obtained 11 to 38 days after the onset of illness. All of these paired serum samples showed demonstrable rises in the titers of heterologous IgG by an enteroviral IgG ELISA based on heated virions as antigen (described below).

Another serum panel from 12 patients with culture-proven enteroviral infections was used for evaluation of the IgM reactivities of the synthetic peptides. The patients were infected with echovirus 4 (n = 2), echovirus 9 (n = 2), echovirus 11 (n = 1), echovirus 30 (n = 1), coxsackievirus A9 (n = 1), coxsackievirus B3 (n = 1), coxsackievirus B4 (n = 2), or coxsackievirus B5 (n = 2). By an enteroviral IgM-capture ELISA based on heated, peroxidase-labeled

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TABLE 1. Sequences and localizations of enterovirus peptides and reactivity with 11 convalescent-phase serum samples from patients infected with enteroviruses of different serotypes

Peptide	Sequence[a]	Viral protein	Amino acid range[b]	No. from serum panel showing reactivity (n = 11)
E1	PALTAVETGATNPL	VP1	42–55	11
E2	PALTAAETG	VP1	42–50	10
E3	PSDTMQTRHVKNYHSRSES	VP1	57–75	1
E4	SRSESSIENF	VP1	71–80	10
E5	RFDLELTFVIT	VP1	129–139	1
E6	QIMYVPPGGP	VP1	156–165	0
E7	WQTSTNPSVF	VP1	175–184	0
E8	NAYSNFYDG	VP1	203–211	0
E9	GYSDRVRSITLGNS	VP2	8–21	2
E10	ITLGNSTITTQE	VP2	16–27	0
E11	GYTIHVQCNASKFHQG	VP2	105–120	0
E12	INLRTNNSATIV	VP2	198–209	1
E13	QFLTSDDFQSP	VP3	12–22	0
E14	HVIWDVGLQSS	VP3	153–163	1
E15	SACNDFSVRMLRDT	VP3	215–228	0
E16	GAQVSTQKTGAHE	VP4	1–14	0
E17	QDPSKFTEPVKD	VP4	47–58	1
E18	IHESIRWTKDP	Polymerase 3D		0

[a] The underlined amino acids indicate variation among different enterovirus types included in this study (see Materials and Methods).
[b] Amino acid numbering according to alignment with poliovirus type 1 Mahoney (GenBank release 67 [accession number, J02281]).

virions as antigen (described below), all sera showed heterotypic IgM responses.

Enteroviral ELISA for heterologous-antibody detection. (i) Enteroviral cross-reactive IgG ELISA. The indirect ELISA test used for the characterization of the heterologous IgG response of our serum panel has been described elsewhere (36). Briefly, three different serotypes of enteroviruses, echoviruses 9 and 30 and coxsackievirus B5, were prepared by differential centrifugation, heated at 56°C, and used as group-reactive antigens. The plates were coated with antigens of the three different serotypes. After the plates were washed three times, patient serum diluted 1/500 was added to duplicate wells. Following incubation overnight at room temperature, bound IgG was detected by rabbit anti-human IgG conjugated with alkaline phosphatase (incubation for 90 min at 37°C). Substrate was added and incubated at room temperature for 45 min, and the A_{405} was read. A heterotypic rise in titer of IgG antibody was defined as an absorbance difference between acute- and convalescent-phase sera of >0.200 against a serotype(s) other than the one isolated. Of the 11 patients tested, 1 showed a rise in titer of antibody to antigen of one serotype, five patients showed a rise in titer of two of the antigens, and five patients showed a rise in titer of antibody to all three antigens.

(ii) Enteroviral cross-reactive IgM ELISA. The μ-capture ELISA used to characterize the IgM activities of our serum panel has been described elsewhere (36). Briefly, two serotypes of enteroviruses, echovirus 6 and coxsackievirus A9, were prepared by differential centrifugation, heated at 56°C, and then conjugated with horseradish peroxidase. Polystyrene microtiter plates (Maxisorp; Nunc AS, Roskilde, Denmark) were coated with rabbit anti-human IgM (Dakopatts, Copenhagen, Denmark). After the plates were washed, 100 μl of patient serum, diluted 1/200, was added. After being incubated for 1 h at 37°C, the plates were washed, 100 μl of peroxidase-conjugated antigen was added, and the plates were further incubated for 2 h at room temperature. After the plates were washed and after the substrate step, the A_{490} was read.

Cutoff serum representing 2.5 times the absorbance value of that of the negative control was included in each test. A positive reaction was defined as an optical density (OD) value above the cutoff value. An IgM response was considered heterotypic when a positive reaction against a serotype(s) other than the one isolated was detected.

Peptide synthesis. The peptide sequences from the structural and nonstructural proteins were selected from regions that are conserved among enteroviruses according to the sequence data for different enteroviruses (GenBank release 67), coxsackievirus A9 (1), and coxsackievirus A21 (19). The sequence of one peptide (E1) was chosen from the literature (34). The peptide sequences are given in Table 1.

The solid-phase peptide synthesis was performed with an Applied Biosystems 430A peptide synthesizer (Applied Biosystems, Foster City, Calif.). An amino-terminal cysteine was added to all peptides to facilitate coupling to carrier protein. The peptides were synthesized by using the t-Boc synthesis protocol as suggested by the manufacturer. All solvents were obtained from Applied Biosystems. Side chain-protected amino acids used were from Nova Biochem (Laufelfingen, Switzerland) and Applied Biosystems. As solid phase, the polymer *p*-methylbenzhydrylamine resin (Peptides International, Louisville, Ky.) was used. Following each amino acid coupling, a sample was taken and a quantitative ninhydrin assay was performed.

After completion of synthesis, peptides were cleaved from the resin by treatment with trifluoromethane sulfonic acid (Applied Biosystems), and amino acid side chains were deprotected by acidic hydrolysis with anisole and ethanedithiol (Merck, Darmstadt, Germany) as scavengers. The amino acid sequence of each peptide was confirmed by sequencing with Applied Biosystem's protein sequencer 473A.

Solid-phase peptide ELISA for antibody detection. (i) Peptide ELISA for detection of IgG antibodies. Synthetic peptides dissolved at a concentration of 1 mg/ml in 10% acetic acid were covalently coupled to bovine serum albumin (BSA) fraction V (Boehringer, Mannheim, Germany) at a

10:1 (peptide-BSA) molar ratio with *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP; Pharmacia, Uppsala, Sweden) as described by the manufacturer. Polystyrene microtiter plates (Maxisorp; Nunc AS) were used in the ELISAs. For coating of the plates, 4 μg of peptide-BSA conjugate per ml in a volume of 100 μl was added to each well and allowed to bind at 4°C overnight. Remaining free-binding sites in the wells were blocked for 1 h at 37°C with 1% BSA in phosphate-buffered saline (PBS).

To identify the reactive peptides, convalescent-phase sera from patients showing a rise in the titer of heterologous IgG antibody were used. Sera diluted 1:50 in PBS containing 1% BSA and 0.05% Tween 20 (100 μl per well) were tested against each peptide. After incubation for 2 h at 37°C in a humidifier, the plates were washed four times in PBS containing 0.05% Tween 20. Subsequently, 100 μl of horseradish peroxidase-conjugated goat-anti-human IgG (Jackson Laboratory, Bar Harbor, Maine), diluted 1/30,000, was added and allowed to react for 90 min at 37°C in a humidifier. Following six washes, 100 μl of *ortho*-phenyl-diamine was added. After 5 min at room temperature, the substrate reaction was stopped by the addition of 50 μl of 2 M H_2SO_4 and the plates were read at 490 nm. All sera were run in duplicate, and the mean OD values were used for further calculations. The background level was the mean OD value obtained with the 11 convalescent-phase serum samples tested against an unrelated synthetic peptide (a peptide from human T-cell leukemia-lymphotropic virus type II [HTLV-II]). A reaction was considered positive when the OD value was equal to or exceeded three standard deviations of the mean background level established. The mean OD values of the unrelated peptide tested against convalescent-phase sera in 10 peptide ELISAs were calculated to be 0.114 to 0.140, and the standard deviations were found to range from 0.032 to 0.047.

(ii) Peptide ELISA for detection of IgM. For the indirect IgM ELISA, the serum samples from patients with heterotypic enteroviral IgM responses were assayed against the different synthetic peptides. The sera were treated with Protein A-Sepharose (Pharmacia) (41). Subsequently, the treated sera were tested at dilutions of 1/50 and 1/200. Goat anti-human IgM conjugated to peroxidase (Jackson) was used as detector antibody. The net absorbance of each sample was the absorbance to the indicated peptide minus the absorbance to BSA. A cutoff serum with a net absorbance value 2.5 times higher than the net absorbance value of the negative control was included in each test. A positive reaction was defined as an OD value above the cutoff value. In all other respects, the assay was similar to the peptide IgG ELISA described above.

Adsorption test. Since E1 and E2 are similar except for an additional sequence on the carboxy terminus of E1 (Table 1), we wanted to know whether E1 and E2 contain the same epitope. Three patient serum samples that reacted against both peptides were diluted from 1/50 to 1/6,400. For adsorption, 100 μl of each dilution was added to an ELISA plate coated with E1 and another plate coated with E2, at a concentration of 4 μg of peptide-BSA conjugate per ml, and the plates were incubated for 30 min at room temperature for adsorption of specific antibodies. The adsorbed sera were transferred for testing of IgG activity against E1 and E2 as described above in the solid-phase peptide ELISA. To control nonspecific adsorption, the sera were adsorbed under conditions identical to those described above with a plate coated with an unrelated peptide (HTLV-II); after that, the adsorbed sera were transferred for testing of IgG activity against E1 and E2. As a second control, the titers of IgG against measles virus were measured by ELISA before and after adsorption of the sera with the E1 and E2 peptides, respectively.

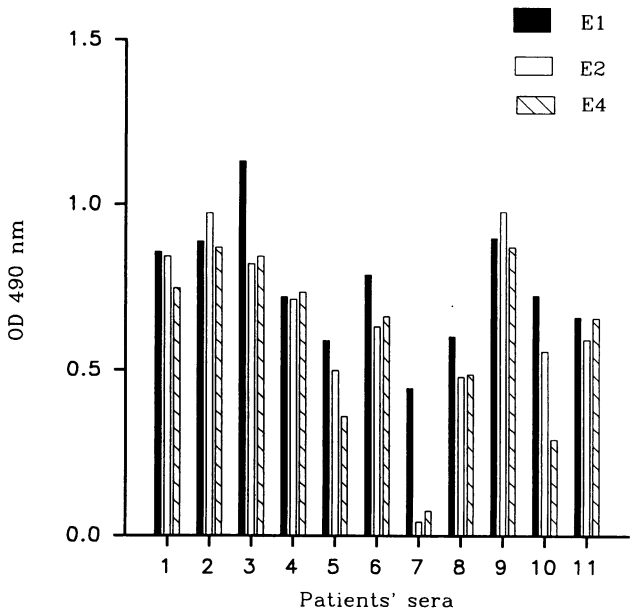

FIG. 1. Serum reactivity profiles obtained with convalescent-phase sera from 11 patients with heterotypic IgG antibody responses to enterovirus infections as measured by ELISA at an OD of 490 nm against the E1, E2, and E4 peptides. Absorbance values obtained with sera at a dilution of 1/50 and with coated peptide-BSA conjugate at a concentration of 4 μg/ml are presented.

Nucleotide sequence accession numbers. The GenBank release 67 accession numbers for the different enterovirus sequences included in this study are as follows: poliovirus type 1, J02281; poliovirus type 2, M12197; poliovirus type 3, K01392; coxsackievirus B1, M16560; coxsackievirus B3 M16572; and coxsackievirus B4, D00149.

RESULTS

Eighteen peptides representing different conserved regions of the structural and nonstructural proteins of enteroviruses were synthesized. The reactivities of synthetic peptides were determined by an ELISA with peptide-BSA adsorbed to solid phase. Sera from patients with culture-proven enterovirus infections and heterotypic enteroviral antibody responses were used for evaluation of the peptides.

In the IgG ELISA, all serum samples reacted with the E1 peptide, whereas both E2 and E4 were recognized by 10 of 11 serum samples (Table 1). The OD values obtained with these three immunoreactive peptides were between 0.4 and 1.13. The absorbance profiles are shown in Fig. 1. The mean absorbance value for the serum panel was 0.889 for E1, 0.765 for E2, and 0.723 for E4. One peptide (E9) reacted with two serum samples, whereas five peptides (E3, E5, E14, E12, and E17) were recognized by only one serum sample (Table 1). However, the absorbance values obtained against these peptides were low, ranging between OD values of 0.300 and 0.600. No reactivity could be detected with the remaining peptides.

The most-reactive serum sample was from one of the patients infected with echovirus 30; it exhibited reactivity to six peptides (E1, E2, E3, E4, E9, and E12). A serum sample

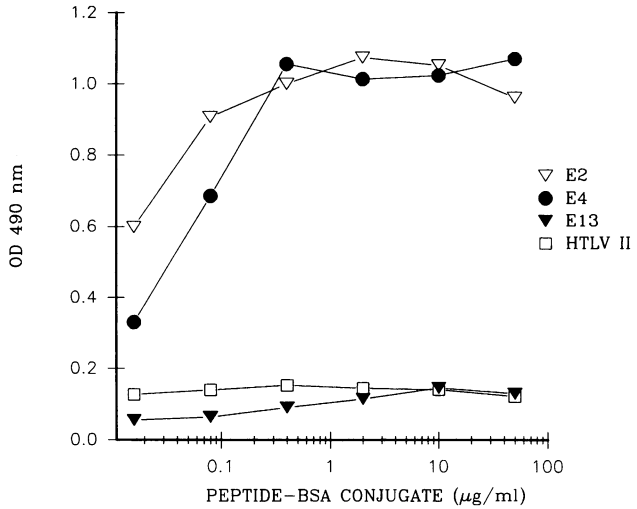

FIG. 2. IgG reactivity of a convalescent-phase serum from a patient with heterotypic IgG antibody response to enterovirus infection against three enterovirus peptides (E2, E4, and E13) and one unrelated peptide (HTLV-II) analyzed by ELISA. Absorbance values obtained with the serum at a dilution of 1/50 and with coated peptide-BSA conjugate at fivefold dilutions from 0.016 to 50 μg/ml are presented.

from another patient infected with echovirus 30 reacted against five peptides (E1, E2, E4, E5, and E17). The serum sample from one patient infected with coxsackievirus B4 reacted only with the E1 peptide. The rest of the serum samples recognized E1, E2, and E4; moreover, sera from three patients (two infected with echovirus 30 and one infected with echovirus 6) recognized one more peptide.

The influence of the concentration of the peptide-BSA conjugates (4 μg/ml) used in the assays was evaluated. A concentration range between 0.016 and 50 μg of each conjugate per ml was used. A peptide from HTLV-II was used to evaluate nonspecific binding. Three convalescent-phase serum samples from patients that showed a rise in titer of antibody to all three antigens by our enteroviral cross-reactive IgG ELISA were used to test the reactivity of all our peptides. Figure 2 shows OD values as a function of the peptide-BSA conjugate concentrations for one serum sample, three synthetic enterovirus peptides, and the control peptide. At conjugate concentrations in the range of 0.4 to 10 μg/ml, the absorbance values reached a plateau. Similar findings were obtained with other sera and peptides. As shown in Fig. 2, an increase in the concentration of the peptide-BSA conjugate neither influenced the activity of already reactive peptides, such as E2 and E4, nor enhanced the reactivity of the nonreactive peptide, such as E13. The differences in reactivities among the peptides are attributable not to quantitative but to qualitative differences.

When acute- and convalescent-phase sera were tested in parallel against the highly reactive peptides E1, E2, and E4, significant increases in IgG activity were seen for most of the patients (8 of 11 patients; data not shown).

For comparison and evaluation of the epitopes in E1 and E2, patient sera reactive with both peptides were adsorbed with one peptide and tested by ELISA against both peptides. After adsorption with either peptide, there was a fourfold decrease in the titer of antibody against both antigens, whereas no change was observed when the sera were absorbed with the HTLV-II peptide (data not shown). Moreover, IgG titers of antibody against measles virus were the same before and after adsorption with either E1 or E2. These results indicate that E1 and E2 contain the same epitope.

The peptides were also tested by indirect IgM assays with the same format as that used for IgG. However, the experimental system suffered from background problems, which were difficult to eliminate, although several different blocking procedures were tried. No specific activity with the 12 serum samples known to be highly reactive by the μ-capture enteroviral IgM test was discernible.

DISCUSSION

In this report, a range of immunoreactivities was observed when synthetic peptides representing conserved regions of enterovirus proteins were tested by ELISA with sera from patients with a heterotypic enteroviral IgG response. Two of our selected peptides from VP1, as well as the E1 peptide chosen from the literature (34), showed consistent reactivity with most of the sera in our panel. It seems likely that these peptides represent epitopes located on VP1 which have been shown to contain cross-reactive determinants by the immunoblotting technique (25, 32, 33). In contrast, the number of sera reacting with the other peptides representing regions of VP2, VP3, and VP4 capsid proteins was very low, and the absorbance values obtained were lower than those obtained with the most-immunoreactive peptides of VP1. Our results are consistent with the data from previous studies performed by Western blot showing that the cross-reactivity of IgG antibodies is directed almost exclusively to VP1 antigens (25, 33). Other investigators using peptide-scanning techniques to identify antigenic regions of poliovirus type 3 (Sabin) have found that a large number of peptides representing regions in VP1, VP2, and VP3 capsid proteins bound significant amounts of IgG antibodies. However, also in this study, regions with the highest activity were found in VP1 (34). Although VP1 seems to have a predominantly antigenic role, antigens from VP2, VP3, and VP4 are also capable of eliciting an IgG response. This was shown in our study by the reactivity of some sera to E9, E12, E14, and E17 synthetic peptides. The extent of cross-reactivity observed in patient sera and the number of epitopes involved may be proportional to the number of enterovirus infections experienced by the individual (21). It can also be expected that because of the genetic variability of the immune system, antibodies in sera derived from different individuals may not be directed to the same epitope and may differ in quality and quantity.

Our results are in agreement with those of Roivainen et al. who detected the presence of the highest titers of antibody to the epitope consisting of residues 40 to 53 of VP1 that has the same amino acid sequence as the E1 peptide. The pattern of reactivity of E2 was similar to that of E1. Since its sequence, PALTAAETG, is very similar to the first stretch of that of E1, PALTAVETG, it may be inferred that the heterotypic antibodies are directed to this conserved linear epitope. The adsorption results support this assumption. Thus, the finding that serum from one patient (serum sample no. 7; Fig. 1) reacted only with the E1 peptide and not with the E2 peptide might be explained by a lower sensitivity of the E2 peptide for detecting antibodies.

Besides, the amino acid sequence of E1 near the carboxy terminus, GATNPL (Table 1), is the less-conserved part of this peptide, which speaks against the possibility that this part of the peptide is directly involved in the induction of heterologous antibodies.

The peptide E4, which was recognized by most sera, is similar to peptides resembling the amino-terminal part of VP1 of poliovirus type 1 previously reported to prime or induce neutralizing antibody response in experimental animals (3, 5). Also, a moderate reactivity in the region of poliovirus type 3 (Sabin) coinciding with the E4 sequence was detected by using the peptide-scanning technique (34). It is known that after attachment of poliovirus type 1 to the cell, the amino terminus of VP1, which is internal in the native virion (16), is exposed and may be available for immune recognition (9). From our results, it seems that a similar conformational change occurs with other enteroviruses during the infection of humans, since a significant increase of IgG activity against three peptides corresponding to conserved parts of the amino terminus of VP1 was detected in our panel of sera.

To demonstrate that antibodies directed to our reactive peptides were specific to enterovirus structures, affinity chromatography was used to obtain antipeptide antibodies from human gamma globulin. By indirect ELISAs, these purified antipeptide antibodies were individually tested against the following antigens: poliovirus types 1, 2, and 3; coxsackievirus types B1, B3, B5, A9, and A16; echovirus types 6, 9, 15, 17, 19, and 21; cytomegalovirus; herpes simplex virus; varicella virus; and measles virus. Reactivity against all enterovirus antigens was detected, whereas no reactivity to the other viral antigens was observed (data not shown).

Since antibodies directed to the E1, E2, and E4 peptides were part of the human response to a variety of infecting enteroviral serotypes, these epitopes or very similar sequences seem to be part of the structures of many enteroviruses that have not yet been sequenced.

The E3 peptide, located between E1 and E4, was recognized by only one serum. By the scanning technique, peptides similar to the E3 sequence were found to be reactive, showing several peaks. The scanning technique, in which a peptide length of 14 amino acids was used, also demonstrated activity for regions of VP1, VP2, and VP3 covered by E5, E7, E8, E9, E10, E11, E12, and E15 (34). These peptides, however, were poorly reactive or nonreactive by our ELISA. This disagreement can be explained in different ways. First, the formats of the tests were different. Second, although the peptides have the same critical amino acids, shorter or longer peptides may adopt a disadvantageous conformation for binding of specific antibodies. Third, the detection of a low level of cross-reactivity is augmented when the peptide-scanning method is used (43).

On the other hand, the sequences of the peptides selected in this study varied in some residues compared with the different enterovirus sequences reported. It has been shown that a change in a single amino acid can dramatically alter the reactivity of a peptide (12). Therefore, we cannot rule out the possibility of having chosen some poorly reactive or nonreactive peptides. A set of analogous peptides, in which the variable residue is replaced by the alternative amino acid(s), could be synthesized in order to find other possible reactive peptides.

The lack of reactivity of some of our peptides cannot be due to a low concentration of peptide-carrier conjugate on the solid phase since, as shown in Fig. 2, an increase in the conjugate concentration above 4 μg/ml did not improve the sensitivity of the ELISA. A small number of peptide molecules on the carrier protein may also cause low sensitivity. However, a ratio of 5 to 20 mol of peptide per mol of carrier protein is considered suitable for the detection of antibodies by ELISA (30).

Although antigenicity prediction methods are popular for predicting the locations of viral epitopes (17, 27), the selection of linear epitopes in our study was done only by choosing conserved regions of enteroviruses. There is evidence that none of the prediction methods achieves a high level of correct prediction (10, 44). Besides, it was found that none of three predictive algorithms predicted better than randomly at a peptide length of less than 15 residues (10).

The rise in titer of antibody against the E1, E2, and E4 peptides observed with sera from patients with enteroviral infections and the heterotypic rise of IgG antibody, plus the fact that affinity-purified human antibodies against these peptides recognize different serotypes of enteroviruses, confirm that these epitopes are part of conserved structures which elicit a heterotypic IgG response. However, this approach was not successful for the measurement of IgM activity. Other formats for the assay may be attempted, e.g., a μ-capture assay with biotinylated peptide.

In conclusion, our attempt to characterize broadly reactive peptides may, if extended, have serodiagnostic implications, since there is a demand for a test that could detect antibodies against a wide range of enterovirus infections.

ACKNOWLEDGMENTS

We thank Bo Johansson for expert assistance with computer searching and Nancy Nenonen for correcting the English and for many helpful suggestions. We also thank Eva Skoog for her excellent technical assistance.

This work was supported by the Medical Faculty of the University of Göteborg, the Karolinska Institute, and Syntello AB.

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