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A Novel Neutralizing Epitope of Human Cytomegalovirus Glycoprotein M Screened by Phage Display*

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Abstract Human cytomegalovirus glycoprotein complex Ⅱ (gC Ⅱ) consists of two glycoproteins, gM and gN. Although gC Ⅱ specific IgG purified from HCMV positive patient sera can neutralize HCMV, there has been no report on the generation of virus-neutralizing antibodies by immunizing with one epitope of gM. The epitope, termed MAD, was screened from random phage peptide library by subtractive strategy. The peptide sequence of MAD was highly homologous with 32～38 amino acids of HCMV gM. Mice immunized with MAD coupled with keyhole limpet hemocyanin (KLH) could produce specific antibodies against MAD, and the antibodies obtained could bind not only native HCMV particles, but also the recombinant gM30～78 peptide. ELISA analysis results showed that MAD could specifically bind HCMV-positive human serum samples. Virus-neutralizing assay results demonstrated that the antibodies against MAD could inhibit HCMV strain AD169 entering the human embryonic lung cells. The results suggested that MAD could be used as a new potential protective antigen in the development of HCMV vaccine.

Key words human cytomegalovirus, glycoprotein M, neutralizing epitope
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Although human cytomegalovirus (HCMV), a β herpesviridae, commonly causes subclinical infections in healthy adults, HCMV infection remains of considerable importance as a cause of disease in infants infected in utero and immunocompromised populations including patients with acquired immune deficiency syndrome (AIDS) and recipients of organ transplantation[1]. Until now, the treatment of HCMV infection with antiviral agents is far from ideal[2,3], so the development of HCMV vaccine has been placed as one of the higher priorities.

To determine the neutralizing epitopes of the antigens is the pivotal task of developing vaccine. HCMV structural glycoproteins have been proved to contain major antigenic determinants of the virus although they remain incompletely defined. To date three major glycoprotein complexes (designated gC Ⅰ to Ⅲ) have been identified. The gC Ⅰ complex is formed by disulfide-linked homodimeric molecules of glycoprotein B (gB, also called gpUL55)[4]. The gC Ⅱ complex is composed of heterodimeric molecules gM (gpUL100) and gN (gpUL73). The gC Ⅲ complex is constituted by gH (gpUL75), gL (gpUL115), and gO (gpUL74). Previous studies revealed that gB is the major neutralizing antigen[5] which has three important antigen domains (ADs): the extracellular immunodominant antigenic domain 1 (AD-1) is located between aa 552 and 635; AD-2 (aa 50～86) at the N terminus containing two sites, aa 50～54 (site Ⅰ) and aa 67～86 (site Ⅱ); and AD-3 is from aa 798 to 805 at the extreme cytoplasmic C terminus. Of the three domains, only AD-1 and site Ⅱ of AD-2 have been shown to induce virus-neutralizing antibodies[6]. Studies also have validated that a gB-based subunit vaccine could protect individuals from infections of some HCMV isolates[7,8].

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However, gM rather than gB is the most abundant glycoprotein of HCMV envelope glycoproteins[9]. Glycoprotein M is highly conserved among different members of the herpes virus family[10]. Previous studies suggested that the gC Ⅱ complex could serve as a protective antigen for subunit HCMV vaccine [11]. Recently, it is reported that gM or gM/gN complex expressed by eukaryotic expressing vector could induce neutralizing antibodies which could reduce virus infection obviously [12,13]. These results indicate that gM may contain neutralizing epitopes. But until now, there has been no further report about the epitopes of gM.

In this study, we identified a novel epitope of gM, termed MAD, by using a phage-display based subtractive screening strategy. The peptide sequence of MAD was highly homologous with the sequence of amino acids 32～38 of gM. The anti-MAD specific antibodies obtained from mice neutralized virus and inhibited the infection of HCMV virus to HEL cells. Our results suggested that MAD might be used as a new potential protective antigen for developing HCMV vaccine.

1 Materials and methods

1.1 Cells and virus

HCMV strains AD169 was kindly provided by Dr. LI Changgui (National Institute for The Control of Pharmaceutical and Biological Products, China) and propagated in human embryonic lung (HEL) cells (our laboratory stock) as described[11].

1.2 Antibodies and ELISA kit

The goat anti-HCMV polyclonal antibody B65275G was purchased from Biodesign International Inc., USA. The mouse monoclonal antibody MAB8129 against HCMV IEp68 protein was obtained from Chemicon International Inc., USA. HCMV IgG ELISA kit (serial number: 20050917) was purchased from Jingmei Biotechnological Corporation.

1.3 Phage library and screening

The Ph.D.-12™ phage display peptide library kit was the product of New England Biolabs,Inc. The library of 1×10^{11} random peptides was screened for peptides that bound to antibody B65275G by using microtiter plates according to the manufacturer's instructions with modifications. In brief, wells coated with anti-HCMV antibody B65275G were blocked with 3% BSA diluted in phosphate-buffered saline (PBS), and the 1 μl phage peptide library was incubated with normal goat IgG binded on protein A sepharose beads for one hour to remove any non-HCMV-antibody-binding phage before it was added to the antibody B65275G-coated well. The phages were amplified and titrated and DNA sequencing was performed by procedures similar to those described previously[14].

1.4 Peptide synthesis and immunization of Balb/c mice

Peptide (KLSLSNVPPSGSC) was synthesized chemically and purified by HPLC. The purity of the peptide was more than 95% . Then the peptide was conjugated to keyhole limpet hemocyanin (KLH).

Ten female Balb/c mice of 6～8 weeks old were purchased from Beijing Research Center of Animals. Each mouse was injected intraperitoneally with 100 μg peptide-KLH complex for the primary immunization and then 80 μg for the following three boosts. The sera were collected 1 week after the forth immunization.

1.5 ELISA

To determine whether the prepared anti-MAD sera could recognize the native virus particles, ELISA was performed. Microwell plates (Coster) were coated with 100 μl/well of B65275G antibody at concentration of 1 mg/L in PBS overnight at 4℃ and subsequently blocked with 3% BSA for 1 h. Then HCMV strain AD169-infected cell culture filtrates were transferred in duplicated to the coated blocked wells, and incubated for 1 h at 37℃ . The wells were washed 5 times with PBS containing 0.05% Tween-20 (PBST), and 100 μl/well of diluted MAD antibodies was added and incubated for 1 h at 37℃ . After the plates were washed, 100 μl goat-anti-mouse antibody conjugated to horseradish peroxidase (HRP, Pharmacia) at a 1∶8 000 dilution was added to each well to incubation for 1 h. After further washing, the bound viruses were detected with TMB (Sigma).

In order to assess the ability of polyclonal antibody against MAD and B65275G antibody at binding the HCMV particles, Competitive ELISA was performed as follows, 1 mg/L polyclonal antibodies against MAD were coated onto the surface of microwells. After blocking step, changeless concentration of AD169-infective cell culture media containing various concentrations of B65275G antibody (0.1 μg, 0.3 μg, 1.0 μg, 5.0 μg, 10 μg) was transferred and then incubated for 1 h at 37℃ respectively. After washing, 100 μl/well HRP conjugated rabbit-anti-goat antibody at a 1∶8 000 dilution was

added and developed by TMB.

1.6 Preparation of recombinant gM30-78 peptide

1.6.1 Construction of vector expressing gM30～78. gM30～78 gene was obtained by PCR using HCMV genome as DNA template, and then cloned into pET28a vector (Novagen). The gene coding gM30～78 peptide was amplified with primers CMVgM-1(5′ CATGCCATGGGCAGCGTGCATCTAGTGCTGAG 3′) and CMVgM-2 (3′ TAGACTCGGTGCAGTTGGTGCTCGAGCGG 5′). The inserts were digested with restriction enzymes *Nco* Ⅰ and *Xho* Ⅰ and then ligated into the corresponding sites in vector. The correct clones were confirmed by sequencing.

1.6.2 Expression and purification of recombinant gM30～78 peptide. The correct plasmids containing gM30～78 gene were transformed into *E. coli* BL21 (DE3) and induced with 0.1 mmol/L IPTG for 6 h at 30℃. After ultrasonication the supernatant was added to the Ni^{2+} chromatographic column (Farmacia), and gM30～78 peptide was purified and lyophilized according to the manufacturer's instructions.

1.6.3 Western blot. gM30～78 peptides dissolved in PBS were subjected to SDS-PAGE and then blotted onto PVDF membrane (Bio-Rad). The membrane was blocked with 5% defatted milk and incubated with sera from MAD-KLH immunized mice at 1∶100 dilution for 1 h. Subsequently, the membrane was washed with PBST and then reacted with HRP conjugated goat anti-mouse IgG at a 1∶2 000 dilution for 45 min. After the final washing, ECL substrate (Applygene) was applied to the membrane for 2 min. Kodak films were exposed to the membrane and developed.

1.7 Microneutralization assay

The microneutralization assay was conducted as previously described[15]. Briefly, flat bottomed 96-well microtiter plates were seeded with 2×10^4 HEL cells each well. IgG against MAD and control IgG were mixed with $100TCID_{50}$ of AD169 virus and incubated for 1 h at 37℃ respectively. Guinea pig serum was added to samples as a source of complement to a final concentration of 5% (*v*/*v*) for 1 h at 37℃. The virus-IgG mixtures were then incubated with the monolayer for 4 h at 37℃. The mixtures were replaced with fresh medium for 16 h incubation. The infected cells were fixed with absolute ethanol for 20 min followed by re-hydration with PBS (pH 7.2) and reacted for 40 min with 0.1 ml of antibody MAB8129 that is against to the IE protein (IEp68) of HCMV. After washing, 0.1 ml of FITC conjugated goat anti-mouse IgG (ZS Biotechnology, Beijing, China) diluted at 1∶80 in PBS was added and incubated for 40 min. Following counterstaining with 0.02% Evans blue and sealing with 0.2 ml of 30% glycerol in PBS, the plate was observed under a fluorescence microscope. Fluorescence stained nuclei were counted and neutralizing activities were valued.

1.8 MAD epitope reactivity to sera from HCMV-positive individuals

According to the instruction, 40 serum samples collected from healthy individuals from Shandong province were detected by standard HCMV-IgG ELISA kit , and the HCMV-seropositive samples were selected. To further detect the MAD epitope reactivity to HCMV-positive sera, ELISA was performed. Microtiter plates were coated with 100 μl/well MAD at the concentration of 1 mg/L overnight at 4℃ and subsequently blocked with 3% BSA for 1 h. Serapositive samples and negative ones at a 1∶100 dilution was transferred into the wells, and incubated for 1 h with shaking. The wells were washed 5 times with PBST, and 100 μl HRP conjugated goat-anti-human IgG antibody at a 1∶5 000 dilution was added to each well. After 1 h incubation and washing as described above, the bound viruses were detected with TMB.

2 Results

2.1 Screening for HCMV epitope

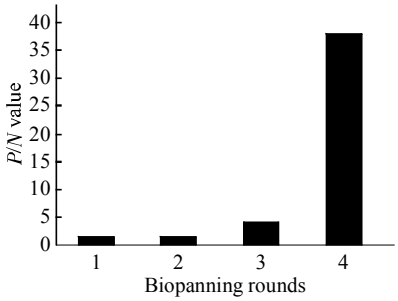
Subtraction by normal goat antibodies, peptides reacting with anti-HCMV antibodies B65275G were isolated after four rounds of panning with a 12-amino-acid random linear peptide library. To select the peptides that bind with a high affinity, the stringency of washing was increased with the successive rounds of panning. Increased numbers of bound phages were detected in the fourth round of panning.

After three rounds of subtractive screening, the specific phage clones binding the anti-HCMV antibodies B65275G were enriched about 40-fold more than those binding the negative control goat antibodies (Figure 1a). The 48 in 60 clones from the round 4 of panning were identified positive to bind anti-HCMV specific antibody B65275G by ELISA analysis.

By DNA sequencing of 20 in 48 positive clones selected from the forth round screening (Figure 1b), fifteen in twenty clones had the same amino acid sequence K-SLSQ/N. However the ELISA values of

peptide K-SLSQ/N-P (clone *1*, *9* and *13*) binding the anti-HCMV antibodies B65275G were higher than the peptide K-SLSQ/N (clone *11*, *20*, *21* and *39*, data not shown). It is indicated that the proline residue had an important role in enhancing the activity of peptide binding antibodies. Moreover, considering the first leucine (L) residue of KLSLSQ/N-P (clone *9*, *13*) appeared 7 times in 20 clones, and the same characteristics of glutamine (Q) with asparagines (N), we regarded the L-LSN-P was the motif sequence. By computer alignment with original sequence of gM, we found that the motif sequence was highly homologous with the sequence of amino-acids 32～38 of gM, that is LVLSNFP.

(a)



(b)

Clone number	Amino acid sequence	Copies
11	NTFVQ K T SLSQ V	×3
9	K L SLSQ V P PSSH	×5
13	K L SLSN V P PSPA	×2
1	H K M SLSQ I P NES	×2
21	NFFSQ K T SLSQ V	×1
20	GPMHA K S SLSQ T	×1
39	ALEKT K T SLSQ S	×1
14	LP K H SVTN L P AF	×1
57	QSHQYT K D SIRQ	×1
28	HH K M SLT*VPIL	×1
10	GHSA* K V SLQ QS	×1
19	SPNHT K M SLS*T	×1

Fig. 1 Selection and enrichment of specific phage clones to antibodies B65275G against HCMV by subtractive biopanning

(a) The phage clones from the forth round were selected and sequenced. (b) Alignment of sequences of phage clones selected from the forth round. The consensus sequence was marked with a single underline.

Although clone *14* and clone *57* had not the motif, they had similar sequence S--N-P and S--Q respectively.

2.2 Anti-MAD antibodies and their binding activity to HCMV

Ten Balb/c mice were given MAD-KLH immunization. Before immunization, sera from each mouse were prepared to be used as negative controls. After about two months' immunization, the titer of anti-MAD antibodies reached 1∶5 000 determined by ELISA (data not shown). Then the antibodies were purified by protein A affinity chromatograph.

The ability of polyclonal antibodies against MAD binding HCMV was confirmed by ELISA. HEL cell culture media containing HCMV particles were incubated with antibodies B65275G which precoated on the wells, then polyclonal antibodies against MAD were added. Our result showed that the antibodies against MAD were shown to specificially bind the native virus (Figure 2). Through competitive ELISA analysis, the ability of the antibodies against MAD binding HCMV particles reduced sharply with the increasing amount of anti-HCMV antibodies B65275G (Figure 3). It is suggested that the MAD polyantibody shares common epitope with B65275G antibody.

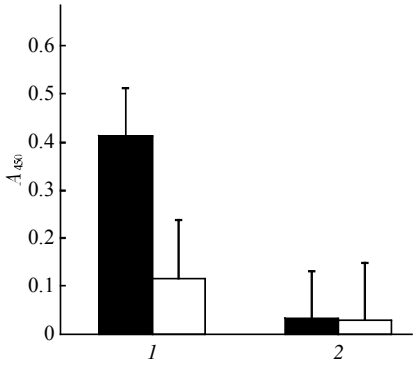


Fig. 2 ELISA result of antibody against MAD binding HCMV particles

All microtiter plate wells were coated with antibody B65275G against HCMV. HEL cells culture medium containing HCMV AD169 and negative control HEL cell culture medium were added to wells respectively and incubated. After washes mice sera against MAD or negative control sera were added and incubated in the same way. ■: Incubated with HCMV AD169. □: Incubated with negative control HEL cell lysates. *1*: Addition mice sera against MAD with 1∶100 dilution. *2*: Addition negative control sera.

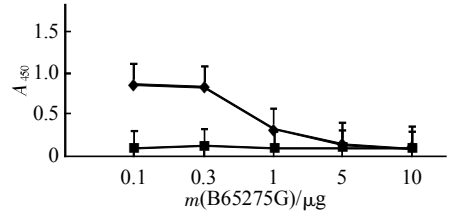


Fig. 3 Competitive-ELISA assay of antibodies against MAD inhibited antibodies B65275G against HCMV binding virus

Polyclonal antibodies against MAD (1 mg/L) were coated onto the surface of microwells. After the blocking step, a constant concentration of AD169-infective cell culture medium was transferred and then incubated with various concentrations of comercial polyclonal antibodies B65275G (0.1 μg, 0.3 μg, 1.0 μg, 5.0 μg, 10 μg) for 1 h at 37℃ respectively. After washes, 100 μl/well HRP conjugated goat-anti-mouse antibody at a 1∶8 000 dilution was added and developed by TMB. ◆—◆: Anti-MAD; ■—■: Negative control.

To test the specificity of the antibody against MAD, the polypeptide correspondence to gM30～78 of HCMV was expressed in *E. coli* and purified. Western blot results showed the recombinant gM30～78 peptide bound not only the antibody against HCMV B65275G, but also the antibody against MAD specifically (data not shown).

2.3 MAD Antibody binding recombinant gM30～78

To test the specificity of the antibody against MAD, the polypeptide correspondence to gM30～78 of HCMV was expressed in *E. coli* and purified. Western blot results showed the recombinant gM30～78 peptide bound not only the antibody against HCMV B65275G (Figure 4a), but also the antibody against MAD (Figure 4b) specifically.

2.4 Neutralizing activities of antibody against MAD to HCMV infected HEL cells

Neutralizing activities of anti-MAD antibodies purified by protein A at its peak level (1 week after the forth immunization) was examined by a microneutralization assay. In this assay, the neutralizing capacity was quantified by determining its capacity to reduce the number of HCMV infected HEL cells. Infection was detected by the expression of the immediate-early gene product with molecular mass 68 ku of HCMV in the nuclei of infected cells 24 h after infection. Result showed that the antibodies against MAD with 1∶8 dilution inhibited about 50% of input virus infectivity as compared with negative control antibodies with 1∶2 dilution purified from mouse or antibodies with 1∶2 dilution purified from goat or PBS control . However the positive control antibodies B65275G with 1∶128 dilution inhibited almost 100% of input virus infectivity (Figure 5).

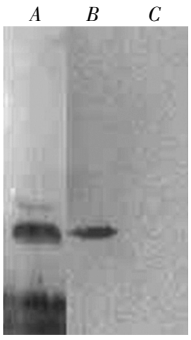


Fig. 4 Mouse polyclonal anti-MAD antibodies binding recombinant gM30～78 determined by Western blot

Recombinant gM30～78 was subjected to SDS-PAGE gel and then assayed by Western blot as described in **Materials and methods**. *A*: The recombinant gM30～78 peptide binding polyclonal antibody B65275G. *B*: The recombinant gM30～78 peptide binding polyclonal antibody against MAD. *C*: Control plasmid pET28 without fusion peptide.

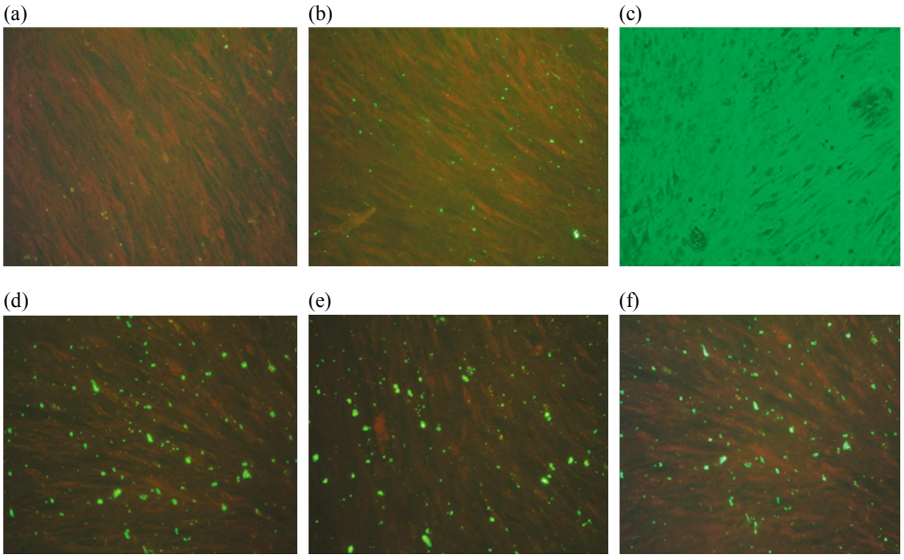


Fig. 5 Neutralization of HCMV AD169 infection to HEL by the standard microneutralization assay

(a) Polyclonal antibody B65275G against HCMV diluted at 1∶128 was pre-incubated with the virus. (b) Polyclonal antibody against MAD with 1∶8 dilution was pre-incubated with the virus. (c) Phase contrast picture of Panel (b). (d) Virus was pre-incubated with the mouse control antibody at dilution of 1∶2. (e) Virus was pre-incubated with the goat control antibody at dilution of 1∶2. (f) PBS control.

2.5 MAD epitope reactivity to sera from HCMV-positive individuals

More than forty sera samples collected from healthy individuals were detected by HCMV-IgG ELISA kit. Compared to negative control, 8 samples were HCMV seropositive. Then the 8 HCMV-

seropositive samples and other HCMV-seronegative ones were transferred into wells precoated with MAD epitope, it was found that 3 in 8 HCMV-seropositive samples could specifically bind the MAD epitope (Table 1). On the other hand, no one in HCMV-seronegative samples binded the MAD epitope (data not shown).

Table 1 MAD epitope reactivity to sera from HCMV-seropositive individuals

Samples	A_{450}	
	Standard kit	MAD
1	0.203±0.02	0.189±0.01
2	0.123±0.03	0.134±0.01
3	0.172±0.01	0.09±0.01
4	0.213±0.05	0.129±0.02
5	0.134±0.03	0.2±0.01
6	**0.701±0.10**	**0.642±0.09**
7	0.205±0.02	0.135±0.03
8	0.132±0.01	0.198±0.04
9	0.2±0.01	0.19±0.01
10	**0.601±0.11**	0.206±0.02
11	**0.749±0.10**	**0.808±0.11**
12	**0.21±0.01**	0.110±0.01
13	0.121±0.02	0.203±0.01
14	0.135±0.04	0.124±0.05
15	0.213±0.01	0.194±0.02
16	0.12±0.08	0.132±0.03
17	**0.628±0.13**	0.17±0.01
18	**0.589±0.10**	0.151±0.03
19	0.07±0.01	0.98±0.02
20	0.152±0.05	0.14±0.05
21	0.168±0.03	0.112±0.08
22	0.08±0.02	0.112±0.01
23	0.23±0.01	0.154±0.04
25	0.167±0.04	0.2±0.02
26	0.147±0.02	0.152±0.02
28	0.173±0.02	0.131±0.09
29	0.159±0.01	0.162±0.01
30	0.18±0.03	0.122±0.02
31	0.087±0.12	0.064±0.09
32	0.052±0.1	0.095±0.12
33	0.147±0.05	0.193±0.01
34	0.051±0.08	0.078±0.01
35	0.181±0.01	0.167±0.03
36	0.209±0.03	0.187±0.02
37	0.156±0.01	0.143±0.05
38	0.193±0.02	0.187±0.04
39	0.132±0.01	0.135±0.02
40	**0.539±0.09**	0.102±0.01
41	**0.801±0.08**	**0.525±0.05**
42	**0.725±0.07**	0.216±0.01

MAD epitope was precoated on wells and to detect the sera samples which has been determined by standard HCMV-IgG ELISA kit. The HCMV-IgG positive samples and MAD-antibody positive ones were all signed with blodface types.

3 Discussion

Anti-HCMV polyclonal antibodies B65275G obtained from goat received native HCMV particles immunization contained many antibodies against specific antigens, including gB[16], gH[17], gM and other membrane proteins. In this study we obtained and identified an epitope of gM, named MAD, by subtractive screened from random phage library using B65275G as the target.

Until now, there is not any epitope of gM that have ever been reported. In this research, through immunized by the epitope MAD, the specific anibodies were obtained successfully. They could specifically bind not only the native HCMV particles, but also the recombinant gM30～78. Since the antibody against MAD shown the ability to neutralize the activity of HCMV to bind their permissible cells, MAD was proved to be a neutralizing epitope.

In order to prove that polyclonal antibody against MAD also exit in the sera from HCMV-seropositive individuals, 40 samples from healthy people were collected and subjected to bind the MAD epitope. ELISA results demonstrated that the MAD epitope could specificially bind part of the HCMV-seropositive samples, and have no cross reaction to any one of the seronegative samples. Our result suggested that some individuals infected with HCMV could generate polyclonal antibodies against the MAD epitope actually and MAD epitope may palys an important role in activation the humoral immune response.

Through subtractive screened from random phage library, the major peptide sequence K-SLSQ/N appeared with a high frequency among the phage clones (15 of 48) that showed reactivity with the goat anti-HCMV antiserum, which indicated that the epitope was a preponderant one. Since the polyclonal antibodies against MAD also exited in the sera from HCMV-seropositive individuals, whether similar results might have been obtained with sera from HCMV-seropositive individuals need further investigated.

Hydrophibicity analysis of gM protein revealed the presence of 6～8 potential transmembrane segments[18]. The sequence of MAD was located at the first extracellular region of gM protein By Blast analysis and DNAMAN software alignment, which suggested that the N terminus of gM protein may have important roles in HCMV vaccine development

although the carboxyl terminus of gM protein had higher hydrophilicity.

To deeply understand the difference of MAD after single residue mutation, some conservative residues were mutated into glycine residue (G) respectively. Once glutamine (Q) residue was mutated, the activity of MAD to bind the antibodies against HCMV was reduced obviously. Otherwise other residues had not the same characteristics (data not shown). The results suggested that the bioactivity of MAD to bind antibodies may be effected by the conformation of glutamine residue.

Previous studies confirmed that anti-gM and anti-gM/gN specific antibodies were able to neutralize multiple laboratory passaged HCMV strains [19]. Considering that gM is the most abundant glycoprotein in HCMV [9, 18], gM may have more poteintial role in HCMV vaccine and could be used as a new protective antigen in the development of HCMV vaccine.

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应用噬菌体展示技术筛选人巨细胞病毒糖蛋白 M 新的中和抗原表位 *

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摘要　人巨细胞病毒(HCMV)糖蛋白复合物Ⅱ包括两种蛋白，即糖蛋白 M(gM)和糖蛋白 N(gN). 尽管来自于 HCMV 阳性病人血清中的糖蛋白复合物Ⅱ的 IgG 抗体能够中和 HCMV 粒子，但迄今为止，还没有 gM 中和性抗原表位的相关研究. 应用消减杂交技术，通过噬菌体肽库筛选获得 gM 抗原的一个表位，即 MAD. MAD 氨基酸序列与 gM 第 32～38 位序列高度同源. 将 MAD 与钥孔血蓝蛋白偶联免疫小鼠可产生抗 MAD 多抗，该多抗不仅结合天然 HCMV 病毒粒子，而且特异结合重组表达的 gM30～78 多肽. ELISA 结果表明 MAD 能够特异结合 HCMV 阳性的病人血清. 病毒中和实验结果进一步证明抗 MAD 多抗能够抑制 HCMV AD169 株病毒感染人胚肺细胞. 总之，MAD 表位有可能成为 HCMV 病毒疫苗潜在的保护性抗原.

关键词　人巨细胞病毒，糖蛋白 M，中和表位
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