

**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES**

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HUMBERTO RUBIO CAMACHO

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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E. 14117

Patente de Invención ☒

Vía Nacional ☐

Vía PCT (Fase Nacional) ☒

Patente de Modelo de Utilidad ☐

Cap. I ☒

Cap. II ☐

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

Expediente 09-81236 1er Art 45

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 lo 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 termino "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 epítipo 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 esenciales	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 SEQ ID NO 7, ó SEQ ID NO 17, o SEQ ID NO 39	Anticuerpo de 50 a 130KD	Región variable de cadena pesada SEQ ID NO 1
Región variable de cadena ligera SEQ ID NO 8, ó SEQ ID NO 18, o SEQ ID NO 40		Región variable de cadena ligera 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:

Ítem	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 07 de AGO. 2017

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

Human Monoclonal Antibodies Neutralizing Human Cytomegalovirus

By YASUHIKO MASUHO,¹* YOH-ICHI MATSUMOTO,¹
TOHRU SUGANO,¹ SHIGEKI FUJINAGA¹ AND
YOICHI MINAMISHIMA²

¹Teijin Institute for Biomedical Research, Asahigaoka, 4-3-2, Hino-city, Tokyo 191 and
²Department of Microbiology, Miyazaki Medical College, Miyazaki, Japan

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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 MAb required complement for neutralization but the other did not. Both MAbs recognized viral proteins of *M*_r 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 (Quinnan *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 (MAb) 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-AgU1 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 κ chain and the others had λ chains.

The ability of each MAb 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

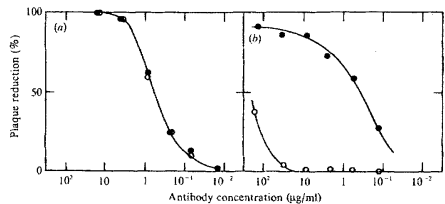


Fig. 1. Dose responses in CMV neutralization by (a) C23 and (b) C41. Reduction of viral plaques (strain AD169) was assessed in the presence (●) and absence (○) of guinea-pig complement by the method described previously (Matsumoto *et al.*, 1986).

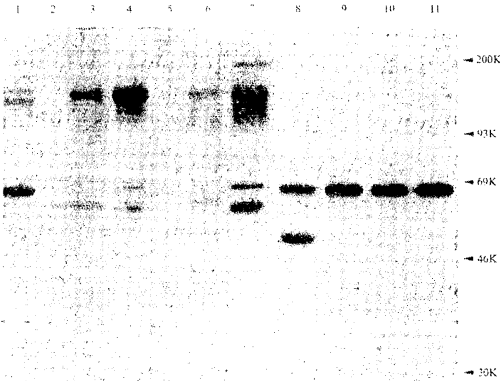


Fig. 2. Autoradiograph of CMV antigens recognized by human MAbs. Human embryonic lung (HEL) cells were infected with the AD169 strain (lanes 1, 4, 7, 8, 9, 10 and 11) or the HI-4 strain (lanes 3 and 6), or were mock-infected (lanes 2 and 5); all HEL cells were labelled with [³⁵S]methionine. Cell lysates were immunoprecipitated with C23 (lanes 2 to 4), C41 (lanes 5 to 7), C1 (lane 8), C3 (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

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 the 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%.

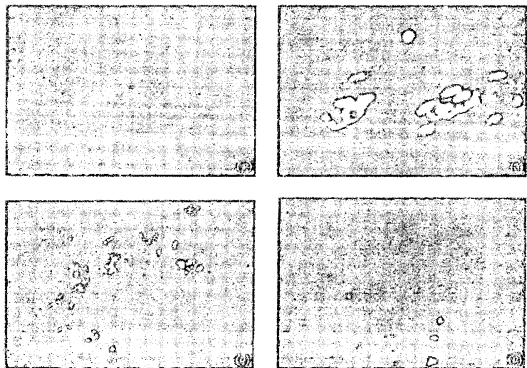
CMV antigens recognized by these human MAbs were determined by immunoprecipitation (Fig. 2). Both of the neutralizing MAbs, C23 and C41, precipitated two proteins of *M*_r 130000 and 130K. Both of the neutralizing cell lysates but not from a mock-infected cell lysate. On the

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 (%)
AD169	> 100	42	95
HI-1	38	150	98
No. 12	8	30	95
Onoda	8	49	98
Omura	6	54	94
YAN-3	5	89	97

* CMV strains were neutralized with 10 µg/ml of C23 or C41 and guinea-pig complement. Phage reduction (%)

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 C3 are shown as the representatives of these two groups. Cells fixed with acetone and methanol (1:1) were allowed to react sequentially with the C23 (a) or C3 (c). Cells fixed with acetone and methanol (1:1) were allowed to react sequentially with the C23 (b) or C3 (d). In the case of C3 (d) were visualized with fluorescein-labelled anti-guinea-pig complement. In the case of uninfected cells, the reactions of C23 (a) and C3 (d) were visualized with fluorescein-labelled anti-human



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other hand, all four of the non-neutralizing MAbs precipitated a protein of 64K, which may be a segment protein (Tarrar & O'Brien, 1984). Serum antibodies from the donors 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 64K protein 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 (Kierling *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 of infected cells (Fig. 3a). 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 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 characterised using mouse MAbs (Tervita, 1984; Rasmussen *et al.*, 1985; Britz & Anger, 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

M. Takekoshi^a, F. Maeda^a, H. Tachibana^b, H. Inoko^a, S. Kato^c, I. Takakura^c,
T. Kenjyo^d, S. Hiraga^e, Y. Ogawa^f, T. Horiki^g, S. Ihara^{a,*}

^a Departments of Molecular Life Science, Tokai University School of Medicine, Isehara, Kanagawa 259-1193, Japan

^b Department of Infectious Diseases, Tokai University School of Medicine, Isehara, Kanagawa 259-1193, Japan

^c Department of Pediatrics, Tokai University School of Medicine, Isehara, Kanagawa 259-1193, Japan

^d Department of Obstetrics and Gynecology, Tokai University School of Medicine, Isehara, Kanagawa 259-1193, Japan

^e Department of Transplantation I, Tokai University School of Medicine, Isehara, Kanagawa 259-1193, Japan

^f Department of Internal Medicine, Tokai University School of Medicine, Isehara, Kanagawa 259-1193, Japan

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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 4×10^6 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 $1 \mu\text{g}/\text{ml}$ 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-

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 (Gallant 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 (Snydman 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 (gH), 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 chimeric antibody (Boulianne 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; Lonberg 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; Burioni 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

* Corresponding author. Tel.: +81 463 93 1121; fax: +81 463 94 8884; e-mail: shira@ris.icu.tokai.ac.jp

Heavy chain 5' primer
Not I
VH1a: AAGGCCCAACCGGCCATGGCCAGGTGACAGTGGTGCAGTCTGG
VH1b: AAGGCCCAACCGGCCATGGCCAGRTY CAGTGGTGCAGTCTGG
VH2a: AAGGCCCAACCGGCCATGGCCAGSTRCAGTGCAGSAGTCRGG
VH3a: AAGGCCCAACCGGCCATGGCCSARGTGCAGTGGTGGAGTCTGG
VH3b: AAGGCCCAACCGGCCATGGCCAGTGTGAGGTGCAGTGTGTGG
VH4c: AAGGCCCAACCGGCCATGGCCAGGTGCAGTACAGSAGTGGGG

Heavy chain 3' primer
Not I
FDG1: CCGGCGCCGCTGTGTGAGTCTTTGTACAAGATTT
FDG2: CCGGCGCCGCTGTGTGAGTCTTTGTGTCCAC
FDG3: CCGGCGCCGCTGTGTGAGTGTGTACAACAAATGG
FDG4: CCGGCGCCGCTGGGGACCATATTGTGACTCAAC

L-chain (κ chain) 5' primer
Nhe I
VK1: CCGCTAGCGMCATY CAGWTGACCCAGTCTCC
VK2a: CCGCTAGCGATRTTGTGATGACYCAGWCTCC
VK3a: CCGCTAGCGAAATTTGTGWTGACCGAGTCTCC
VK4: CCGCTAGCGACATCGWGHGTGACCCAGTCTCC

L-chain (κ chain) 3' primer
Ase I
VKC: TTGGCGCGCTACACTCTCCCTGTTGAAGTCTTT

L-chain (λ chain) 5' primer
Nhe I
VL1a: CCGCTAGCCAGTCTGYSCTGACTCAGCCW
VL1b: CCGCTAGCCAGTCTGTGYTGACGACGCCG
VL2a: CCGCTAGCCAGTCTGTATATGACTCAACCG
VL2b: CCGCTAGCCAGTCTGTGTAATGACTCAGGAG
VL3a: CCGCTAGCTCTATGAGTGTGACTCAGCCA
VL3b: CCGCTAGCTCTTCTGAGTGTGACTCAGGAC

L-chain (λ chain) 3' primer
Ase I
VLC: TTGGCGCGCTGAAMATKCTGTAGSGGCCACTGT

Fig. 1. Primers used for PCR amplification of the human immunoglobulin heavy and light genes. Primer sequences are aligned from 5' to 3'. Restriction sites are underlined. Degenerate nucleotides are indicated as follows: R = A or G, Y = C or T, S = C or G, W = A or T, K = T or G, M = A or C, and H = A or C or T.

an individual with a high serum neutralizing titer against HCMV, and lymphocytes were isolated by centrifugation in Ficoll/Paque (Pharmacia). Total RNAs were prepared using a commercial kit (RNeasy Total RNA Purification kit; Qiagen). Using an RNA PCR KIT (Takara Biochemicals), total RNA was used to synthesize first-strand cDNAs with random nonamers, and DNAs of the antibody Fab portion were amplified in the presence of corresponding 5'- and 3'-specific primers

for the antibody heavy- and light-chain genes (Fig. 1). The VH-CH1 region of the γ heavy chain and the κ and λ light chains were amplified separately. PCR was carried out by the 'touchdown' protocol (Don et al., 1991): 11 cycles of denaturation at 95°C for 1 min, annealing at a temperature decreasing from 65 to 55°C over 1 min, and elongation at 72°C for 2 min. Touchdown PCR was followed by 25 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and

elongation at 72°C for 2 min. Amplified DNA fragments were digested with the appropriate restriction enzymes, and purified by electrophoresis in 2% agarose gels. The relevant DNA bands were excised from the gels and extracted using a commercial kit (Qiaex II Gel Extraction Kit; Qiagen). Purified DNAs were cloned into the pRPLS/Fab1 vector (Fig. 2). In this construction, the heavy chain DNA was fused to the phage gIII gene. Following transformation of *E. coli* DH12S cells by electroporation (Gibco/BRL) the helper phage, VCS M13, was infected and the cells were grown overnight. Antibody Fab fragments, fused to the coat protein gIII protein, were displayed on the phage surface. Phage particles were precipitated by adding PEG to 4% and NaCl to 3%, and the resulting pellet was re-suspended in PBS containing a cocktail of protease inhibitors (Complete; Boehringer-Mannheim). Human embryonic lung (HEL) cells infected with HCMV for 5 days were harvested, extracted in buffer P (20 mM phosphate buffer, pH 6.4, 10 mM NaCl, 1.5 mM MgCl₂, 1% NP-40, 1% sodium deoxycholate and Complete) on ice for 20 min, and sonicated. Insoluble debris was pelleted and the supernatant was used for panning, to select anti-HCMV phage antibodies. ELISA plates Costar 3590 were coated with the extract of the HCMV-infected cells and allowed

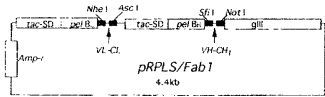


Fig. 2. Plasmid map of the phage display vector, pRPLS/Fab1. The vector has two cloning sites. At the NheI/AseI site and at the AseI/NotI site, immunoglobulin genes are ligated for light and heavy chains, respectively. These restriction enzymes do not cut, or rarely cut, in the immunoglobulin genes. Tac-SD represents the tac promoter Shine-Dalgarno sequence, purchased from Pharmacia Biotech. PelB₁ and pelB₂ were derived from the peptate lyase gene of the *Erwinia carotovora* strains MAFF301629 and MAFF301614, respectively. After the final round of panning, the plasmid DNAs were purified, the NotI site was digested and blunt-ended, and the universal translation terminator sequence (Pharmacia Biotech) was ligated.

to stand overnight at 4°C. Plates were blocked with blocking agent (Block Ace; Dai-Nippon Seiyaku) at 37°C for 1 h. Phage solution was poured onto the plate, and allowed to react for 2 h at room temperature. After washing, the bound phages were eluted with 0.1 N HCl-glycine (pH 2.3) containing 1 mg/ml of bovine serum albumin. These phages were used for reinfection of fresh *E. coli* DH12S. After three rounds of panning, the plasmid DNAs were purified from the bacteria and digested with NotI. The universal translation terminator sequence (Pharmacia Biotech) was inserted upstream of the gIII gene by using a DNA Blunting kit (Takara Biochemicals) to generate a soluble Fab form, by removal of a gIII protein.

2.2. Selection and screening of anti-HCMV neutralizing phage antibodies from the combinatorial library

Ligated DNAs were introduced into electrocompetent *E. coli* DH12S. A number of colonies were selected and Fab antibody fragments were induced by treatment with 0.1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) as previously described (Sanna et al., 1995). The cells were recovered by centrifugation, and re-suspended in PBS containing Complete. Fab antibody fragments were extracted by osmotic shock and sonication, and used for testing on ELISA plates.

2.3. ELISA for Fab

HEL cells infected with the Towae strain of HCMV were harvested 5 days post-infection (pi) and extracted by sonication in buffer P. Fifty microliter aliquots of 10 µg/ml HCMV-infected cell extracts were used to coat ELISA plates. Soluble Fab antibodies were reacted with the cell extracts, which contained HCMV antigens, for 1 h at room temperature. A goat anti-human Fab₂ conjugated to peroxidase (Sigma) was used as the secondary antibody and 3,3',5,5'-tetramethylbenzidine base was used as the substrate. The optical density was determined at 650 nm.

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 F(ab')₂ 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 Sequenase; 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

HIEC 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 HIEC 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 F(ab')₂ 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 HIEC 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

HIEC cells infected with HCMV at an MOI of 5 were incubated at 37°C in a CO₂ incubator, labelled with ³⁵S-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). Thioredoxin fusion proteins were induced, denatured by guanidine hydrogen chloride and affinity-purified according to the manufacturer's protocol.

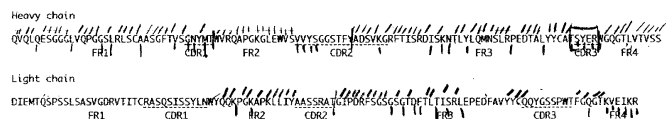


Fig. 3. Amino acid sequences of heavy and light chains of Fab13-3. Amino acid sequences were derived from the DNA sequences of the Fab 13-3 inserts.

3. Results

3.1. Cloning of an anti-HCMV Fab gene

The mixture of PCR-amplified κ/λ -chain products that had been digested by the appropriate restriction enzymes were ligated to the pRPLS/FabI 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 8×10^5 independent clones. DNAs were extracted from the library and ligated to PCR-amplified γ chain products to generate a phage display Fab library with 4×10^6 clones. The library was panned to select for HCMV antigen binding clones. After three rounds of panning, phagemid DNAs were prepared and digested with *Not*I, 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 λ chain (Fig. 3).

3.3. Immunofluorescence analysis

To detect the HCMV antigen that was recognized by the Fab13-3 products, HIEC cells in the immediate-early, early and late phases after HCMV infection were prepared and examined by an immunofluorescence test. HIEC 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 F(ab')₂ 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 μ g/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 (Takekoshi 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

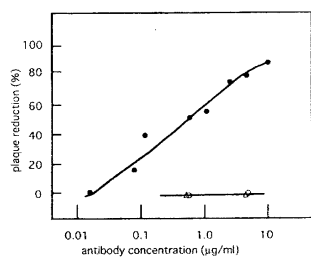


Fig. 5. Neutralizing activity of Fab13-3. Purified Fab13-3 was mixed with virus solution containing approximately 300 PFU of HCMV, Towne strain, and plaque numbers were counted 14 days after plating. Fab13-3 neutralized HCMV infection (●) at 1.0 μ g/ml, but did not affect HSV-1 (○) and -2 (○) infection.

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 ^{35} 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 (Burioni et al., 1994) and anti-HSV gB antibodies (Sanna et al., 1995). Williamson et al. (1993) isolated Fab antibodies specific for

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 μ g/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 IgGs of isotypes 1 and 3 are the dominant subclasses formed in response to HCMV infection, of which IgG1 has been found at higher titers (Joassin 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 (Mullinax 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 μ g 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

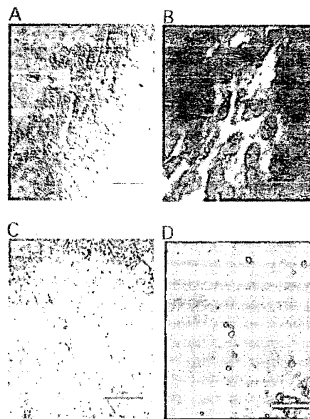


Fig. 4. Immuno-staining of the HCMV infected HEp-2 cells with Fab13-3. At the time indicated, uninfected and infected HEp-2 cells were fixed with acetone and the slides were subjected to indirect immunofluorescence with Fab13-3. The slides were analyzed using both transmission, as well as fluorescence, in the same visual fields under a confocal laser microscope. Infected HEp-2 cells at 96 h pi (A, B), uninfected HEp-2 cells (C, D). Transmission mode (A, C). Fluorescence mode (B, D).

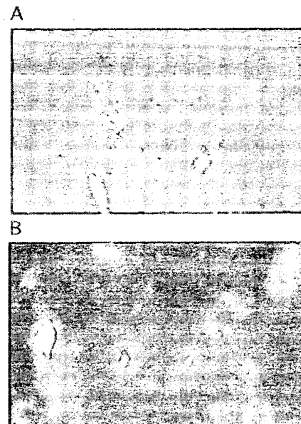


Fig. 6. Effect on virus growth of Fab13-3. HCMV-infected HEp-2 cells were cultured for 96 h in the presence (A) or absence (B) of 10 μ g/ml of purified Fab13-3 and stained. Fab13-3 was added every 12 h.

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

Dai Wang and Thomas Shenk*

Department of Molecular Biology, Princeton University, Princeton, NJ 08544
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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, polysarcomatous, 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 FLX 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 UL25-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 transmembrane 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

cellular membrane proteins interact with gB (27–29), and these interactions likely facilitate entry and activate cellular signaling pathways. The gC11 complex contains the UL100-coded gM and UL73-coded gN (30, 31), and it is the most abundant of the glycoprotein complexes (32). The complex binds to heparan sulfate proteoglycans, suggesting it might contribute to the initial interaction of the virion with the cell surface (33). It also could perform a structural role during virion assembly/envelopment, similar to the gM-gN complex found in some α -herpesviruses (34). The gC111 complex is composed of UL75-coded gH, UL115-coded gL, and UL74-coded gO (15, 35–38). All known herpesviruses encode gH-gL heterodimers (39), which mediate fusion of the virion envelope with the cell membrane. Antibodies to HCMV gH do not affect virus attachment but block penetration and cell-to-cell spread (40, 41). Expression of gH-gL in the absence of infection was sufficient to induce syncytia, and inclusion of gO in the assay did not enhance or block the fusion (42). A gO-deficient mutant of AD169 shows a significant growth defect (18). Recently, it was reported that gH binds to integrin α vB3 (43).

We report here that pUL128 and pUL130 form a complex with gH/gL that is incorporated into virions. Clinical HCMV isolates produce virions containing two gH-gL complexes; one includes gO, and the other includes pUL128-pUL130. The gH-gL-pUL128-pUL130 complex is required to infect endothelial and epithelial cells but not fibroblasts.

Materials and Methods

Biological Materials. Human MRC-5 embryonic lung fibroblasts and ARPE-19 retinal pigmented epithelial cells (American Type Culture Collection) were cultured in DMEM with 10% FBS or DMEM/Ham's F-12 medium (1:1) with 10% FBS, respectively, and both cell types were used at passage 24–30. Human umbilical vein endothelial cells (HUVECs) were obtained by collagenase digestion of umbilical veins grown on gelatin-coated plates in RPMI medium 1640 with 10% FBS, endothelial cell growth supplements (50 μ g/ml, Biomedical Technologies, Stroughton, MA) and heparin (75 μ g/ml) and used at passage 3–6. HUVECs were maintained without endothelial growth supplements or heparin during virus adsorption and after infection. Human foreskin fibroblasts at passage 10–15 were grown in DMEM with 10% newborn calf serum.

The AD169 strain of HCMV (2) contains a frame-shift mutation in UL131 (44, 45). BADw (10, 46) is produced from a bacterial artificial chromosome (BAC) clone of AD169. BADrUL131 (10) is a derivative of BADw in which the UL131 mutation has been repaired so that the ORF is identical to that in the TR HCMV clinical isolate (47). BADrUL131-128 is a derivative of BADw that was constructed by replacing the

Conflict of interest statement: No conflict is declared.

Abbreviations: HCMV, human cytomegalovirus; HUVEC, human umbilical vein endothelial cells; HMF-6A, Human Herpesvirus 6A.

* To whom all correspondence should be addressed. E-mail: tshenk@princeton.edu.

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UL131-UL128 locus, base pairs 174865–176806 (44), with a marker cassette containing kanamycin resistance and *LucZ* genes by using linear recombination (48, 49). The BAC-cloned derivative of the VR1814 clinical isolate of HCMV (58) is termed FIX (51), and we term virus reconstituted from that clone BFXw. 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 IMP41-3/1 (30), and anti-gH 14-4b (53) and AP56 (54) monoclonal antibodies were gifts from W. Britt (University of Alabama, Birmingham), and 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 72 h after infection at a multiplicity of three plaque-forming units per cell; 200 μ Ci/ml (1 Ci = 37 GBq) of ^{35} S 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 precleared with protein A Sepharose (Amersham Pharmacia Bioscience) 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-sucrose gradients (56–58). The purified virions were boiled 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, 200- μ 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; foci of GFP-expressing cells

were counted 2–3 weeks later. Neutralization of FIX 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: BADw, an isolate of the AD169 strain of HCMV with a nonfunctional UL131 ORF; BADrUL131–128, a derivative of BADw that lacks the UL131-UL128 locus; and BADrUL131, a derivative of BADw with a repaired UL131 ORF (10). At 72 h postinfection, cells were treated for 1 h with ^{35} S-labeled methionine and cysteine, the label was chased for 20 or 120 min, and then viral 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 BADw- 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 reprecipitated with anti-pUL130 or pUL128 antibodies (Fig. 1D, Center and Right). The 33- to 35- and 16-kDa proteins were specifically precipitated 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 coimmunoprecipitation 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 precipitated 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.

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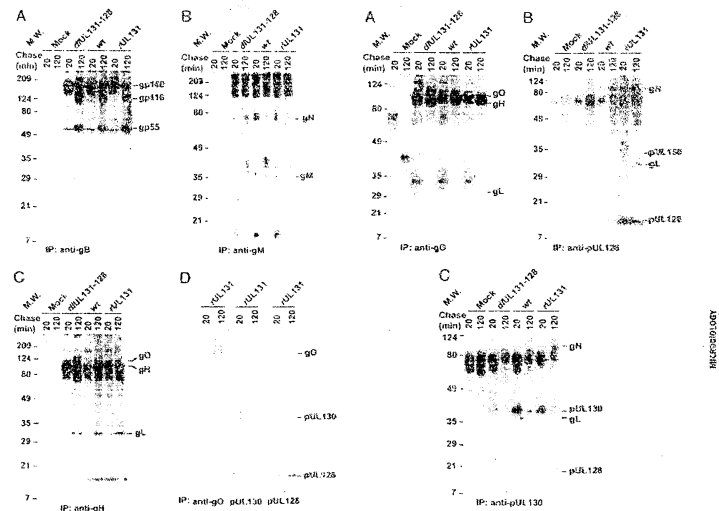


Fig. 1. pUL128 and pUL130 form a complex with gH. MRC-5 cells were infected with BADVUL131-128, BADVUL131-128, or BADVUL131-128. Cells were radiolabeled for 1 h and chased for 20 or 120 min. Proteins were immunoprecipitated (IP) from cell lysates and analyzed by electrophoresis in an SDS-containing 12% polyacrylamide gel followed by autoradiography. Immunoprecipitations used anti-gH (A) or anti-gO (B) (1:100), anti-gH 14-10 (C), or anti-gH 14-10 (D). The positions at which marker proteins migrated are identified by their molecular weights (MW).

pUL128 and pUL130 are present in virions. Because pUL128 and pUL130 are associated with gH in infected cells, the complex might be incorporated into virions. To test this possibility, purified BADV and BADVUL131 virions were assayed for gH, pUL128, and pUL130 (Fig. 3A). As expected, gH was present in both virion preparations. BADVUL131 but not BADV virions contained pUL130, consistent with the failure of pUL130 to interact with gH in a BADV-infected cell lysate (Fig. 1C). Surprisingly, pUL128 also was present only in BADVUL131 virions, even though it interacted with gH independently of pUL130 and pUL131 within extracts of BADV-infected cells (Fig. 1C). We interpret this to indicate that only a complete gH-gL-pUL130-pUL128 complex is incorporated into virions.

To ascertain that pUL128 associates with gH in virions, BADVUL131 virion proteins were immunoprecipitated with pUL128-specific antibody and analyzed by Western blot with anti-gH or anti-pUL130 antibody (Fig. 3B). Both gH and pUL130

Fig. 2. pUL128-pUL130 and gO form separate complexes with gH. MRC-5 cells were infected with BADVUL131-128, BADV, or BADVUL131. Cells were radiolabeled for 1 h and chased for 20 or 120 min beginning at 74 h postinfection. Proteins were immunoprecipitated (IP) from cell lysates and analyzed by electrophoresis in an SDS-containing 12% polyacrylamide gel followed by autoradiography. Immunoprecipitations used anti-gH (A) or anti-gO (B) (1:100), anti-gH 14-10 (C), or anti-gH 14-10 (D). The positions at which marker proteins migrated are identified by their molecular weights (MW).

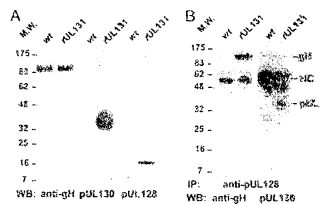


Fig. 3. pUL128 and pUL130 are in virions. (A) BADV and BADVUL131 virion proteins were analyzed by Western blot (WB) by using anti-gH (A) or anti-pUL130 (B) (1:100) antibody. (B) Virion proteins were immunoprecipitated (IP) with anti-pUL128 (B551A) and analyzed by Western blot by using anti-gH (A) or anti-pUL130 (B) antibody. The positions at which marker proteins migrated are identified by their molecular weights (MW). Antibody heavy chains (HC) are designated.

proteins were captured with anti-pUL128 antibody, confirming that the three proteins are complexed in virions as in cell extracts.

Characterization of gH Complexes. Disulfide bonds link gH to gL and gL to gB, gC, gD, and gE. We tested the possibility that gH interacts with pUL128 and pUL130 in the same manner. BADVUL131 virion proteins were resolved by electrophoresis in reducing or nonreducing gels, transferred to membranes, and probed with anti-pUL130 or anti-pUL128 antibodies. As shown in Fig. 4A (left), the reducing agent did not change the mobility of pUL130, suggesting that it is not linked to other proteins through disulfide bonds. In contrast, in the absence of 2-mercaptoethanol treatment, the anti-pUL128 antibody recognized a 125-kDa protein, and treatment with the reducing agent released monomeric pUL128 (Fig. 4A, right). The complex presumably includes gH and gL in addition to pUL128, because both gH and gL were precipitated from extracts of infected cells with pUL128-specific antibody (Fig. 2B). Its 125-kDa size is consistent with the interpretation that

it contains one molecule each of gH (86 kDa), gL (31 kDa), and pUL128 (16 kDa). Because pUL128 and gO form separate disulfide-bonded complexes with gH, we then compared the gH-gL-gO and gH-gL-pUL128 complexes present in BADV and BADVUL131 virions. Under reducing conditions (Fig. 4B, left), only monomeric, 86-kDa gH was observed. However, in the absence of reducing agent, more slowly migrating bands were evident (Fig. 4B, right). 2-mercaptoethanol, which previously represented disulfide-bonded complexes. In BADV, major gH-containing complexes migrated at 100 and 220 kDa. The 220-kDa moiety was previously shown to be a partially modified gH-gL-gO complex (25), and the 100-kDa moiety corresponds to the mature gH-gL-gO (gH) complex (35, 36, 60). An additional gH-containing complex was present in BADVUL131 but not BADV virions. It migrated with an apparent molecular weight of 135 kDa, the same mobility as the complex recognized by antibody to pUL128 (Fig. 4), suggesting that it might be a gH-gL-pUL128 complex. Consistent with earlier reports (36), monomeric gH was observed in the absence of reducing agent in virions, indicating that a portion of it is not covalently bound to other glycoproteins.

We performed additional Western blot assays on the same set of virion samples. As anticipated, gO-specific antibody recognized the 300- and 220-kDa complexes but not the 135-kDa complex (Fig. 4B, center). In contrast, the anti-pUL128 antibody reacted with the 135-kDa moiety but not the larger complexes (Fig. 4B, right). We conclude that two gH complexes are in BADVUL131 virions: gH-gL-pUL128-pUL130 and gH-gL-gO. BADV virions contain only one gH complex, gH-gL-gO.

pUL128 and pUL130 Antibodies Block Infection of Epithelial and Endothelial Cells. BADVUL131 virions, which contain the gH-gL-pUL128-pUL130 complex, can efficiently infect endothelial cells, epithelial cells, and fibroblasts (19). BADV, which lacks the complex, is restricted to fibroblasts. Accordingly, we performed neutralization assays to test the idea that this complex is required to infect epithelial or endothelial cells. Affinity-purified antibodies were used, and no complement was added to the assay. As shown in Fig. 5A, pUL130-specific 3B3 monoclonal antibody inhibited BADVUL131 infection of

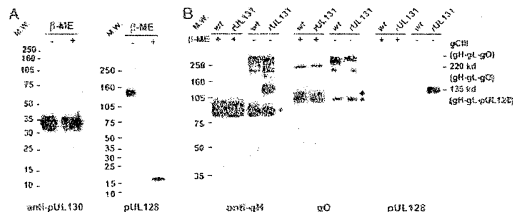


Fig. 4. Characterization of gH-gL complexes. (A) Disulfide linkage of pUL128 with gH. Purified BADVUL131 proteins in buffer with or without 2-mercaptoethanol (2-ME) were subjected to electrophoresis in an SDS-containing 12% polyacrylamide gel and analyzed by Western blot (WB) by using anti-pUL128 (A) or anti-pUL130 (B) (1:100) antibody. (B) Comparison of complexes in BADV and BADVUL131 virions. Virion proteins were separated by electrophoresis in SDS-containing, reducing or nonreducing, 8% polyacrylamide gels and analyzed by Western blot (WB) assay by using anti-gH (C) or anti-pUL128 (D) (1:100) antibody. In (C), pUL128 (100 kDa) antibody + virion (1:100) and (D) pUL130 (100 kDa) antibody + virion (1:100) are designated. The positions at which marker proteins migrated are identified by their molecular weights (MW).

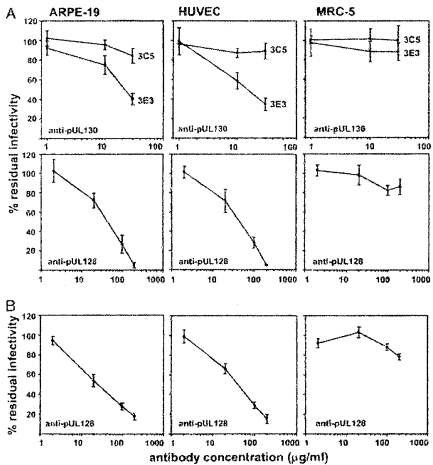


Fig. 5. Neutralization of HCMV infectivity. ARPE-19 epithelial cells, HUVEC endothelial cells, and MRC-5 fibroblasts. BADpUL131 (A) or BFKwt (B) were incubated with various concentrations of anti-pUL130 3C5 or 3E3 or anti-pUL128 8551A antibody, and residual infectivity was determined on different cell types.

ARPE-19 or HUVEC cells but not MRC-5 cells. Fifty percent neutralization was achieved at $\sim 20 \mu\text{g/ml}$ antibody. The 3C5 antibody, which recognizes a different pUL130 epitope (data not shown), did not block infection. Rabbit polyclonal antibody to pUL128 also neutralized the ability of BADpUL131 to infect ARPE-19 and HUVEC cells but not MRC-5 cells. The patterns of inhibition were the same for endothelial and epithelial cells, suggesting that BADpUL131 utilizes the same mechanism to infect the two cell types. The antibody to pUL128 also blocked infection of ARPE-19 and HUVEC cells by the BFKwt clinical strain of HCMV and again did not notably inhibit infection of MRC-5 cells (Fig. 5B).

Discussion

Earlier work demonstrated that the UL130/UL128 locus is a primary determinant of HCMV cell host range (5, 10). Now we have determined that pUL128 and pUL130 are present in a complex with gH and gL (Fig. 1–4). Glycoprotein II is a component of the viral membrane fusion machinery that facilitates viral entry (41, 61, 62), and gL may function as a chaperone-like protein, targeting gH to the cell surface (63). By complexing with gH/gL, pUL128 and pUL130 likely participate in the entry of HCMV into epithelial and endothelial cells. Consistent with this interpretation, antibodies to either protein neutralized the ability of HCMV to infect endothelial or epithelial cells, but not fibroblasts (Fig. 5).

The function of pUL131 is not clear. We anticipated that all three proteins encoded by the UL130/UL128 locus would form a complex, because mutations in any of the three ORFs can abolish

epithelial and endothelial cell tropism (5, 10). However, we did not observe a band potentially corresponding to pUL131 after immunoprecipitation with antibodies to gH (Fig. 1) or pUL128 (Fig. 2). It is conceivable that pUL131 is part of the complex, but its association is not stable to the sample preparation conditions that we used. Alternatively, pUL131 might not be a component of the complex but might serve to stabilize pUL130 or deliver pUL130 to the gH/gL-pUL128 complex, because pUL130 was not detectably incorporated into the complex in pUL131-deficient BADwt-infected fibroblasts (Fig. 1C). It also could play a role in the incorporation of the complex into virions, because the gH/gL-pUL128 complex accumulates in BADwt-infected cells (Fig. 1C) but is not incorporated into virions (Fig. 3).

Two distinct gH complexes were found in BADpUL131 virions: gH/gL-pUL128-pUL130 and gH/gL-gO (Figs. 3 and 4B). In contrast, BADwt virions contain only the gH/gL-gO complex. The different complexes likely form the basis for the host range differences between the two viruses. We propose that, whereas the gH/gL-gO complex is sufficient to mediate infection of fibroblasts, the gH/gL-pUL128-pUL130 complex is required to infect epithelial and endothelial cells.

The HCMV gH complexes resemble the Epstein-Barr virus (EBV) and human herpesvirus 6A (HHV-6A) gH complexes. Like HCMV, EBV virions contain two gH complexes: gH/gL and gH/gL-gp42 (64, 65). EBV uses different gH and gL complexes to infect B and epithelial cells (66). Fusion with B cells is mediated by the gH/gL-gp42 complex, which binds to cell surface B1A class II (67–69). In contrast, entry into an epithelial cell does not require

gp42 but does require interaction of the gH/gL complex with an unknown receptor (64, 66, 70). HHV-6A also produces two gH complexes: gH/gL-gO (71) and gH/gL-gO1-gO2 (72). The HHV-6A gH/gL-gO1-gO2 complex serves as a viral ligand for the entry receptor, CD46 (73–75). HHV-6A, but not HHV-6B, mediates fusion in a variety of cells expressing CD46 (73). The presence of gH/gL-gO1-gO2 on the virion envelope or sequence divergence

of its constituents might be responsible for differences in host cell tropism between HHV-6A and -6B (76).

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