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Identification of Human Cytomegalovirus Strain with Immediate-Early (IE) Antigen-Specific Monoclonal Antibody Is Prevented by Point Mutation in IE Gene

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In an AIDS patient with a disseminated human cytomegalovirus (HCMV) infection, presence of HCMV in blood was repeatedly excluded by the shell vial culture method with the HCMV immediate-early (IE) antigen-specific monoclonal antibody (MAb) 5D2 currently employed for rapid HCMV identification, whereas it was repeatedly confirmed by all other assays (conventional virus isolation from blood, antigenemia, and DNAemia). Sequence analysis of the HCMV strain revealed a point mutation in exon 2 of the IE gene, which led to a serine-to-proline substitution at position 20 of the corresponding protein. Cloning and expression of a region of the IE gene containing the mutation showed that this was responsible for the lack of reactivity of MAb 5D2. A pool of IE antigen-reactive MAbs instead of a single MAb must be used for rapid HCMV identification to detect all viral strains.

Human cytomegalovirus (HCMV) represents one of the most important opportunistic pathogens in immunocompromised hosts, namely, transplant recipients and AIDS patients (3). In the last decade, the availability of specific antiviral drugs has required early and rapid diagnosis of HCMV infections. Among several assays developed for this purpose, one of the most useful has been the rapid identification of HCMV isolates in cell cultures by the shell vial culture method with a monoclonal antibody (MAb) to the major immediate-early antigen (IEA) (9). This method has been widely used for rapid determination and quantitation of HCMV viremia in immunocompromised patients (7). In the last few years, an IEA-specific MAb (5D2) selected from a panel of IEA-specific MAbs developed in our laboratory and reactive with different epitopes of the protein was used for rapid identification of several hundred HCMV isolates and compared with a widely used commercially available IEA-specific MAb (15). The two MAbs were shown to be equally reactive with all tested HCMV isolates (7).

Recently, during a clinical trial for the evaluation of the effectiveness of foscarnet for treatment of HCMV retinitis in AIDS patients, a patient enrolled in the study (PFA71) and suffering from a disseminated HCMV infection was shown to be highly positive for HCMV antigenemia (6) and for leukoDNAemia (5), as revealed by PCR. The same patient, however, was repeatedly negative for viremia when tested with MAb 5D2. On the other hand, conventional virus isolation following inoculation of the patient's polymorphonuclear leukocytes onto human embryonic lung fibroblast (HELF) cell monolayers was successful. Subsequent attempts to identify the virus isolate (VR4414) by using MAb 5D2 and the shell vial assay were unsuccessful on multiple sequential cell culture passages.

VR4414 was then analyzed on HCMV-infected HELF cells by an indirect immunofluorescence assay using a panel of six different IEA-specific MAbs (5D2, 6B1, 5B2, 2A1, 1A1, and 2C3) and a MAb (5A11) directed to a late structural protein (7). As shown in Table 1, when identified by indirect immunofluorescence in cell cultures, VR4414, unlike the reference strain AD169, was negative for MAbs 5D2 and 6B1 whereas it was positive, like AD169, for the remaining four IEA-specific MAbs. On the other hand, a MAb to a late viral protein stained both AD169 and VR4414 strains by the conventional identification method (96 h postinoculation) and did not stain the 2 strains by the shell vial method (16 to 24 h postinoculation). Competitive binding assays by the immunoperoxidase technique showed that of the six IEA-specific MAbs tested, two (5D2 and 6B1) were reactive with the same epitope whereas each of the other four was reactive with a different IEA epitope (Table 1). These data suggested that a mutation in the major IE gene could be responsible for the lack of reactivity of MAbs 5D2 and 6B1 with VR4414.

In preliminary experiments, B. Plachter mapped the IE epitope reactive with MAbs 5D2 and 6B1 to an 85-amino-acid stretch encoded by exons 2 and 3 of the major IE gene (11), using procaryotically expressed polypeptides and Western blot (immunoblot) analysis, as already reported for MAb E-13 (10). Moreover, in radioimmunoprecipitation experiments we observed that both MAbs 5D2 and 6B1 reacted with both p72 and p86 proteins (4). It is well-known that, within the major IE gene, differential splicing of exons 1, 2, 3, and 4 and exons 1, 2, 3, and 5 leads to synthesis of IE-1 (p72) and IE-2 (p86) proteins, respectively. Since the translation initiation codon is in exon 2, which, along with exon 3, is shared by the two transcription units IE-1 (1, 18) and IE-2 (19, 20), the IE-1 and IE-2 proteins have the same N-terminal 85-amino-acid sequence encoded by exons 2 and 3 (17).

In order to investigate the molecular basis for the lack of reactivity of MAbs 5D2 and 6B1 with VR4414, the viral isolate was propagated on HELF cell cultures and, in order to avoid sequencing of introns, RNA was extracted from 2×10^5 infected cells, retrotranscribed, and amplified as previously reported (8). PCR was performed with a forward primer, CR1,

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TABLE 1. MAb reactivity with respect to identification in cell cultures and Western blot analysis of HCMV reference strain AD169 and the mutated strain VR4414

MAb	HCMV antigen	IEA epitope	Result for HCMV identification in cell cultures by indirect immunofluorescence assay[a]:				Result for Western blot analysis of Cro–β-Gal IE protein[a]	
			Conventional method (>4 days p.i.[b])		Shell vial method (16 to 24 h p.i.[b])			
			AD169	VR4414	AD169	VR4414	AD169	VR4414
5D2	IE	A	+	−	+	−	+	−
6B1	IE	A	+	−	+	−	+	−
1A1	IE	B	+	+	+	+	+	+
5B2	IE	C	+	+	+	+	−	−
2A1	IE	D	+	+	+	+	−	−
2C3	IE	E	+	+	+	+	−	−
5A11	Late	NA[c]	+	+	−	−	−	−

[a] +, positive reaction; −, negative reaction.
[b] p.i., postinoculation.
[c] NA, not applicable.

localized in exon 1 (nucleotides [nt] 34 to 53), and a reverse primer, CR2, localized in exon 4 (nt 1547 to 1528) (1). Thus, an amplification product covering part of exon 1, all of exons 2 and 3, and part of exon 4 was obtained. As shown in Fig. 1, cDNA was then sequenced by means of the AmpliTaq Cycle Sequencing Kit (Roche Molecular Systems, Alameda, Calif.) according to the manufacturer's instructions by using the oligonucleotide CRP (nt 961 to 980), spanning the initiation codon of the IE gene, and the oligonucleotide CRS (nt 1213 to 1234) as primers.

Comparison of the obtained sequence with those of reference strains AD169 (1) and Towne (18) revealed four nucleotide substitutions in the amplified IE-gene coding region of isolate VR4414. Three of these mutations, affecting codons 40, 49, and 65, caused no amino acid substitutions. Mutation TCC→CCC, in codon 20 of exon 2, caused a serine-to-proline substitution in the corresponding IE proteins (Fig. 2).

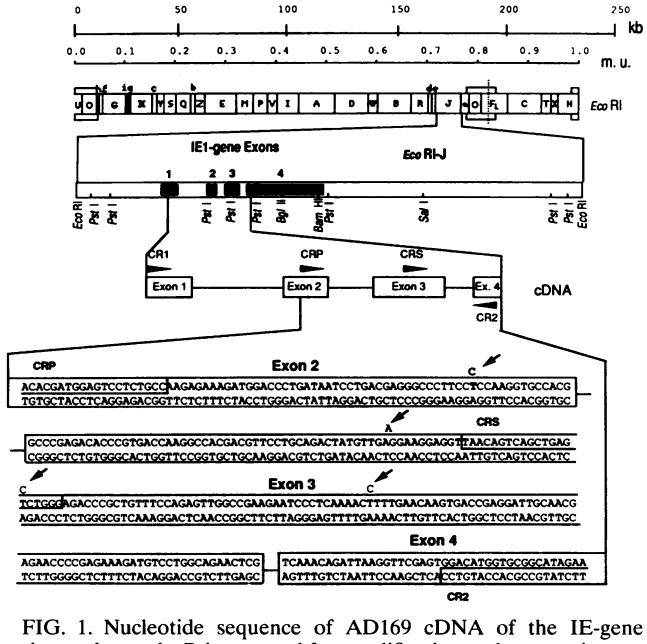

FIG. 1. Nucleotide sequence of AD169 cDNA of the IE-gene region under study. Primers used for amplification and sequencing are indicated by arrowheads in the middle part of the figure (cDNA) and boxed in the cDNA sequence. The four mutations found in VR4414 are indicated by arrows; of these, the significant mutation is indicated on the top row of the sequence in boldface type. m.u., map units; Ex., exon.

In order to verify whether the same mutations were present in vivo, DNA, rather than RNA (because of the difficulty in recovering viral RNA from polymorphonuclear leukocytes), was extracted from 50,000 polymorphonuclear leukocytes of the same AIDS patient and amplified by using the same set of primers (CR1 and CR2). Direct sequencing of PCR products confirmed all four mutations observed in the IE-gene coding region of VR4414, thus excluding the possibility that the mutations arose ex novo during propagation in cell cultures.

Finally, to verify that the detected amino acid substitution was indeed responsible for the lack of reactivity of MAbs 5D2 and 6B1 with HCMV isolate VR4414, a cDNA fragment of 303 bp spanning the coding regions of exon 2, exon 3, and part of exon 4 of the IE gene, and containing the site of the significant nucleotide substitution, was cloned from reference strain AD169 and from viral isolate VR4414 into the bacterial expression vector pEX3 (16). The resulting Cro–β-Gal fusion protein, containing the N-terminal region (amino acids 1 to 99) of the IE protein(s), was then analyzed by Western blotting with MAbs 5D2 and 6B1, along with the other four IEA-specific MAbs (Table 1).

The data obtained showed that MAbs 5D2 and 6B1 were reactive with the fusion protein containing the polypeptide from the wild-type form of the IE gene, whereas they were not reactive with the fusion protein including the amino acid sequence from the mutated IE gene (Fig. 3). Among the other MAbs tested, MAb 1A1 was reactive with both the wild-type and mutated forms of the IE gene products (Fig. 3) whereas the remaining three MAbs (5B2, 2A1, and 2C3) were not reactive with either variant (Table 1). Finally, all six MAbs showed no reactivity with the Cro–β-Gal protein used as a control (Fig. 3).

Our study shows that a nucleotide substitution in codon 20 of exon 2, leading to a serine-to-proline substitution in the relevant IE proteins, is responsible for the lack of reactivity of IEA-specific MAbs 5D2 and 6B1 with VR4414. This amino acid substitution could induce a structural alteration in this region of the protein and, possibly, in the epitope recognized by MAbs 5D2 and 6B1. Computer peptide analysis showed that the region surrounding the mutation is acidic and hydrophilic, is presumably located on the external surface of the

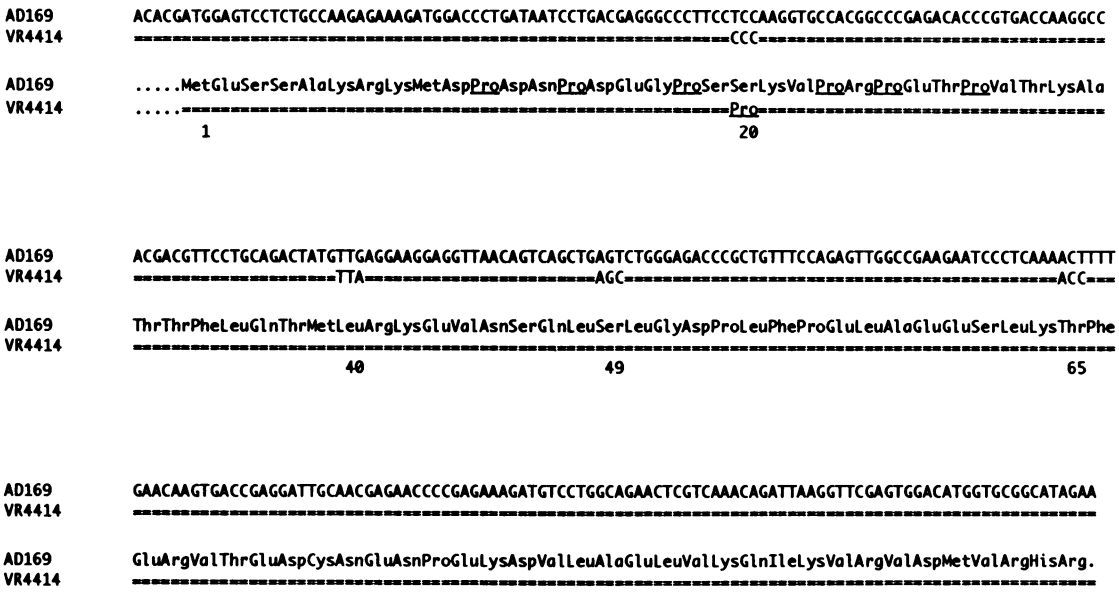

FIG. 2. Comparison of nucleotide (upper line) and amino acid (lower line) sequences of the IE-gene coding region of HCMV reference strain AD169 and isolate VR4414 under study. Numbers indicate the positions of mutated codons. Proline residues surrounding the significant mutation (Ser→Pro, codon 20) are underlined.

protein, and contains neither α-helix nor β-sheet structures. The presence of a large number of proline residues (n = 6) should confer a relative stiffness to this region of the protein. The insertion of a further proline residue could conceivably lead to a relevant structural modification.

Recently, by using MAbs generated against recombinant IE polypeptides, at least 10 different linear epitopes were identified on the IE proteins of HCMV (12). Among these, one was mapped to exon 2-encoded sequence (amino acids 1 to 24 shared by p72 and p86) and is likely to correspond to the epitope recognized by MAbs 5D2 and 6B1. This conclusion seems to be strengthened by the finding that all three of the IEA-specific MAbs most widely used for rapid HCMV identification by the shell vial method (MAb 9221 [DuPont Co., Billerica, Mass.], MAb E-13 [Biosoft, Paris, France], and MAb CH160 [L. Pereira, San Francisco, Calif.]) were reactive with the same exon 2-encoded polypeptide (12).

Thus far, the reported mutation has been detected in a single HCMV strain among more than 500 strains examined in our laboratory (estimated frequency, 0.1 to 0.2%). Commercially available IEA-specific MAbs have been reported to have different degrees of efficiency in staining HCMV isolates. Indeed, MAb 9221 was shown not to recognize 5 of 59 (8.5%) HCMV strains (13), while MAb L14 (14) was reported not to recognize 2 of 21 (9.5%) strains (2). These percentages appear much higher than the estimated percentage of strains missed by our IEA-specific MAb, 5D2.

The reactivity of MAb 1A1 with both the wild-type and mutated forms of the IE gene products was interpreted as due to an epitope other than that recognized by MAbs 5D2 and 6B1. Finally, the lack of reactivity of the remaining three MAbs with both the wild-type and mutated viruses could be due to reactivity either with conformational epitopes or with a region not contained in the cloned fragment.

In conclusion, our results suggest that a pool of IEA-specific MAbs reactive with different epitopes should be used for virus strain identification as well as for determination and quantitation of viremia and that multiple assays, including quantitation of antigenemia and PCR, should preferably be performed for correct monitoring of HCMV infections in AIDS patients.

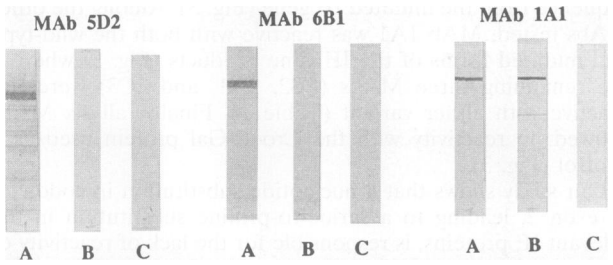

FIG. 3. Western blot analysis of fusion proteins containing the N-terminus region (amino acids 1 to 99) of wild-type strain AD169 (A) and mutated strain VR4414 (B) by using MAbs. Lanes C refer to the Cro–β-Gal protein used as a control. MAbs 5D2 and 6B1 both react only with the wild-type form of the protein, whereas MAb 1A1 reacts with both wild-type and mutated forms of the protein.

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