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DIRECCIÓN DE NUEVAS CREACIONES**

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Abogado  
**HUMBERTO RUBIO CAMACHO**

|        |            |            |
|--------|------------|------------|
| ASUNTO | Radicación | 09 - 81236 |
|        | Trámite    | 011        |
|        | Evento     | 378        |
|        | Actuación  | 519        |
|        | Oficio     |            |
|        | Folios     | 014        |

**E 14117**

|                         |                                     |                               |                          |
|-------------------------|-------------------------------------|-------------------------------|--------------------------|
| Patente de Invención    | <input checked="" type="checkbox"/> | Patente de Modelo de Utilidad | <input type="checkbox"/> |
| Vía Nacional            | <input type="checkbox"/>            |                               |                          |
| Vía PCT (Fase Nacional) | <input type="checkbox"/>            | Cap. I                        | <input type="checkbox"/> |
|                         |                                     | Cap. II                       | <input type="checkbox"/> |

No. Solicitud Internacional PCT/IB2008/001111  
Fecha de Presentación Internacional 03 enero 2008

Como resultado del Examen de Fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

### 1. Documentos estudiados

|                   |                    |                |
|-------------------|--------------------|----------------|
| Descripción:      | Folios 27 a 69     | 04 agosto 2009 |
| Reivindicaciones: | Folios 70 a 78     | 04 agosto 2009 |
|                   | Folios 120 a 124   | 13 abril 2011  |
| Dibujos:          | Folios 80 a 85, 99 | 04 agosto 2009 |
| Secuencias:       | Folios 86 a 98     | 04 agosto 2009 |
| Prioridad:        | GB 0700133.2       | 04 enero 2007  |

### 2. Análisis de la Prioridad

Se acepta la reivindicación de Prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486), y su contenido técnico corresponde con el de la prioridad reivindicada.

### 3. Objeto de la invención

Título de la solicitud: "Anticuerpos neutralizantes del citomegalovirus humano y su uso"

El problema planteado en esta solicitud consiste en la necesidad de anticuerpos neutralizantes del citomegalovirus humano con alta capacidad neutralizante que permita una menor administración.

Para resolver este problema técnico la solicitud en estudio proporciona un anticuerpo monoclonal neutralizantes del citomegalovirus humano que comprende una región variable de cadena pesada que comprende la secuencia SEQ ID NO 7, SEQ ID NO 17, ó SEQ ID NO 39 y una región variable de cadena ligera que comprende la

secuencia SEQ ID NO 8, SEQ ID NO 18 ó SEQ ID NO 7,

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C12N 15/13; A61K 39/42; A61P 31/22

#### 4. No es una invención (Art 15 D 486 literal (b))

El objeto de las reivindicaciones 15 y 16 no se considera una invención puesto que hace referencia a un anticuerpo humano, de acuerdo con el Art citado.

#### 5. Reivindicaciones relativas a un uso o a un segundo uso (Art 14 y 21 D 486)

El uso de las reivindicaciones 19, 26 a 28 no es patentable, de acuerdo con el Art 14.

#### 6. De la Solicitud de Patente

##### Título

El título hace referencia a el uso de los anticuerpos reclamados, debido a que los usos no son patentables se sugiere al Solicitante hacer la corrección necesaria.

##### Reivindicaciones (Art 30 D 486, Art 22 PCT)

Las reivindicaciones que se refieren a un anticuerpo deben caracterizarlo a partir de su estructura completa, mencionando las secuencias que lo conforman en la **cadena ligera y cadena pesada**, o las secuencias variables de las cadenas ligera y pesada o las secuencias de los 6 CDR's que conforman **ambas cadenas**. La referencia a estructuras parciales del anticuerpo no son válidas puesto que no es una estructura activa, probada ni definida. Se sugiere establecer la caracterización del anticuerpo tal como se aprecia en la reivindicación 14.

Las reivindicaciones 1 a 5, 17 a 19 hacen referencia a la concentración necesaria para neutralizar la infección con hCMV mediante el uso de un anticuerpo, y al hecho de que este se une a un epítipo específico, lo cual no caracteriza adecuadamente el anticuerpo reclamado y corresponden a un resultado a alcanzar puesto que la reivindicación incluiría no sólo la solución propuesta por el solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado. Se sugiere reemplazarlo por las características esenciales, estructurales o funcionales, que contribuyen a la obtención de ese efecto.

En el capítulo reivindicatorio, especialmente las reivindicaciones 6 a 12 existen numerosas reivindicaciones que reclaman un anticuerpo definido a partir de diversas y numerosas opciones de anticuerpo, dependiendo opciones de cadena ligera ó cadena pesada. Estas reivindicaciones dan lugar cada de la combinación de secuencias empleada y pocas de ellas presentan sustento en la descripción se sugiere hacer una generalización razonable de secuencias de acuerdo a lo divulgado en la descripción tales como los anticuerpos con VH SEQ ID NO 7 y VL SEQ ID NO 8.

Las reivindicaciones 6, 11, 12 contienen el término "al menos" el cual es impreciso, por lo cual no permite distinguir la invención del estado de la técnica. Se sugiere sustituirlo por un término preciso o un rango concreto.

La reivindicación 15 se refiere a una denominación particular que se le dio al anticuerpo reclamado, sin embargo esta denominación no hace claro el anticuerpo que desea reclamarse en la reivindicación. Se recuerda al solicitante que las reivindicaciones deben ser claras por sí mismas.

El objeto reclamado en las reivindicaciones 13 y 14 es el mismo se sugiere eliminar alguna de ellas.

Las reivindicaciones 6, 11, 12, 21 contienen la expresión "que tiene una identidad de secuencia de al menos un 85%...", o con otros porcentajes. Dicha expresión debe eliminarse, puesto que no permite establecer a que secuencia se refiere y en el caso de las regiones CDR o variables de un anticuerpo es necesaria esta definición para obtener actividad en un anticuerpo, puesto que el cambio de un solo aminoácido puede alterar la especificidad del mismo. es así que estas no permiten definir la invención ni permiten que pueda ser reproducida por el experto en la materia.

Las reivindicaciones 20, 21 y 23 y dependientes se refieren a polipéptidos. Sin embargo, dado que el problema que resuelve la solicitud es proveer anticuerpos que se unen a hCMV, los polipéptidos aislados no hacen parte de la invención. Lo mismo se aplica para los ácidos nucleicos reclamados. De igual manera no se definen dichos péptidos y ácidos nucleicos por su estructura por tal razón no se encuentran adecuadamente caracterizados.

No se encuentra soporte para composiciones que contengan mas de un anticuerpo dentro de sus componentes tal como se reclama en la reivindicación 25, por tal razón se sugiere eliminar dicha reivindicación.

## **7. Unidad de invención (Art 25 D 486)**

La Solicitud no cumple con el requisito de unidad de invención porque se refiere a 5 Grupos Inventivos los cuales reclaman un anticuerpo definido a partir de diversas opciones de anticuerpo, dependiendo opciones de región variable de cadena ligera ó cadena pesada sin ninguna relación estructural entre si.

Los Grupos Inventivos están definidos por las siguientes características **esenciales**

**Grupo 1:** Reivindicaciones 7 a 15, las cuales corresponden a anticuerpos monoclonales humanos que comprenden las regiones variables de cadena pesada de SEQ ID No. 7 y región variable de cadena ligera SEQ ID No. 8. Designado como anticuerpo 1F11.

**Grupo 2:** Reivindicaciones 7 a 14, las cuales corresponden a anticuerpos monoclonales humanos que comprenden las regiones variables de cadena pesada de SEQ ID No. 17 y región variable de cadena ligera SEQ ID No. 18. Designado como anticuerpo 2F4.

**Grupo 3.** Reivindicaciones 7 a 14, las cuales corresponden a anticuerpos monoclonales humanos que comprenden las regiones variables de cadena pesada de SEQ ID No. 39 y región variable de cadena ligera SEQ ID No. 40. Designado como anticuerpo 5A2.

**Grupo 4.** Reivindicaciones 20 y 21 que corresponden a un ácido nucleico que codifica para cualquiera de los anticuerpos reclamados.

**Grupo 5.** Reivindicaciones 23 y 24 referente a un péptido inmunogénico aislado o purificado que comprende un epítopo que se une al anticuerpo

Se sugiere presentar las Solicitudes divisionales que considere necesarias, ó limitar la solicitud inicial a una sola invención, eliminando de ella las otras invenciones.

**8. Determinación del Estado de la Técnica**

El Estado de la Técnica se determinó con base en la fecha de presentación de la solicitud de prioridad (04 enero 2007) y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

| Ítem | Documento                                      | Título                                                                                            | Fecha de Publicación* |
|------|------------------------------------------------|---------------------------------------------------------------------------------------------------|-----------------------|
| D1   | J. Gen. Virol. (1987), 68, 1457-1461           | Human monoclonal antibodies neutralizing human cytomegalovirus                                    | 28 enero 1987         |
| D2   | PNAS. Vol. 102; No. 50, Pág. 18153-18158       | Human cytomegalovirus virion protein complex required for epithelial and endothelial cell tropism | 21 octubre 2005       |
| D3   | Journal of virological methods 74 (1998) 89-98 | Human monoclonal anti HCMV neutralizing antibody from phage display libraries                     | 28 abril 1998         |

**D1** (folio135) divulga anticuerpos monoclonales humanos anti-HCMV que neutralizan su capacidad virulenta en una concentración entre 0.5 y 0.3ug/ml

**D2** (fólios 136-138) divulga anticuerpos monoclonales anti-hCMV obtenidos a partir de una librería genéticas de fagos que expresan proteínas de dicho virus.

**D3** (fólios 139-140) divulga anticuerpos anti-hCMV los cuales están dirigidos directamente a las proteínas UL-130 y UL131A las cuales son importantes para virulencia del virus.

**9. Examen de Patentabilidad (Art 14 D486)**

**Novedad (Art 16 D486)**

La **reivindicación 1** consiste en “un anticuerpo o un fragmento de unión a antígeno del mismo, que neutraliza una infección de células endoteliales, células epiteliales, células retiniana, células mieloides o células dendríticas por citomegalovirus humano (hCMV), en que la concentración de anticuerpo necesaria para una neutralización del 50% de hCMV es de 0.3ug/ml o menos

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

| Características <b>esenciales</b>                                                                                                                                                                                                    | D1                                                                                                                          | D2                                                                                                                          | D3                                                                                                                          |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------|
| Un anticuerpo o un fragmento de unión a antígeno del mismo, que neutraliza una infección de células endoteliales, células epiteliales, células retiniana, células mieloides o células dendríticas por citomegalovirus humano (hCMV), | Un anticuerpo o un fragmento de unión a antígeno del mismo, que neutraliza una infección por citomegalovirus humano (hCMV), | Un anticuerpo o un fragmento de unión a antígeno del mismo, que neutraliza una infección por citomegalovirus humano (hCMV), | Un anticuerpo o un fragmento de unión a antígeno del mismo, que neutraliza una infección por citomegalovirus humano (hCMV), |

### Frente a D1

Las características **esenciales** del anticuerpo de la reivindicación 1 habían sido divulgadas previamente en D1, el cual enseña anticuerpos monoclonales humanos anti-hCMV (folio ,pagina 1457, resumen) de un peso aproximado de 50 y 130KD que neutralizan una infección por citomegalovirus humano (hCMV), dichos anticuerpos tienen una concentración de neutralización entre un 0,5 y un 0.3 ug/ml. (folio 135, pagina 1459)

En consecuencia, la reivindicación 1 carece de novedad, según lo divulgado en el documento D1.

Las reivindicaciones dependientes 2 a 5, 15 a 19 no mencionan alguna característica que haga nueva a la composición de la reivindicación 1, por lo cual se considera que no son nuevas.

### Frente a D2

Las características **esenciales** anticuerpo de la reivindicación 1 habían sido divulgadas previamente en D2, el cual enseña anticuerpos monoclonales humanos obtenidos a partir de una biblioteca de fagos (folio 136, pagina 89, resumen) los cuales exhiben una actividad neutralizante de 1ug/ml (folio 137, pagina 95, figura 5). Dichos anticuerpos se unen a las proteínas de superficie gpUL55 y gpUL75 del virus las cuales son importantes para la actividad vírica del virus (folio 136, pagina 90, ítem 1).

Asimismo se revela la secuencia de aminoácidos de la región variable de cadena ligera y la región variable de cadena pesada la cual tiene una correspondencia del 95 con las secuencias SEQ ID NO 7 y SEQ ID NO 8 de la presente solicitud (folio 137, pagina 94, figura 3); y la secuencia de nucleótidos que codifica dicho anticuerpo.

En consecuencia, la reivindicación 1 carece de novedad, según lo divulgado en el documento D2.

Las reivindicaciones dependientes 2-3, 6, 11, 12, 15 a 22 no mencionan alguna característica que haga nueva a la composición de la reivindicación 9, por lo cual se considera que no son nuevas.

### Frente a D3

Las características **esenciales** anticuerpo de la reivindicación 1 habían sido divulgadas previamente en D3, el cual enseña anticuerpos monoclonales los cuales exhiben una actividad neutralizante menor de 20ug/ml (folio 139, pagina 18156). Dichos anticuerpos se unen a las proteínas de superficie UL128 y UL130 del virus las cuales son importantes para la actividad vírica del virus (folio 139, pagina 18153, resumen, ítem 1).

En consecuencia, la reivindicación 1 carece de novedad, según lo divulgado en el documento D2.

Las reivindicaciones dependientes 2 a 5 y 15 a 22 no mencionan alguna característica que haga nueva a la composición de la reivindicación 9, por lo cual se considera que no son nuevas.

Nivel Inventivo (Art18 D486)

La **reivindicación 14** consiste en un anticuerpo, o fragmento de unión a antígeno del mismo, en el que el anticuerpo comprende...(folio 122)

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

| Características esenciales                                                      | D1                       | D2                                              |
|---------------------------------------------------------------------------------|--------------------------|-------------------------------------------------|
| Anticuerpo                                                                      | Anticuerpo               | Anticuerpo                                      |
| Región variable de cadena pesada<br>SEQ ID NO 7, ó SEQ ID NO 17, o SEQ ID NO 39 | Anticuerpo de 50 a 130KD | Región variable de cadena pesada<br>SEQ ID NO 1 |
| Región variable de cadena ligera<br>SEQ ID NO 8, ó SEQ ID NO 18, o SEQ ID NO 40 |                          | Región variable de cadena ligera<br>SEQ ID NO 2 |

Los documentos D1 y D2 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 1 porque:

El documento D1 enseña anticuerpos monoclonales humanos anti-hCMV (folio ,pagina 1457, resumen) de un peso aproximado de 50 y 130KD que neutralizan una infección por citomegalovirus humano (hCMV), dichos anticuerpos tienen una concentración de neutralización entre un 0,5 y un 0.3 ug/ml. (folio 135 , pagina 1459)

La diferencia entre la invención, definida en la reivindicación 1 y el anticuerpo divulgado en el documento D1 consiste en que se definen las secuencias de las regiones variables pesada y ligera.

El hecho de especificar las secuencias tanto de la región variable de cadena pesada como la región variable de cadena ligera no evidencia un efecto técnico sorprendente con respecto a los divulgados en D1, puesto que no se presenta neutralización en la mayor concentración (>2ug/ml) para tejidos de tipo fibroblasto con respecto a lo reportado por D1 (0.3ug/ml).

Por lo tanto, el Problema Técnico Objetivo que pretende resolver esta invención se puede formular así: "cómo obtener anticuerpos monoclonales humanos anti hCMV alternativos a los divulgados en D1 con secuencias de las regiones variables pesada y ligera específicas?

Por su lado el documento D2 hace referencia a anticuerpos monoclonales humanos obtenidos a partir de una biblioteca de fagos (folio 136, pagina 89, resumen) los cuales exhiben una actividad neutralizante de 1ug/ml (folio 137, pagina 95, figura 5). Dichos anticuerpos se obtienen luego de obtener 6 clones correspondientes a fagos que contienen el virus hCMV. A partir de dichos clones se escogen las proteínas que influyen en la capacidad vírica y dichas proteínas se establecen como epítopes (folio 138), generándose así un anticuerpo anti-hCMV que presenta la secuencia de aminoácidos de la región variable de cadena ligera y la región variable de cadena pesada la cual tiene una correspondencia del 90% con las secuencias SEQ ID NO 7 y SEQ ID NO 8 de la presente solicitud (folio 137, pagina 94, figura 3); y la secuencia de nucleótidos que codifica dicho anticuerpo.

En consecuencia, la persona del oficio normalmente versada en la materia, que conozca el estado de la técnica en donde se divulga un anticuerpo con actividad neutralizante anti hCMV menor a 0.3ug/ml (documento D1) y tenga acceso a la generación de librerías de genes o fagos que expresen proteínas específicas del virus hCMV , estaría motivado a generar clones, ácidos nucleicos y anticuerpos con una

actividad neutralizante optima de secuencia similar a la divulgada en D2 para llegar así al objeto de la reivindicación 1 de la solicitud. Por lo cual se considera que carece de nivel inventivo.

## 10. Conclusión

Los requisitos de Patentabilidad de la presente Solicitud están afectados así:

| Item | Documento                                      | Reivindicaciones afectadas | Requisito que afecta     |
|------|------------------------------------------------|----------------------------|--------------------------|
| D1   | J. Gen. Virol. (1987), 68, 1457-1461           | 1-24                       | Novedad /Nivel inventivo |
| D2   | PNAS. Vol. 102; No. 50, Pág. 18153-18158       | 1-24                       | Novedad /Nivel inventivo |
| D3   | Journal of virological methods 74 (1998) 89-98 | 1 a 5 y 15 a 22            | Novedad                  |

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante y presentarla por escrito

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.

  
**JOSÉ LUIS SALAZAR LÓPEZ**  
 Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha  
17/4 AGO. 2012

Elaborado por: Ana Delia Pinzón García  
 Revisado por: Consuelo Leguizamón Leguizamón 

# Human Monoclonal Antibodies Neutralizing Human Cytomegalovirus

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(Accepted 28 January 1987)

## SUMMARY

Hybridomas producing human monoclonal antibodies (MAbs) against human cytomegalovirus (CMV) were generated by fusion of human spleen cells and mouse myeloma cells. Two of the six MAbs obtained neutralized viral infectivity even at concentrations lower than 1 µg/ml. One MAh required complement for neutralization but the other did not. Both MAbs recognized viral proteins of *M*<sub>r</sub> 130 000 and 55 000. Furthermore, these neutralizing MAbs bound to the surface membrane of CMV-infected cells. These results suggest that human MAbs may provide a new means of passive immunization against CMV infection in humans.

Cytomegalovirus (CMV) has been associated with significant morbidity and mortality in immunocompromised hosts such as organ transplant recipients (Meyers *et al.*, 1983) and patients with acquired immunodeficiency syndrome (Quinn *et al.*, 1984). Condie & O'Reilly (1984) showed that high doses of human serum immunoglobulin with a high antibody titre to CMV completely protected bone marrow transplant recipients from fatal CMV pneumonia. Their results suggest that if a human monoclonal antibody (MAh) with an even higher antibody titre was available, it would have a potent protective effect against CMV infection. Many laboratories have attempted to develop human MAbs, but to date there has been only marginal success (Cole *et al.*, 1984; Burnett *et al.*, 1985; Sawada *et al.*, 1985). The major obstacle is the difficulty in obtaining human B lymphocytes from individuals highly immunized with a pertinent antigen. To overcome the restriction on human immunization, we recently established a method for efficient generation of hybridomas that produce human MAbs against a predefined antigen (Masuho *et al.*, 1986; Matsumoto *et al.*, 1986). In this study, we describe human MAbs capable of neutralizing CMV infectivity.

Hybridomas were generated by the fusion of human lymphocytes and mouse myeloma cells as described previously (Matsumoto *et al.*, 1986). In brief, human B lymphocytes were obtained from the spleen of a CMV-seropositive patient with idiopathic thrombocytopenic purpura. These were cultured for 6 days with 50 ng protein/ml of inactivated CMV and 1/10 vol. of B cell growth factor (Cellular Products Inc., Buffalo, N.Y., U.S.A.) and fused with mouse myeloma P3-X63-AgU cells. The fused cells were grown in selection medium containing hypoxanthine, aminopterin and thymidine. Four weeks after cell fusion, macroscopic hybridoma colonies appeared in most of the culture wells and 20 to 50% of them were positive for IgG antibody to CMV. We succeeded in cloning six different hybridomas stably producing antibodies by limiting dilution and obtained human MAbs from their culture fluids. All of these MAbs, designated C1, C3, C4, C7, C23 and C41, bound to CMV strains but not to other herpesviruses such as herpes simplex virus and varicella-zoster virus. Their isotypes were found all to be IgG1. C23 had a  $\kappa$  chain and the others had  $\lambda$  chains.

The ability of each MAh to neutralize viral infectivity was determined by measuring the reduction in the number of viral plaques. C23 and C41 neutralized viral infectivity (Fig. 1) but

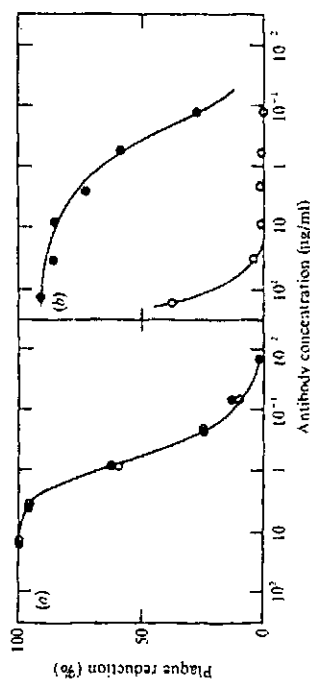


Fig. 1. Dose responses in CMV neutralization by (a) C23 and (b) C41. Reduction of viral plaques (strain AD169) was assessed in the presence (●) and absence (○) of guinea-pig complement by the method described previously (Matsumoto *et al.*, 1986).

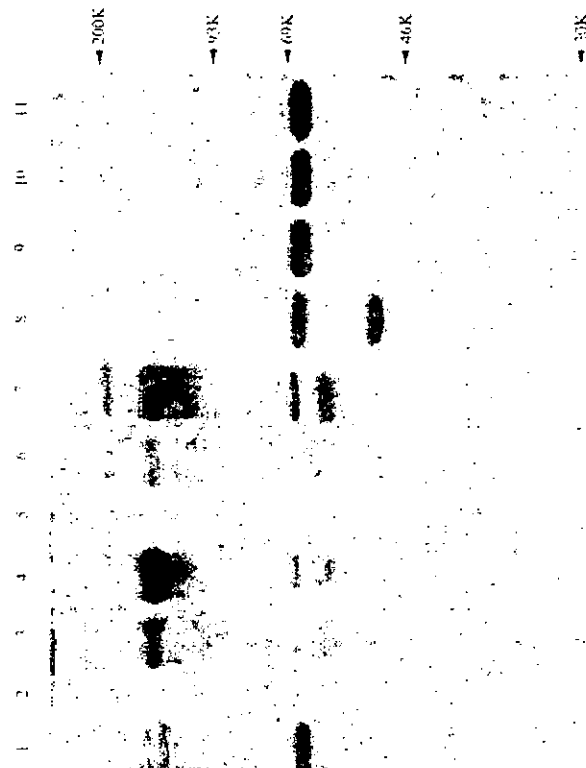


Fig. 2. Autoradiographs of CMV antigens recognized by human MAbs. Human embryonic lung (HEL) cells were infected with the AD169 strain (lanes 1, 4, 7, 8, 9, 10 and 11) or the Hfi-1 strain (lanes 3 and 6), or were mock-infected (lanes 2 and 5); all HEL cells were labelled with [<sup>35</sup>S]methionine. Cell lysates were immunoprecipitated with C23 (lanes 2 to 4), C41 (lanes 5 to 7), C3 (lane 8), C7 (lane 9), C4 (lane 10), C7 (lane 11) and serum from the donor of the lymphocytes used for the hybridomas (lane 1). The precipitated antigens were analysed by SDS-PAGE as described by Laemmli (1970) and autoradiographed with a Kodak X-ray-Omat film.

the other MAbs did not, even at concentrations higher than 75 µg/ml. Addition of complement did not affect the neutralization by C23 (Fig. 1a) but greatly augmented the neutralization by C41 (Fig. 1b). The concentrations of C23 and C41 necessary for 50% reduction in the viral

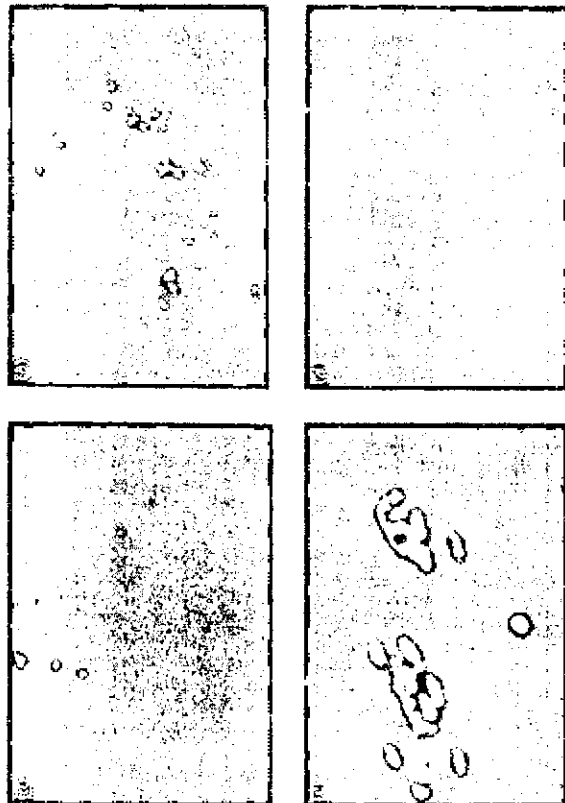


Fig. 3. Photomicrographs of immunofluorescence reactions of human MAbs with CMV-infected cells. There were no differences in the immunofluorescence profiles of the two neutralizing MAbs or the four non-neutralizing MAbs. Photomicrographs for C23 and C41 are shown as the representatives of these two groups. Cells fixed with acetone and methanol (1:1) were allowed to react sequentially with C23 (a) or C41 (b), guinea-pig complement and fluorescein-labelled anti-guinea-pig complement. In the case of unfixed cells, the reactions of C23 (b) and C41 (d) were visualized with fluorescein-labelled anti-human IgG.

Table 1. Neutralization of CMV clinical and laboratory strains by C23 and C41\*

| CMV strain | Tissue culture passage |    | P.f.u. in control | Plaque reduction (%) |     |
|------------|------------------------|----|-------------------|----------------------|-----|
|            | > 100                  | 38 |                   | C23                  | C41 |
| AD169      |                        |    | 42                | 95                   | 83  |
| Hr-1       |                        |    | 150               | 98                   | 99  |
| No. 12     |                        |    | 30                | 95                   | 20  |
| Onoda      |                        |    | 49                | 98                   | 80  |
| Onine      |                        |    | 54                | 94                   | 94  |
| YAN-3      |                        |    | 89                | 97                   | 64  |

\* CMV strains were neutralized with 10 µg/ml of C23 or C41 and guinea-pig complement.

plaque count were 0.5 µg/ml and 0.3 µg/ml, respectively, in the presence of complement. It is known that CMV strains possess different antigenicities (Rasmussen *et al.*, 1984). Thus, the neutralization by C23 and C41 was examined using five clinical CMV isolates (Table 1). C23 reduced the plaque counts of all the tested CMV strains by more than 90%. On the other hand, C41 showed considerably less plaque reduction in the cases of the No. 12 and YAN-3 strains. Even when the concentration of C41 was increased to 50 µg/ml, the plaque counts of the No. 12 strain were reduced by only 20%.

CMV antigens recognized by these human MAbs were determined by immunoprecipitation (Fig. 2). Both of the neutralizing MAbs, C23 and C41, precipitated two proteins of *M<sub>r</sub>* 130000 (130K) and 55K from CMV-infected cell lysates but not from a mock-infected cell lysate. On the

other hand, all four of the non-neutralizing MAbs precipitated a protein of 64K, which may be a tegument protein (Farrar & Oran, 1984; Pereira, 1984). Serum antibodies from the donor whose lymphocytes were used in the production of these MAbs precipitated much larger amounts of 64K protein than of 130K or 55K proteins (Fig. 2, lane 1). These results suggest that the minor antigens of 130K or 55K rather than the major 64K antigen have important epitopes recognized by neutralizing antibodies.

Binding of the human MAbs to CMV-infected cells was examined by an immunofluorescence test described previously (Kettering *et al.*, 1977). Both of the neutralizing MAbs C23 and C41 bound to the surface membrane of infected cells (Fig. 3b). On the other hand, none of the four non-neutralizing MAbs bound to the surface membrane, though they bound to the nuclei and cytoplasm of infected cells (Fig. 3c, d). These results imply that the epitopes on the 130K or 55K proteins recognized by C23 and C41 are exposed on the surface of infected cells and that the epitopes on the 64K protein recognized by the non-neutralizing MAbs are in internal, non-exposed portions of infected cells.

We have described human MAbs capable of neutralizing CMV infectivity. Both C23 and C41 had a very high neutralization activity. From the viewpoint of passive immunization, however, C23 is more useful than C41 because C23 neutralized all the CMV strains tested but C41 did not strongly neutralize some of the strains. These MAbs recognized 130K or 55K antigens, which have an electrophoretic profile almost identical to that of the envelope glycoprotein complex characterized using mouse MAbs (Pereira, 1984; Rasmussen *et al.*, 1985; Britt & Auger, 1986). In addition, C23 and C41 were capable of binding to the surface membrane of infected cells. Therefore, these MAbs may protect a host from CMV infection not only through viral neutralization but also through antibody-dependent cellular cytotoxicity and complement-mediated cytotoxicity. In contrast to the MAbs against 130K or 55K antigens, none of the four MAbs against the 64K antigen had neutralizing activity. Recently, Redmond *et al.* (1986) reported that human MAbs against an antigen similar to our 64K antigen neutralized CMV. This study suggests that these human MAbs capable of neutralizing viral infectivity may provide a new means of passive immunization against CMV infection in immunocompromised patients. Large-scale production of these MAbs is now in progress.

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# Human monoclonal anti-HCMV neutralizing antibody from phage display libraries

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## Abstract

Human cytomegalovirus (HCMV) infection in immunocompromised patients causes considerable morbidity and mortality. Although ganciclovir prophylaxis reduces the incidence of HCMV disease, severe side effects raise serious problems. Thus, the development of new strategies for prophylaxis are clearly needed, and human monoclonal antibodies offer a potential alternative. We describe the cloning, using the phage display system, of a recombinant human Fab fragment against HCMV. A phage display library with  $4 \times 10^6$  clones was panned three times against lysates of HCMV-infected cells, and screened by ELISA. Of six antigen-binding clones, one monoclonal antibody reacted strongly to HCMV. In immunostaining analysis, this Fab was able to stain HCMV-infected cells from 24 h post-infection (pi) through to 96 h pi, but not at 6 h pi. In the presence of cytosine arabinoside, HCMV-infected cells were not stained, even at 24 h pi. These results indicate that an HCMV protein that was recognized by the Fab was synthesized in the late phase of infection. In addition, this Fab exhibited neutralizing activity; at  $1 \mu\text{g/ml}$  it reduced HCMV plaque formation by 50%. The Fab was able to neutralize three HCMV strains, but it did not neutralize HSV-1 or -2 infection. © 1998 Elsevier Science B.V. All rights reserved.

**Keywords:** Human cytomegalovirus; Human monoclonal antibody; Neutralizing antibody; Phage display

## 1. Introduction

Human cytomegalovirus (HCMV) is a common infection and disease in immunocompromised in-

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dividuals, such as allograft recipients and AIDS patients. In allogeneic bone marrow transplantation (BMT), reactivation of latent HCMV infection is a well-documented and a relatively common phenomenon under the immunocompromised conditions caused by receiving immunosuppressive treatment, and it results in morbidity and mortality. In the absence of ganciclovir or acyclovir prophylaxis, approximately half of BMT recipients develop life-threatening HCMV disease. A mortality rate of 30% is associated with HCMV pneumonia, the most common life-threatening HCMV disease (Reed et al., 1988) and 25% of patients with AIDS acquire HCMV chorioretinitis (Callani et al., 1992). Ganciclovir prophylaxis reduces the incidence of HCMV disease directly following BMT, but severe side-effects, such as neutropenia in 30% of patients, and an increase in HCMV disease occurring more than 100 days after BMT, have been observed (Li et al., 1994). High-dose intravenous acyclovir therapy has reduced the incidence of HCMV infection, but more than 25% of BMT recipients still develop HCMV disease (Meyers et al., 1988; Prentice et al., 1994). Consequently, research is now concentrating on clinical prevention and treatment of these late side effects.

Both humoral and cellular responses of the immune pathway are likely to be involved in resistance to HCMV disease. The administration of human immunoglobulins possessing high anti-HCMV neutralizing titer has been used as a prophylactic measure to protect against HCMV disease (Snyderman et al., 1993), but it has been reported that only partial protection is afforded by these antibodies, possibly because neutralizing or other protective antibodies are present only as minor components of the natural immune response. The use of human monoclonal antibody preparations for HCMV prophylaxis may overcome many of the problems inherent in the use of pooled sera.

The virion surface proteins, gpUL55 (gB) and gpUL75 (gL), contain the major neutralization epitopes of HCMV. gB has two neutralization epitopes at its C- and N-terminal portions. By peptide mapping, the C-terminal epitope was shown to consist mainly of amino acids 570–579 and 606–619 (Ohlin et al., 1993). The epitope of the

N-terminal portion of gB is located between amino acid residues 68 and 84 (Ayata et al., 1994). The neutralization epitope of gH is located between amino acid residues 34 and 43 (Urban et al., 1992).

Several technologies have been developed to generate human monoclonal antibodies, such as a hetero-hybridoma (James and Bell, 1987), a chimera antibody (Boutin et al., 1984; Morrison et al., 1984), a humanized antibody (Jones et al., 1986), a knockout mouse carrying the human immunoglobulin genes (Green et al., 1994; Lönberg et al., 1994; Mendez et al., 1997), and a Fab (Barbas et al., 1992) or single chain Fv (Bird et al., 1988; McCafferty et al., 1990) fragment displayed on a filamentous phage. A new technology for the generation of Fab and single chain Fv fragments of human monoclonal antibodies has been developed in recent years which provides an easy and efficient alternative means of selection of diverse human monoclonal antibodies. The production of antigen-specific human monoclonal antibodies generated by the phage display system has been reported (Barbas et al., 1992; Burton et al., 1994; Sanna et al., 1995). In this procedure, cDNA libraries are constructed from human B cells, derived from peripheral blood or bone marrow, by RT-PCR using primers specific for the Fab fragments of antibodies. DNA fragments are cloned into phagemid vectors, Fab fragments are expressed on the surface of a filamentous bacteriophage, and phage antibodies of interest are selected by binding them to specific antigens. The amino acid sequences of the antibodies thus generated are human, making possible frequent use of these antibodies for prophylaxis and therapy against diseases.

The construction and isolation of a human monoclonal anti-HCMV neutralizing antibody produced by using the phage display system are described.

## 2. Materials and methods

### 2.1. RNA preparation and construction of the phage display library

A 10 ml volume of blood was withdrawn from



## 2.4. Purification of Fab antibody

Phagemid clones reacted against HCMV antigens were cultured on a large scale in the presence of 0.1 mM IPTG. *E. coli* DH12S cells were collected by centrifugation and cell pellets were suspended in PBS containing 1 mg/ml lysozyme and Complete. The cells were incubated for 60 min at room temperature and sonicated using a Branson sonifier. Insoluble materials were pelleted by centrifugation and the supernatants were used for further purification. Fab antibody fragments were affinity-purified using goat anti-human Fab<sub>2</sub> antibody (Sigma)-conjugated protein G Sepharose 4 FF (Pharmacia). Eluted Fab fragments were concentrated using a centrifugal concentrator (Centricon 30; cut-off, 12 kDa) and were repeatedly washed with PBS in the concentrator.

## 2.5. DNA sequencing

The heavy and light chains of Fab13-3 were re-cloned into sequencing vectors CV-1 and CV-2, respectively. Nucleic acid sequencing was undertaken with an automated DNA sequencer (LI-COR model 14000), and a fluorescent dideoxynucleotide terminator cycle sequence kit (Thermo Sequase; Amersham). The DNA sequences described in this paper have been deposited in the DNA Data Bank of Japan under the accession numbers AB004303 and AB004304.

## 2.6. Immunofluorescence test

HEp-2 cells were cultured on four-chambered plastic slides (Corning) and infected with HCMV at a multiplicity of infection (MOI) of 0.5 plaque forming units (PFU) per cell. The infected HEp-2 cells were fixed with acetone at 2, 6, 24, 48, 72 and 96 h pi. This was followed by incubation with 10% normal goat serum in PBS for 15 min at room temperature, and then with each purified Fab antibody for 30 min at 37°C. After three washes with PBS, the cells were incubated with 10% goat serum, as described above, and then with a 1:10 dilution of fluores-

cein isothiocyanate-conjugated goat anti-human Fab<sub>2</sub> antibody (Cappel) for 30 min at 37°C. After being washed in PBS the cells were mounted and examined under a confocal laser microscope.

## 2.7. Neutralization tests

Purified Fab antibodies were tested for neutralizing activity against three strains of HCMV: the Towne and AD169 strains, and a clinical isolate, the Tanaka strain (Takekoshi et al., 1987). About 300 PFU of the virus were incubated with serial dilutions of each purified Fab fragment for 1 h at 37°C, and used to infect the HEp-2 cells. After adsorption for 1 h at 37°C, the inoculum was assayed for plaques by counting as described previously (Takekoshi et al., 1987).

## 2.8. Immunoprecipitation of HCMV antigen with Fab antibody

HEp-2 cells infected with HCMV at an MOI of 5 were incubated at 37°C in a CO<sub>2</sub> incubator, labelled with <sup>35</sup>S-methionine for 2 h on day 4 and chased for 2 h. The cells were sonicated and then pelleted by centrifugation to remove the insoluble material. The cell extracts (100 µl) were incubated with 5.0 µg of a purified Fab antibody for 1 h at 37°C. Immune complexes were purified on an anti-human Fab antibody-conjugated Sepharose 4B column, separated by SDS-PAGE and detected by autoradiography.

## 2.9. Cloning and expression of neutralization epitope portions of gB and gH

DNA fragments which included the neutralization epitope portions of gB (encoding amino acid residues 28–93, and 552–633 of gB gene) and gH (encoding amino acid residues 24–94) were amplified by PCR and cloned into the pET32 expression vector (Novagene). Thio-doxin fusion proteins were induced, denatured by guanidine hydrogen chloride and affinity-purified according to the manufacturer's protocol.

## Heavy chain

QVQSPGGLVQPGSLRLCAASGFTVSPNPKVQKQKGLGKLEWYSSGSGTITKDKGRFTTSKDTLSQTLVQMSLPEDIALYCAIETKGGTLYVSSFR2CDR2FR3CDR3

## Light chain

DIETWSPSSLSASVGVDTTTCRASQSTSSSYLMYVQVQVQKGLITVAASSTRAVTPKQFTLSRLPEDEAVYTCQYGGSSMTFCQCTQVKKRCDR1CDR2CDR3

Fig. 3. Amino acid sequences of heavy and light chains of Fab13-3. Amino acid sequences were derived from the DNA sequences of the Fab 13-3 inserts.

## 3. Results

### 3.1. Cloning of an anti-HCMV Fab gene

The mixture of PCR-amplified  $\kappa/\lambda$ -chain products that had been digested by the appropriate restriction enzymes were ligated to the pRPLS<sub>2</sub>/Fab1 phagemid vector (Fig. 2) and introduced into *E. coli* DH12S by electroporation. Titration of the ampicillin-resistant clones indicated that the light chain library contained  $8 \times 10^5$  independent clones. DNAs were extracted from the library and ligated to PCR-amplified  $\gamma$  chain products to generate a phage display Fab library with  $4 \times 10^6$  clones. The library was panned to select for HCMV antigen binding clones. After three rounds of panning, phagemid DNAs were prepared and digested with *NorI*, and the cleaved fragments were ligated to the universal translation terminator sequence, which prevents the Fab molecule from fusing with the gH protein, to make soluble Fab proteins. Phagemid DNAs thus obtained were introduced into *E. coli* DH12S and each clone was tested by ELISA; six clones expressed material bound to the HCMV (Towne strain) late antigens in the ELISA. These six clones were tested using both immunofluorescence and neutralization tests. One of the six clones (Fab13-3) was gave positive results in both assays and was characterized further as described below.

### 3.2. Amino acid sequence analysis

The amino acid sequences of the heavy and light chains, predicted from the DNA sequences of Fab13-3, showed that the heavy chain had high homology to the human IgG1 subclass VH3, and that the light chain was a  $\lambda$ 1 chain (Fig. 3).

### 3.3. Immunofluorescence analysis

To detect the HCMV antigen that was recognized by the Fab13-3 products, HEp-2 cells in the immediate-early, early and late phases after HCMV infection were prepared and examined by an immunofluorescence test. HEp-2 cells infected with HCMV were fixed with acetone at 2, 6, 24, 48, 72 and 96 h pi. The cells were incubated with purified Fab13-3 antibody products and then with fluorescein-conjugated anti-human Fab<sub>2</sub>; goat serum. No fluorescence was observed in uninfected cells (Fig. 4D). In addition, no positive signal was observed either at 24 h pi in infected cells that had been incubated in the presence of cytosine arabinoside (AraC) or at 2 and 6 h pi in cells incubated in the absence of AraC (data not shown). It is well known that AraC inhibits the expression of late genes, but not of immediate-early and early genes, of HCMV. By contrast, cytoplasmic fluorescence appeared at 24 h pi in cells without AraC, and strong staining was observed in the cytoplasm and nuclear membrane at 96 h pi (Fig. 4B). The strength of staining increased with time up to the end of the observation period (96 h pi). These data indicated that the HCMV antigen recognized by the Fab13-3 antibody was not synthesized in the immediate-early and early phases after infection, but was synthesized in the late phase of infection.

### 3.4. Neutralizing activity of Fab13-3

The neutralizing activity associated with Fab13-3 was estimated by both plaque reduction and plaque growth assays. Purified Fab13-3 product exhibited marked neutralizing activity (Fig. 5).

Plaque reduction by the Fab13-3 product was specific for HCMV, for which 50% inhibition was attained at 1 µg/ml Fab, no reduction was observed when the Fab13-3 antibody fragment was tested against HSV-1 or -2 (Fig. 5). This neutralizing ability was also observed with other HCMV strains: Fab13-3 neutralized the AD169 strain and a clinical isolate, Tanaka (Takakoshi et al., 1987), with a similar level of activity as for the Towne strain (data not shown).

In the plaque growth assays (Fig. 6), the cells were stained at 96 h pi and the size and number of plaques was determined by immunofluorescence assay. In this experiment, Fab13-3 was added to the culture medium of HCMV-infected cells. The

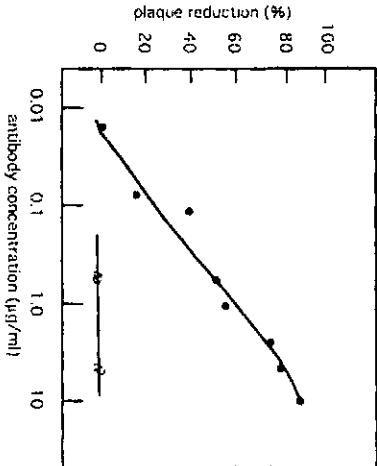


Fig. 5. Neutralizing activity of Fab13-3. Purified Fab13-3 was mixed with virus solution containing approximately 300 p.f.u. of HCMV, Towne strain, and plaque numbers were counted 11 days after plating. Fab13-3 neutralized HCMV infection (●) at 1.0 µg/ml, but did not affect HSV-1 (○) and -2 (□) infection.

number of plaques was lower in the presence of Fab13-3 than in its absence, indicating that re-infection by free viruses was inhibited by Fab13-3. By contrast, plaque size continued to enlarge during incubation by the presence of Fab13-3 indicating that Fab13-3 did not prevent cell-to-cell virus transmission.

3.5. Neutralization epitope

Judging from the specificity of Fab13-3 described above, this antibody recognizes an HCMV protein that is related to neutralization epitopes. To identify the HCMV protein recognized by Fab13-3, Western blotting and immuno-precipitation analyses were performed using <sup>35</sup>S-labelled HCMV-infected HEp-2 cells, but no protein that was reactive for Fab13-3 was identified by these methods. In the next step, we prepared prokaryotic fusion proteins that included the neutralization epitope portions of gB and gH. DNA fragments including each neutralization epitope portion were cloned into the pET32 expression vector. Affinity-purified thioredoxin fusion proteins, denatured by guanidine hydrogen chloride, were subjected to ELISA assays to identify

the epitope recognized by Fab13-3. The results showed that Fab13-3 did not bind specifically to individual fusion proteins, although Fab13-3 reacted with the gH epitope with slightly greater affinity than for the gB epitopes (data not shown).

4. Discussion

A new technology for generating human monoclonal Fab antibodies has been developed recently, based on cloning antibody gene fragments in filamentous phage libraries. This technique has been applied to the isolation of several anti-viral antibodies: anti-HIV gp120 (Barbas et al., 1992), anti-HSV gD (Burton et al., 1994) and anti-HSV gB antibodies (Sanna et al., 1995). Williamson et al. (1993) isolated Fab antibodies specific for

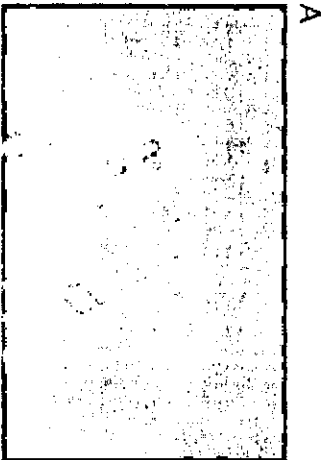


Fig. 6. Effect on virus growth of Fab13-3. HCMV-infected HEp-2 cells were cultured for 96 h in the presence (A) or absence (B) of 10 µg/ml of purified Fab13-3 and stained. Fab13-3 was added every 12 h.

HCMV utilizing the phage display system. Of the 25 clones they examined, some recognized the 65 kDa lower matrix phosphoprotein or the 52 kDa nuclear DNA binding protein, but none efficiently neutralized the activity of the virus. In this study, we isolated one clone, Fab13-3, which recognized the HCMV-infected cells and neutralized the virus. The neutralizing ability of Fab13-3 is broad; three HCMV strains were neutralized at similar levels. In addition, 1 µg/ml Fab13-3 neutralized the HCMV (Fig. 5), suggesting that this Fab antibody is as potent as human monoclonal antibodies produced by hybridoma technology (Masuho et al., 1987; Ohlin et al., 1993). It has been reported that IgG1 of isotypes 1 and 3 are the dominant subclasses formed in response to HCMV infection, of which IgG1 has been found at higher titres (Jasssin et al., 1989). The heavy chain of Fab13-3 belongs to IgG1 subclass VH3 (Fig. 3).

The choice of tissue source is crucial for the preparation of Fab libraries. Bone marrow is the best source of RNA, because it is the richest tissue source of antibody-producing cells (Barbas et al., 1992). Prior boosting has also been reported to be important for isolating antigen-binding Fabs, since boosting elevates not only the number of antibody-producing cells in the peripheral blood, but also the diversity of the antibody clones. To isolate anti-tetanus human Fab antibodies, healthy volunteers were given a booster of antigen and were subjected to leukapheresis of 5 l of blood 3–10 days after boosting (Mallat et al., 1990; Persson et al., 1991). The amounts of total RNA prepared from the tissue sources used in these studies were relatively large, and 10–20 µg of purified mRNA was used to construct the Fab libraries. By contrast, our choice of tissue source restricted yet. We constructed, successfully, a Fab library using a small volume of peripheral blood (10 ml) from a healthy volunteer. In addition, we used total RNA without further purification to synthesize the cDNA, because the final yield of mRNA purified from 10 ml of blood would have been too low for our purpose. When we amplified the cDNA using the standard PCR protocol, we did not obtain any PCR product in most cases, especially those involving the heavy chains. For

this reason, we applied the touchdown protocol (Don et al., 1991) to enhance the annealing efficiency of the primers for the template DNAs. This resulted routinely in yields sufficient to construct Fab libraries with reasonably sized phagemid clones.

To identify the HCMV protein that Fab13-3 recognized, Western blotting and immuno-precipitation analyses using HCMV-infected cells were performed. In addition, fusion proteins that included the neutralization epitope portions of gB and gH were subjected to ELISA assays; however, none of these procedures identified a protein that was reactive for the Fab13-3. This result suggests that Fab13-3 recognizes a conformational, rather than a linear, epitope or that it recognizes an unknown neutralization epitope.

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# Human cytomegalovirus virion protein complex required for epithelial and endothelial cell tropism

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Human cytomegalovirus replicates in many different cell types, including epithelial cells, endothelial cells, and fibroblasts. However, laboratory strains of the virus, many of which were developed as attenuated vaccine candidates by serial passage in fibroblasts, have lost the ability to infect epithelial and endothelial cells. Their growth is restricted primarily to fibroblasts, due to mutations in the UL131-UL128 locus. We now demonstrate that two products of this locus, pUL130 and pUL128, form a complex with gH and gL, but not gO. The AD169 laboratory strain, which lacks a functional UL131 protein, produces virions containing only the gH-gL-gO complex. An epithelial and endothelial cell tropic AD169 variant in which the UL131 ORF has been repaired, termed BADrUL131, produces virions that carry both gH-gL-gO and gH-gL-pUL128-pUL130 complexes. Antibodies against pUL130 and pUL128 block infection of epithelial and endothelial cells by BADrUL131 and the fusion-inducing factor X clinical human cytomegalovirus isolate but do not affect the efficiency with which fibroblasts are infected, glycoprotein complex ( host range

In immunocompromised individuals, human cytomegalovirus (HCMV) infects multiple organ systems, replicating in all major cell types (1). Although clinical isolates replicate in a variety of cell types, laboratory strains, such as AD169 (2) and Towne (3), replicate almost exclusively in fibroblasts (4–10). The restriction in tropism, which results from serial passage of the virus in fibroblasts, is a marker of attenuation (11). Mutations causing the loss of epithelial cell, endothelial cell, polymorphonuclear leukocyte, and dendritic cell tropism in HCMV laboratory strains have been mapped to three ORFs: UL128, UL130, and UL131 (5, 10, 12). Mutation of any one of these ORFs in the V1X clinical isolate of HCMV blocked endothelial cell tropism (5), and repair of a single-nucleotide insertion in the UL131 ORF restored the ability of AD169 to infect endothelial and epithelial cells (10).

Three lines of evidence suggest that the UL131-UL128 proteins are involved in membrane fusion. First, the UL131-UL128 locus mediates the transmission of virus from endothelial cells to leukocytes (5), and the transfer is carried out by transient fusion of plasma membrane patches (13). Second, repair of the defective UL131 ORF in AD169 not only restored its ability to infect endothelial and epithelial cells but also enabled the virus to induce syncytia (10). Third, pUL130 was recently reported to be in virions (14).

Because the UL131-UL128 locus is not sufficient to induce membrane fusion (our unpublished data), we hypothesized that its products might function in cooperation with one or more virus-coded fusogenic glycoproteins. HCMV particles contain three major glycoprotein complexes (15), all of which are required for HCMV infectivity (16, 18). The gC1 complex includes two molecules of the UL55-coded gB (15, 19–20). Each 160-kDa monomer is cleaved (21, 22) to generate a 116-kDa surface unit linked by disulfide bonds to a 55-kDa trans-membrane component. Some antibodies to gB inhibit the attachment of virions to cells, whereas others block the fusion of infected cells (19, 23–26), suggesting that the protein might execute multiple functions at the start of infection. Several

UL131-UL128 locus, base pairs 174865–176806 (44), with a marker cassette containing kanamycin resistance and *LacZ* genes by using linear recombination (48, 49). The BAC-cloned derivative of the VR1814 clinical isolate of HCMV (50) is termed V1X (51), and we term virus reconstituted from that clone B1Xvrf. Virus was prepared by electroporation of BAC DNAs into fibroblasts, and the first passage of the virus was used in this study. Virions were partially purified by centrifugation through a sorbitol cushion for use as virus stocks.

Anti-gB 7–17 (52), anti-gM IMP91-3/1 (20), and anti-gH 14–4b (53) and AF56 (54) monoclonal antibodies were gifts from W. Britt (University of Alabama, Birmingham), rabbit polyclonal anti-gO antibody (35) was a gift from T. Compton (University of Wisconsin, Madison). Murine monoclonal antibodies specific for pUL130 (3E3 and 3C5) and pUL128 (4B10), as well as rabbit anti-pUL128 polyclonal antibody (R551A), were generated by using GST fusion proteins as immunogens.

**Protein Analysis.** For pulse-chase analysis, MRC-5 cells were held for 1 h in medium lacking methionine and cysteine at 37°C after infection at a multiplicity of three plaque-forming units per cell (200 p.f.u./ml [ $1 \text{ C} = 37 \text{ GBq}$ ] of 35S Express Protein Labeling Mix (PerkinElmer) was added for 1 h, and then the radioactivity was removed and cells were maintained for 20 or 120 min in medium with excess unlabeled methionine and cysteine plus 10% FBS. Cells were harvested and lysed in RIPA buffer (50 mM Tris, pH 7.4/150 mM NaCl/1 mM EDTA/1% Nonidet P-40/0.1% SDS/0.5% deoxycholate) containing protease inhibitor mixture (Roche Applied Science, Indianapolis).

Before immunoprecipitation, lysates were incubated with preimmune mouse or rabbit serum overnight at 4°C and then preincubated with protein A Sepharose (Amersham Pharmacia Biosciences) or protein G-agarose (Roche Applied Science) to remove proteins that interact nonspecifically with the beads. Antibodies were added to the precleared lysates, incubated overnight at 4°C, and then protein A Sepharose or protein G-agarose was added for 4 h at 4°C. Immune complexes were collected by centrifugation, washed with RIPA buffer, suspended in reducing sample buffer (50 mM Tris, pH 6.8/10% glycerol/2% SDS/1% 2-mercaptoethanol), boiled for 5 min, and proteins were separated by electrophoresis in SDS-containing polyacrylamide gels. The gM-gN complex was assayed by electrophoresis in urea-containing polyacrylamide gels (30). Sequential immunoprecipitations were conducted as described (55).

For analysis of virion proteins, virions were separated from noninfectious particles by centrifugation through glycerol-lactate gradients (56–58). The purified virions were loaded in reducing or nonreducing sample buffer, and proteins were analyzed by Western blot assay.

**Neutralization Assay.** Anti-pUL130 monoclonal antibodies were purified by affinity chromatography on protein G-agarose. To purify rabbit anti-pUL128 polyclonal antibody, antiserum was first passed through GST-conjugated Sepharose to deplete anti-gST antibodies that resulted from the use of a pUL128-GST fusion protein as immunogen, followed by affinity purification on protein A Sepharose. Neutralization of BADrUL131 was quantified by plaque-reduction assay. Purified antibodies were diluted in DMEM with 5% complement-inactivated FBS and mixed with an equal volume of virus; after 1 h at room temperature, 300- $\mu$ l aliquots (100 plaque-forming units) were used to infect cell monolayers; after adsorption, the inoculum was removed, and cells were overlaid with medium containing 1% agarose; foot of GFP-expressing cells

were counted 2–3 weeks later. Neutralization of V1X virus was quantified by using a microneutralization assay (59).

## Results

**pUL128 and pUL130 Are Present in a Complex with gH.** Products of the UL131–128 locus are required for HCMV replication in endothelial and epithelial cells (5, 10). To test the idea that proteins encoded by this locus might function in cooperation with one or more virus-coded fusogenic glycoproteins, we searched for UL131-UL128-coded proteins in virion glycoprotein complexes. Fibroblasts were infected with three viruses: BADrUL131, an isolate of the AD169 strain of HCMV with a nonfunctional UL131 ORF; BADrUL131–128, a derivative of BADrUL131 that lacks the UL131-UL128 locus; and BADrUL131, a derivative of BADrUL131 with a repaired UL131 ORF (50). At 72 h postinfection, cells were treated for 1 h with 36S-labeled methionine and cysteine, the label was chased for 20 or 120 min, and then virion glycoprotein complexes were examined by immunoprecipitation (Fig. 1).

Consistent with previous reports (20, 22, 53), gB was synthesized and glycosylated to produce a 160-kDa protein at 20 min postlabeling, and it was partially cleaved by 120 min to generate the mature gp55-gp116 gB complex (Fig. 1A). The gM molecule was synthesized as a protein with an apparent molecular weight of 38 kDa, and it was modified by 120 min after its synthesis to migrate as a diffuse 38– to 46-kDa band (30) (Fig. 1B). The ~60-kDa protein that coprecipitated with gM was previously identified as gN (30). No differences in gB or gM-gN were observed among the AD169 variants.

In contrast, gH immunoprecipitates revealed distinct complexes after infection with the different viruses (Fig. 1C). As described (36, 55), gL and gO coprecipitated with gH from fibroblasts infected with all three viruses. In addition, a 16-kDa protein was coprecipitated from extracts of BADrUL131- and BADrUL131-infected cells but not from cells infected with BADrUL131–128. Also, a 33-kDa protein (20 min) and a 33- plus 35-kDa doublet (120 min) were detected in the BADrUL131 gH coprecipitate. Based on their apparent sizes, we suspected that the 16- and 33- to 35-kDa proteins were pUL128 and pUL130. We therefore performed sequential immunoprecipitation assays in which the gH coprecipitating proteins from BADrUL131-infected lysates were coprecipitated with anti-pUL130 or pUL128 antibodies (Fig. 1D Center and Right). The 33- to 35- and 16-kDa proteins were specifically coprecipitated with these antibodies, confirming their identities as pUL130 and pUL128. Antibody to gH also coprecipitated gO from the BADrUL131 lysates (Fig. 1D Left).

**pUL128-pUL130 and gO Form Separate Complexes with gH.** To further verify the gH interactions, we performed immunoprecipitation experiments using a gO-specific antibody (Fig. 2A). This antibody, like the gH antibody, captures the gH-gL-gO complex (55), and the three components were evident in the immunoprecipitates. Neither pUL128 nor pUL130 was coprecipitated at a detectable level with the gO antibody, suggesting that gO and pUL128-pUL130 form separate complexes with gH. Consistent with this interpretation, the pUL128-specific antibody precipitated gH, gL, and pUL130 from BADrUL131 lysates, but gO was not detected (Fig. 2B). In addition, anti-pUL130 antibody coprecipitated gH, gL, and pUL128 from BADrUL131-infected cell lysates, but not gO (Fig. 2C). The identities of gH and pUL130 in the anti-pUL128 immunoprecipitate were confirmed by performing Western blot assays, and once again we did not detect gO (Fig. 2D). Our data argue that pUL128-pUL130 and gO form separate complexes with gH.



