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Human Cytomegalovirus Structural Proteins: Immune Reaction against pp150 Synthetic Peptides

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In the present study, several peptides of the major structural antigen (pp150) of human cytomegalovirus (CMV) have been chemically synthesized and tested by a modified slot blotting procedure for their ability to bind CMV-specific immunoglobulin G (IgG) and IgM present in human sera. The sequences of the peptides were deduced on the basis of either (i) their presence in a fusion protein already known to be frequently recognized by human antibody or (ii) their high content of hydrophilic amino acids as deduced from the published nucleotide sequence. An important IgM-binding epitope was found to be located in the last 38 amino acids at the carboxy terminus of the molecule. This region reacts with anti-CMV IgM present in the great majority (83.3%) of IgM-positive human sera, and adsorption experiments have shown that IgM titers to the entire pp150 decrease 25 to 50% in most sera previously absorbed with this region. The overall results obtained endorse the continued synthesis of other sequences in order to define a group of peptides sensitive and specific enough to replace the virus and infected cells as an antigenic substrate in the serological evaluation of anti-CMV antibody.

Human cytomegalovirus (CMV) particles contain a basic phosphoprotein with an M_r of 150,000 (pp150) which is particularly reactive in Western blot (immunoblot) analyses with anti-CMV human antibody (4, 8, 9). The gene coding for pp150 has been mapped in *Hin*dIII fragments J and N of the AD169 genome and is transcribed into a late 6.2-kb mRNA (3).

The analysis of the humoral immune response elicited during natural infection has repeatedly shown that pp150 is highly immunogenic and is recognized by sera from nearly 100% of the CMV-seropositive subjects tested. The response to this polypeptide persists for years after convalescence, even when antibody to the other CMV proteins has disappeared (4, 8, 9). For these reasons, pp150 seems to be a primary antigenic candidate for developing new diagnostic reagents.

In the last three years, some fusion proteins containing significant epitopes of pp150 have been obtained via recombinant DNA techniques and were shown to react efficiently with human antibody (6, 12, 13). This finding has suggested the possibility of using fusion proteins as antigenic material in CMV serology in order to facilitate antigen standardization.

An alternative approach would be the chemical synthesis of the important epitopes present in pp150. In fact, in recent years, the serological diagnosis of viral infection has been vastly simplified by the invention of synthetic peptide-based serological procedures. The use of site-directed serology is attractive for a number of reasons. The antigen used in the test is biochemically defined and therefore can be standardized readily and can be free of any contaminating proteins. Unlimited amounts can be synthesized, and synthetic peptides are inexpensive compared with materials prepared by cell culture. The application of this technology has proven very useful in several systems, including the diagnosis of infection with Epstein-Barr virus (2), respiratory syncytial virus (11), human immunodeficiency virus (14), and human papillomavirus (1). To the best of our knowledge, there are no published data yet on the use of synthetic peptides in CMV serodiagnosis.

In the present study, several peptides of pp150 were chemically synthesized and analyzed for their reactivity with immunoglobulin G (IgG) and IgM of human sera.

The sequences of the peptides were decided on the basis of either (i) their presence in a fusion protein already known to be frequently recognized by human antibody or (ii) their high content of hydrophilic amino acids as deduced from the published nucleotide sequence (3).

An important IgM-binding epitope was located in the last 38 amino acids at the carboxy terminus of pp150, which reacts with anti-CMV IgM present in the great majority (83.3%) of IgM-positive human sera.

The overall results obtained encourage the continued synthesis of other sequences in order to define a group of peptides showing a reactivity pattern which will replace the virus in the serological evaluation of anti-CMV antibodies.

MATERIALS AND METHODS

Peptides. Peptides were synthesized by the Merrifield solid-phase peptide synthesis method (10). The peptides were synthesized on a DuPont multiple peptide synthesis module (RaMPS) with highly pure-grade chemicals. All solvents were analytical grade and were used without further purification. 9-fluoroenylmethyloxycarbonyl-protected amino acids were obtained from either Bachem Bioscience (Philadelphia, Pa.) or Star Biochemicals (Torrance, Calif.). Standard *N*-α-9-fluoroenylmethyloxycarbonyl-protected amino acids were employed in the synthesis. The side chain groups used were *tert*-butyl for Ser, Thr, Glu, and Asp, *tert*-butyloxycarbonyl for the Σ amino group of Lys, trityl for Cys (SH) and His (imidazole) groups, and N^g-4-methoxy-2,3,5-trimethoxybenezenesulfonamide for Arg. Asparagine and glutamine were used as penta-fluorophenyl esters. The resin used for synthesis was Rapidamine resin (DuPont), a

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p-methylbenzhydrylamine-based resin which, on cleavage, yielded peptide amides.

Fluoroenylmethyloxycarbonyl-protected amino acids were attached sequentially as 1-hydroxybenzotriazole esters (in a 2.5 M excess of amino acid, 1,3-diisopropylcarbodiimide, and 1-hydroxybenzotriazole in *N*,*N*′-dimethylformamide), except Arg and Gln, which were used as pentafluorophenyl esters in *N*,*N*′-dimethylformamide and 1-hydroxybenzotriazole solution.

Chain assembly was monitored by the Kaiser test (5). The assembled peptides were simultaneously cleaved and deprotected with a mixture of trifluoroacetic acid (70%), methylene chloride (25%), and methyl sulfide (5%). For Arg-containing peptides, a mixture of trifluoroacetic acid (90%), thioanisole (5%), dithiothreitol (3%), and anisole (2%) was used. The crude peptides were precipitated with diethyl ether and washed with ethyl acetate. The peptides were then extracted with 60% acetonitrile and 0.1% trifluoroacetic acid and analyzed by high-pressure liquid chromatography (octyldecyl silane, 25 by 4.6 cm) (Rainin; Dynamax).

The designations, the amino acid positions in pp150, and the amino acid sequences of the synthesized peptides are reported in Table 1. The sequences of the peptides to be synthesized were decided on the basis of either their presence in the D1 fusion protein (6, 12), already known to be frequently recognized by human antibody (6, 12), for group 1 peptides or their high content of hydrophilic amino acids, as deduced from the published sequence of pp150 and as suggested to be immunodominant epitopes (3), for group 2 peptides.

In group 1, two series of peptides (covering an area of 78 amino acids [aa]) were made: the first series (89A, 89B, 145B, and 145C) with a leucine (L) at position 1037, as deduced from the published nucleotide sequence (3), and the second series (90A, 90B, 146B, and 146C) with a serine (S) in the same position, as indicated by the sequencing of the CMV fragment inserted in the recombinant lambda gt11 D1 clone (6, 12). Another two peptides (147A and 147B) were sequenced as the 25-mer and the 62-mer that precede the last 25 aa of pp150.

Human sera. Several groups of human sera were used. For the first series of experiments (Table 2), a group of 32 serum samples from healthy adults (mean age, 32) who had a high titer of IgG to CMV over a long period as judged by enzyme immunoassay (EIA) was compared with a group of 36 serum samples from renal transplant recipients (mean age, 39) with a high titer of CMV-specific IgM (as judged by IgM-capture EIA), which correlated with clinical symptoms indicative of CMV infection or with positive virus isolation from urine or both.

In the second series of experiments (Table 3), four groups of sera were used. The first group (25 serum samples) reacted specifically with the D1 fusion protein (6, 12); the second group (23 serum samples) was seropositive to native viral pp150, as determined by immunoblotting with structural polypeptides from purified viral particles; the third group (64 serum samples) was randomly selected in our diagnostic laboratory from subjects who were long-term seropositive for CMV; and the fourth group (8 serum samples) was seronegative for CMV. Serological status with regard to CMV was determined by EIA.

Modified dot blotting for the analysis of peptide reactivity with human sera. Nitrocellulose paper was mounted in a miniblotter chamber (Immunetics, Cambridge, Mass.), and 100 μg of peptide in phosphate-buffered saline (PBS) was deposited in each channel (130 mm long and 1.5 mm wide)

TABLE 1. Designations and amino acid composition of pp150 synthetic peptides

Group and peptide	Position	Amino acid sequence
1		
89A	1024–1048	GGAKTPSDAVQNILQKIEKIKNTEE
89B	1011–1048	KSGTGPQPGSAGM GGAKTPSDAVQNILQKIEKIKNTEE
145B	999–1048	MKTVAFDLSSPQ KSGTGPQPGSAGM GGAKTPSDAVQNILQKIEKIKNTEE
145C	990–1048	SPVKSTTG MKTVAFDLSSPQ KSGTGPQPGSAGM GGAKTPSDAVQNILQKIEKIKNTEE
90A	1024–1048	GGAKTPSDAVQNISQKIEKIKNTEE
90B	1011–1048	KSGTGPQPGSAGM GGAKTPSDAVQNISQKIEKIKNTEE
146B	999–1048	MKTVAFDLSSPQ KSGTGPQPGSAGM GGAKTPSDAVQNISQKIEKIKNTEE
146C	990–1048	SPVKSTTG MKTVAFDLSSPQ KSGTGPQPGSAGM GGAKTPSDAVQNISQKIEKIKNTEE
147A	999–1022	MKTVAFDLSSPQ KSGTGPQPGSAGM
147B	961–1022	APPVASPSILKPGASAALQSRRSTGTAAVG SPVKSTTG MKTVAFDLSSPQ KSGTGPQPGSAGM
2		
151A	517–537	RPLTETRGDLFSGDEDSDSSD
151B	501–537	TKASPGRVRRDSAWDV RPLTETRGDLFSGDEDSDSSD
152A	701–724	LRDQTAESPVTEDSEEEDDSSDTG
152B	692–724	RDAADEVWA LRDQTAESPVTEDSEEEDDSSDTG
153A	420–440	SRRLPGSSADEDDDDDDDEKN
153B	407–440	QQQPPPPARKPSA SRRLPGSSADEDDDDDDDEKN

TABLE 2. Reactivities of pp150 synthetic peptides with a commerical gamma globulin preparation and with immunoglobulins in human sera[a]

Peptide	Reaction with the following amt (μg/ml) of purified anti-CMV IgG:					% Reactivity with:	
	2	1	0.5	0.25	0.125	IgG	IgM
145C	+	+	+	−	−	18.7	38.8
145B	+	+	−	−	−	12.5	16.6
89B	+	+	+	+	−	25.0	83.3
89A	−	−	−	−	−	0	16.6
146C	+	+	−	−	−	6.2	22.2
146B	+	−	−	−	−	6.2	11.1
90B	+	+	+	+	−	18.7	61.1
90A	+	−	−	−	−	12.5	38.8
147A	+	−	−	−	−	0	11.1
147B	+	+	−	−	−	18.7	11.1
151A	−	−	−	−	−	0	5.5
151B	−	−	−	−	−	0	0
152A	−	−	−	−	−	0	0
152B	−	−	−	−	−	0	0
153A	−	−	−	−	−	0	0
153B	−	−	−	−	−	0	0
89A + 90A	+	+	+	+	−	6.2	ND[b]
89B + 147B	+	+	+	+	−	25.0	61.1

[a] Serum samples (32 for IgG and 36 for IgM) which had high ELISA values for CMV-specific Igs were tested.
[b] ND, not done.

and incubated overnight with gentle agitation at room temperature. The peptide-treated nitrocellulose was removed from the miniblotter and incubated for 1 h at room temperature with 3% gelatin–1% bovine serum albumin (BSA) in Tris-buffered saline (TBS). The saturated nitrocellulose was mounted again in the miniblotter chamber after a 90° rotation. Human serum at a dilution of 1:5 in TBS–1% gelatin–0.33% BSA was deposited in each channel (each serum sample reacted with 1.15 μg of peptide) and incubated at room temperature for 6 h. Nitrocellulose was again removed from the miniblotter chamber, extensively washed with TBS–0.05% Tween 20, and then incubated with a peroxidase-conjugated rabbit anti-human γ or μ chain (DAKO, Glostrup, Denmark) diluted 1:30 in TBS–1% gelatin–0.33% BSA for 5 h at room temperature. After extensive washes with TBS-Tween 20, the reaction was developed with ortho-chloro-naphthol–H_2O_2 (Bio-Rad Laboratories, Richmond, Calif.). By this procedure, up to 43 different serum dilutions can be tested simultaneously against 43 different peptide dilutions, for a total of 1,849 different combinations.

TABLE 3. Immune reactivity to peptide 89B of different groups of human sera

Serum	No. of serum samples		% Positivity
	Tested	Positive	
D1 positive	25	21	84
Viral pp150 positive	23	16	69
CMV positive	64	38	59
CMV negative	8	0	0

Absorption of human sera with peptides. In a miniblotter channel (130 mm long and 1.5 mm wide), 100 μg of peptide in PBS was deposited and incubated overnight with gentle agitation at room temperature. The peptide-treated nitrocellulose channel was incubated for 1 h at room temperature with 3% gelatin–1% BSA in TBS. Human serum to be absorbed was deposited in the same channel at a dilution of 1:5 in TBS–1% gelatin–0.33% BSA and incubated at room temperature for 7 h. Serum was then aspirated from the channel and stored at −20°C until used.

Conventional EIA. The evaluation of anti-CMV IgG was carried out with a cytomegalovirus test kit (M. A. Bioproducts, Walkersville, Md.). The plates were read on a microELISA automatic reader (Dynatech Products, Alexandria, Va.). To perform linear regression analysis and to standardize the test run, every plate included three serum calibrators. The results were then interpreted as suggested by the manufacturers.

The evaluation of anti-CMV IgM was carried out with an antibody capture kit (CMV IgM ELA purchased from Technogenetics, Hamburg, Germany). The test was run and the results were interpreted as indicated by the manufacturer.

To exclude the possibility of false-positive IgM reactions due to the presence of rheumatoid factor, all serum samples were routinely checked by latex agglutination for rheumatoid factor detection (Rheuma Wellcotest; Wellcome Research Laboratory, Dartford, England). Any serum sample which had a positive result was not included in this study.

Immunoblotting with purified viral structural proteins. The procedure for immunoblotting has been previously described in detail (9). Briefly, CMV (Towne strain) was purified from the extracellular medium through a sorbitol cushion and a sorbitol gradient. Purified CMV was denatured in the presence of sodium dodecyl sulfate and β-mercaptoethanol, and the proteins were separated in 9% acrylamide gels. Separated polypeptides were electrotransferred to nitrocellulose, and the immune reaction was performed in a miniblotter chamber.

RESULTS

Peptide reactivity with commercial anti-CMV gamma globulins. The two groups of peptides (Table 1) were screened first for their reactivity with purified IgG present in a commercial preparation of anti-CMV hyperimmune gamma globulin (Immuno AG, Vienna, Austria). As shown in Table 2, positive reactions were obtained with most of the peptides in the first group, although they had different degrees of reactivity. Briefly, three peptides (89B, 90B, and 145C) gave a strong reaction, three others (145B, 146C, and 147B) gave a medium reaction, and three peptides (90A, 146B, and 147A) gave a very low reaction. The level of reactivity did not increase when mixtures of two different peptides were used. Another six peptides (belonging to the second group) did not show any significant reactivity.

Peptide reactivity with human IgG and IgM. Table 2 shows the reactivities of pp150 peptides with two groups of human serum samples. The first group was composed of 32 serum samples obtained from long-term-seropositive subjects with a high titer of anti-CMV-specific IgG. The most reactive peptide, 89B, reacted with 25% of the 32 serum samples. Some reactivity was also obtained with 145C, 90B, 90A, and 147B. The second group was serum samples from 36 renal transplant recipients experiencing acute CMV infection and having a positive enzyme-linked immunosorbent assay (ELISA) test for CMV-specific IgM. A very high percentage

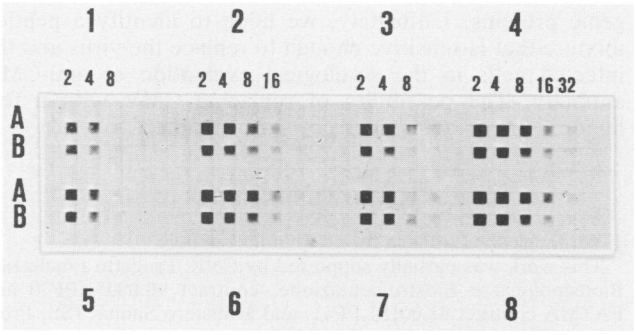

FIG. 1. Titration of total Ig reacting with peptide 89B in eight human serum samples from patients with acute CMV infection. Peptide 89B was used in duplicate experiments (A and B) to determine the reproducibility of our detection system. Serum samples were tested at progressive dilutions from 1:2 to 1:32, as indicated above the blots, to titrate total Ig reactivity with the epitope present in peptide 89B. Serum samples 1 through 3 were from patients suffering from CMV mononucleosis; serum samples 4, 6, 7, and 8 were from renal transplant recipients with positive virus isolation from urine; serum sample 5 was from a liver-transplanted patient with biopsy-proven CMV hepatitis. Under the experimental conditions described in Materials and Methods, antibody titers from 1:4 to 1:32 could be detected in human sera. The results were reproducible as the duplicate reactions were almost identical. Ten CMV-negative serum samples repeatedly tested always gave negative results (data not shown).

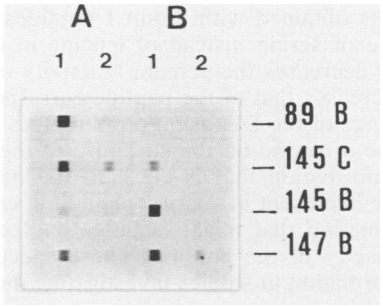

FIG. 2. Absorption experiments of two pools of serum with peptides 89B and 145B. Sera were absorbed as described in Materials and Methods. Serum A was a pool of two serum samples which had strong reactivities to both 89B and 145C. Serum B was a pool of two serum samples which reacted strongly with both 145B and 147B. Pool A was absorbed with 89B, and pool B was absorbed with 145B. Lanes 1 and 2, nonabsorbed and absorbed sera, respectively. Peptide designations are given on the right.

of IgM reactivity (83.3%) was observed with peptide 89B. Other peptides (145C, 146C, 90B, and 90A) were often recognized by human IgM but to a lesser extent. The overall percentage of IgG and IgM reactivities did not increase when mixtures of two different peptides were used.

An example of total Ig reactivity with peptide 89B of a group of eight CMV-positive human sera is shown in Fig. 1. Under our experimental conditions, antibody titers from 1:4 to 1:32 could be detected in human sera. The results were reproducible since reactivities for experiments run in duplicate (Fig. 1) were the same.

The reactivity of peptide 89B with different groups of human sera was then studied in more detail. As shown in Table 3, peptide 89B reacted with 84% of the serum samples that were positive against the D1 recombinant protein, with 69% of the samples positive to the entire pp150, and with 59% of the samples that were CMV positive by EIA. No reaction was obtained with CMV-negative sera.

Absorption experiments. A first absorption experiment was done in order to establish whether the reactivity of 145C was due to the same epitope present in 89B and whether the reactivity obtained with 147B was due to the epitope present in 145B or to other antibody binding sites.

Two pools of serum were used in this experiment. Pool A was composed of two serum samples which had strong reactivities to both 89B and 145C. Pool B contained two serum samples which reacted strongly with both 145B and 147B. Pool A was absorbed with 89B, and pool B was absorbed with 145B.

As shown in Fig. 2, the positivity to peptide 145C, although greatly reduced, was not completely abolished by absorption with 89B and the positivity to 147B was not completely absorbed by 145B. Instead, the reactivities to 89B and to 145B were completely abolished after absorption with the corresponding peptides.

Other experiments were carried out to evaluate the importance of peptide 89B in the total IgM-binding ability of the native viral pp150. For this purpose, we determined the titer of IgM to native pp150 of five serum samples from acutely infected individuals before and after adsorption with peptide 89B. As shown in Table 4, anti-pp150 IgM titers decreased in four of five samples. In one serum sample, no difference between the pre- and postabsorption titers was found.

DISCUSSION

In recent years, serological diagnosis of viral infection has been vastly simplified by the invention of synthetic peptide-based procedures (1, 2, 10, 11, 14).

Although CMV serodiagnosis is hampered by several problems, no attempt to explore the possible use of synthetic peptides in CMV serodiagnosis has been made.

In this study, several peptides of the major CMV structural antigen of 150 kDa were synthesized and tested by a modified slot blotting procedure for their ability to bind CMV-specific IgG and IgM present in human sera.

Two groups of peptides were synthesized and tested. Group 1 peptides were synthesized to reproduce a sequence that is present in a fusion protein that we have produced, characterized, and shown to be frequently recognized by anti-CMV antibodies (6, 12). Group 2 peptides were synthesized because they are present in other highly hydrophilic regions of pp150 and are suggested to be immunodominant epitopes (3). Among group 1 peptides, two series of peptides were made with a leucine or a serine at position 1037, according to the published pp150 nucleotide sequence (3) and the sequencing of the D1 recombinant protein (6, 12), respectively.

TABLE 4. Evaluation of the importance of 89B epitope in the total IgM-binding ability of pp150

Serum	Reciprocal of anti-pp150 IgM titer with 89B	
	Preabsorption	Postabsorption
M26	80	60
M11	60	60
M45	40	20
M18	60	40
M35	60	40

The results obtained with group 1 peptides showed that the presence of serine instead of leucine in position 1037 significantly decreases the percent reactivity of all the peptides but increases that of the peptide corresponding to the 25-aa sequence of the D1 fusion protein. This suggests that the influence of serine on the structure of the 25-mer may favor a conformation that is close to the native epitope. When serine is present in a larger peptide, it could induce a new conformation that might negatively affect the overall binding capacity of the epitope. These speculations need further confirmation in studies investigating the substitution of each residue.

Furthermore, the peptide corresponding to the sequence present in the D1 fusion protein (the last 25 aa at the COOH terminus) had a lower reactivity with human antibodies than we expected on the basis of the good reactivity we obtained with the corresponding sequence present in the fusion protein (6, 12). High reactivity may depend on conformation of that amino acid sequence in the native protein.

A good Ig reactivity was obtained with another peptide composed of the previous one (the last 25 aa at the COOH terminus) and another 13 aa toward the amino terminus (peptide 89B). This region seems to be an important IgM binding site because it reacts with anti-CMV IgM present in the great majority (83.3%) of IgM-positive human serum samples and because absorption experiments have shown that IgM titers to the entire pp150 decrease 25 to 50% in most sera previously absorbed with peptide 89B.

When we tested another peptide composed of the previous one and the next 13 aa, we obtained a much lower reactivity (16% of the IgM-positive serum samples had a positive reaction), indicating that the last 13 aa probably did change the conformation of the peptide, resulting in a less recognizable structure.

Finally, when a peptide containing another 8 aa was tested, more reactivity was obtained, suggesting that either the new amino acid sequence contained a new Ig-binding epitope or the sequence changed the peptide conformation, allowing a better exposition of 89B epitope. Absorption experiments favor the latter possibility.

We were disappointed but not surprised to find that none of the peptides of the second group gave a significant reaction (when they reacted at all) with sera. This group of peptides was synthesized exclusively on the basis of their high content of hydrophilic amino acids as deduced from the nucleotide sequence. Their lack of reaction suggests that information on the chemical characteristics of the secondary structure of a peptide is not sufficient to deduce its immunogenic properties as long as there is no well-proven method that allows construction of possible tertiary structures starting with the sequence of a peptide.

Several peptides reacted with purified IgG present in a commercial preparation of anti-CMV hyperimmune gamma globulin, indicating the wide distribution of antibodies to the epitope(s) present in our peptides. In fact, though commercial gamma globulin is composed of pooled Ig from multiple donors and theoretically only one reactive donor is sufficient to give a positive reaction, we consider it unlikely that positive reactions of pooled Ig may be due to the contribution of only one or a few positive donor sera, because we never found sera with high titers of antibodies to the peptides.

As a whole, the results we obtained indicate that synthetic peptides can be very useful in CMV serodiagnosis. We endorse the continued synthesis of other peptides, both of the same polypeptide and of other well-characterized antigenic proteins. Ultimately, we hope to identify a peptide mixture that is sensitive enough to replace the virus and the infected cells in the serological evaluation of anti-CMV antibody. The possibility of replacing CMV with a few hundred amino acids does not seem remote.

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