Functional antibody response to human cytomegalovirus in immunocompetent and HIV-1 infected individuals with antibodies that inhibit virus penetration into cells and intercellular transmission of viral infection

J. ALBEROLA, A. TAMARIT and D. NAVARRO

Department of Microbiology, School of Medicine, University of Valencia, Blasco Ibáñez 17, Valencia 46010, Spain

Antibodies mediating post-attachment virus neutralisation (PN), inhibition of human cytomegalovirus (HCMV)-induced cell fusion in the glioblastoma cell line U373 (IF) and global neutralising activity (NA) were quantified in sera from healthy immunocompetent individuals, asymptomatic HIV-1-infected subjects and AIDS patients to further characterise the neutralising antibody response to HCMV in these population groups and to assess whether HIV-1-infected individuals exhibited an abnormal functional antibody profile. PN and IF antibodies accounted for a minor fraction of the NA activity of sera from all population groups. Sera from HIV-1-infected individuals (particularly AIDS patients) displayed higher levels of PN and IF antibodies than those from the healthy control group; however, the relative contribution of these antibodies to the global serum NA activity appeared to be lower in the former individuals than in immunocompetent controls. Serum antibodies preventing HCMV cell-to-cell spread (IP) were then measured to determine whether a specific deficiency could be detected in the HIV-1-infected group population. Serum IP antibody titres were significantly higher in HIV-1-infected individuals (particularly in AIDS patients) than in controls. The potential implications of the data for explaining the pathogenesis of HCMV infection are discussed.

Introduction

Human cytomegalovirus (HCMV) infection is a frequent cause of life-threatening disease in severely immunosuppressed individuals such as patients with advanced AIDS [1]. Although cell-mediated immunity appears to determine control of human cytomegalovirus (HCMV) infection, several lines of experimental evidence also support a role for antibodies in affording protection against HCMV, specifically by preventing virus dissemination [2-6]. The ability of sera to neutralise free virions in vitro has been tentatively considered as a surrogate marker of protection against the virus; nevertheless, a correlation between in-vitro neutralisation, as measured by standard assays, and in-vivo protection has not been proven unequivocally.

Neutralisation of enveloped virions by antibodies in the absence of a source of complement may be accomplished by a wide array of mechanisms including virus aggregation, the inhibition of virion attachment to the cell surface, blocking of fusion between the viral envelope and the target cell membrane, and inhibition of early post-entry events [7]. Standard HCMV neutralisation assays cannot discriminate between these potential modes of antibody action. Knowledge of the relative individual contributions of these mechanisms to the process of HCMV neutralisation in vivo may prove to have relevant implications for pathogenesis, as circulating HCMV virions might not be equally susceptible to all of them in vivo. Thus, protection against HCMV infection might be specifically related to the presence (in sufficient quantity) of a particular type of antibody achieving neutralisation of HCMV virions by a defined mechanism, rather than to the global serum neutralising activity. In addition to neutralisation of free virions, other antiviral activities performed by antibodies, such as the inhibition of cell-
to-cell viral spread might also prove to be important in the immune control of HCMV infection, insofar as HCMV is a highly cell-associated virus, and thus viral spread through tissues may be mostly dependent on cell-to-cell dissemination rather than on cell infection by free virions. An earlier study showed that a number of HCMV gB-specific murine neutralising monoclonal antibodies (MAbs) inhibit HCMV infection by preventing virus entry into cells, while exerting no effect upon virus adsorption to the cell membrane [8]. These MAbs were shown to neutralise HCMV infection of cell surface pre-bound virus and to inhibit HCMV-induced cell fusion in the glioblastoma cell line U373 – a process thought to mimic the fusion event between the viral envelope and cell membrane [8–11]. Furthermore, some of these MAbs were found to interfere with HCMV cell-to-cell spread [8]. It was shown later that antibodies with epitope specificities similar to those of the murine MAbs described are generated after natural infection, suggesting that they may perform similar functions in vivo [12, 13]. Nevertheless, to our knowledge, direct assessment of such functional antibody activities in human sera has not been reported to date.

HIV-infected individuals, particularly those with AIDS, are known to display significantly higher serum neutralising antibody titres than immunocompetent control individuals [5, 8, 14, 15]. The significance of this finding is uncertain, inasmuch as AIDS patients do not appear to be protected against dissemination of HCMV active infections. Whether this functional antibody response is qualitatively similar to that seen in immunocompetent individuals is unknown at present. The current study investigated the contribution of antibodies that neutralise HCMV by blocking virus penetration into cells to the total complement-independent neutralising capacity of sera from HCMV-seropositive healthy subjects and HIV-1-infected patients in varying stages of disease. The objectives were to gain further insight into the mechanisms by which serum antibodies neutralise HCMV infection in vivo, and to determine whether the latter individuals display a particular abnormality in their functional antibody profile in comparison with immunocompetent subjects. In addition, serum antibodies preventing HCMV cell-to-cell spread were quantified in individuals from all study groups to determine whether a particular deficiency could be detected in HIV-1-infected persons. Finally, an indirect estimation of the contribution of antibodies to HCMV gB – a highly immunogenic viral envelope glycoprotein – to the functional antibody activities analysed in this study was made.

Materials and methods

Subjects and samples

Serum samples from 19 CMV seropositive immunocompetent subjects (10 males and 9 females; mean age, 28.7 years; range 19–40), as determined by a commercial microparticle immunoassay (MEIA-Imx, Abbott Diagnostics, Germany), and from 51 HIV-1-infected patients also seropositive for HCMV were used. Among the latter patients, 26 were asymptomatic HIV-1-infected individuals (14 males and 12 females; mean age 38.2 years; mean CD4+ cell counts 464.8 cells/mm$^3$) who were naive for anti-retroviral therapy and 25 were AIDS patients (18 males and 7 females, mean age 33.1 years; mean CD4+ cell counts 37.6 cells/mm$^3$), with no HCMV-related end-organ disease who were being treated with Zidovudine at the time of sampling. Formal consent was obtained from individuals enrolled in the study. Five sera from HCMV seronegative subjects were used as negative controls in the experiments. Sera were heat-inactivated at 56°C for 30 min before use.

Virus and cells

The HCMV AD169 laboratory strain (obtained from the ATCC collection) was used for the experiments reported in this study. Virus was propagated in low-passage human foreskin fibroblasts (HFF) grown in Dulbecco’s modified Eagle’s Minimum Essential Medium (DMEM) supplemented with fetal calf serum (FCS) 10% and antibiotics. A glioblastoma cell line expressing full length gB (U373-gB) [10] was kindly provided by Professor L. Pereira (UCSF) and used for immunofluorescence assays [6]. The parental glioblastoma cell line U373GM (ATCC) was propagated in DMEM and used for the experiments involving the inhibition of HCMV-induced cell fusion by human sera and as control for the immunofluorescence assays.

Virus neutralisation assay

Neutralisation titres (NA) of human sera were determined by a rapid neutralisation assay, basically following a previously published procedure [6, 8]. Briefly, human sera were serially diluted from 1 in 10 to 1 in 1280 in a 125-μl volume of DMEM and mixed with an equal volume of AD169 virus stock containing c. 50 plaque-forming units (pfu). The mixtures were incubated at either 4°C for 2 h or at 37°C for 1 h and then plated on to HFF monolayers (in some experiments glioblastoma U373 cells were used) in 24-well plates. After incubation for 60 min (120 min at 4°C), mixtures were washed off and replaced by fresh DMEM. Then, 48 h later, cells were fixed and stained with an IE-1 specific MAb (Chemicon International, Temecula, CA, USA) at 1 in 6000 dilution in phosphate-buffered saline (PBS). Fluorescent nuclei were counted and the neutralising titre was defined as the highest serum dilution that reduced infectivity of the input virus by 50%. Neutralisation assays were performed twice in duplicate.

Post-attachment virus neutralisation assay

These experiments were performed basically following a previously described procedure [8]. Briefly, HFF cells
(in some experiments glioblastoma U373 cells were used) grown on 24-well plates were infected with c. 50 pfu/well of HCMV AD169 in DMEM with FCS 2%. After adsorption for 2 h at 4°C, the infected cell monolayers were washed twice with DMEM without serum. Serially diluted human sera (in DMEM) (1 in 10–1 in 1280) were then added (250 µl total volume) and plates were further incubated for 2 h at 4°C. Cell monolayers were washed twice with DMEM and then incubated for 48 h at 37°C. Subsequently, cells were fixed and stained with an IE-1 specific MAb (Chemi-con International) at 1 in 6000 dilution in PBS. Fluorescent nuclei were counted and the post-attachment neutralising antibody (PN) titre was defined as the highest serum dilution that reduced infectivity of the input virus by 50%. Post-attachment neutralisation assays were performed twice and each serum was run in duplicate wells.

**Inhibition of virus-induced cell fusion assay**

These experiments were performed as described previously [8], with some modifications. Briefly, U373 glioblastoma cells were grown to 70% confluence in 96-well plates and then infected with HCMV AD169 (to yield c. 50 nuclei-fluorescent foci per well when stained with IE-1 MAb in DMEM with FCS 1% at 37°C). Cells were then washed twice with DMEM without serum and incubated for 18 h at 37°C. Serially diluted human sera (1 in 10–1 in 1280) was added to cell monolayers in a 250-µl volume of DMEM with FCS 2% and replenished daily for a further 72 h. Finally, cells were fixed with methanol 70%, dried and stained with Giemsa. Plates were observed by light microscopy and the syncytia in each well were counted (syncytia were considered to be cells containing five or more nuclei). The inhibition of HCMV-induced cell fusion antibody (IF) titre was defined as the highest serum dilution that reduced the number of syncytia by 50%. In a typical experiment, virus control wells contained an average of 30–40 syncytia, while cell control wells (uninfected) contained an average of 30–40 syncytia, while cell control wells (uninfected) contained an average of 30–40 syncytia. Each serum dilution was tested in duplicate wells in two different experiments.

**Inhibition of virus cell-to-cell spread assay**

The ability of sera to block cell-to-cell spread of HCMV was tested as described previously [8]. Confluent HFF cell monolayers grown in 24-well plates were infected with HCMV (50 pfu/well) and allowed to adsorb for 1 h at 37°C. Cells were washed in DMEM without serum and fresh DMEM with FCS 10% was added. At 18 h post-infection, serially diluted human serum (1 in 10–1 in 1280) was added to wells in DMEM with FCS 2% and plates were incubated at 37°C. Medium containing the appropriate dilution of serum was replenished every 48 h until plaques (infectious foci) were fully developed (12–15 days after infection). The titre of antibodies that blocked spreading of HCMV from cell-to-cell (IP) was defined as the highest dilution that achieved reduction of plaque size by c. 50% (read visually) – which roughly corresponded to a 50% reduction of the number of cells forming the plaque. At this time control plaques involved 15–30 infected cells. Only the largest 10 plaques in each well were scored. Experiments were performed twice.

**Quantification of anti-HCMV gB antibodies**

Serum anti-HCMV gB antibodies were measured by an immunofluorescence assay with the cell line U373-gB as antigen source [10], as described previously [6].

**Data analysis**

Comparison of raw data (titres) was performed by the non-parametric Mann–Whitney U-test with the assistance of commercially available software (Instat, San Diego, CA, USA). In this test, the average ranks of two independent samples are compared statistically. Two-tailed p values are given throughout the text. Two-tailed p values of <0.05 were considered to be of statistical significance. Correlations between raw values of different parameters were calculated by the Spearman rank correlation test.

**Results**

**Quantification of antibodies that neutralised infectivity of cell surface pre-bound virions**

An earlier study optimised a biological assay for testing the ability of murine neutralising MAbs to prevent infection by HCMV virions pre-attached to the cell surface (post-attachment neutralising activity, PN) [8]. In this assay, HCMV virions are incubated with cell monolayers at 4°C – a temperature at which virions bind to the cell surface but do not penetrate the cells – and later exposed to human sera before shifting the temperature of infected cell monolayers to 37°C to allow virus entry into the cells. In the first set of experiments the levels of PN antibodies in sera from both immunocompetent HCMV seropositive individuals and HIV-1-infected patients were quantified and correlated with those measured by a standard neutralisation assay (NA). To make both neutralisation assays comparable, virus-antibody incubations were done at 4°C in the NA assay. PN antibody titres correlated significantly with those measured by the NA assay (overall correlation, \( r = 0.546; 95\% \text{ CI: } 0.3463–0.6934; p < 0.0001 \)); however, the median PN antibody titre was significantly lower than the median NA antibody titre in both healthy control subjects (\( p = 0.0460 \)) and overall in HIV-1-infected patients (\( p < 0.0001 \)). Six of 19 sera from immunocompetent controls displayed identical PN and NA antibody levels. In the remaining sera, PN antibody titres were
2–8-fold lower than NA antibody titres. Only three sera from HIV-1-infected asymptomatic individuals and one serum from a patient with AIDS showed equivalent PN and NA antibody levels. Sera from the rest of these patients had NA antibody titres 2–16-fold higher than PN antibody titres. As shown in Table 1, PN antibodies were more abundant in sera from both asymptomatic HIV-1-infected subjects and AIDS patients than in sera from immunocompetent controls – although statistical significance was not reached in either case (Table 2). As can be inferred from Table 1, the relative contribution of PN antibodies to the global neutralising activity of sera (NA) was lower in HIV-1-infected asymptomatic individuals and AIDS patients than in healthy controls (compare mean PN and NA titres).

Quantification of antibodies that prevent HCMV-induced cell fusion in glioblastoma U373 cells

Data from the PN experiments demonstrated that a fraction of antibodies contained in sera from both immunocompetent and HIV-infected patients neutralised HCMV virions pre-bound to the cell surface; however, post-attachment virus neutralisation may potentially be accomplished by several mechanisms [7]. To delineate the mode of action of these antibodies, the capability of sera to inhibit HCMV-induced fusion of the glioblastoma U373 cells (IF) – a process thought to mimic that occurring naturally when the viral envelope fuses with the target cell membrane [8–11] – was studied. To make a comparison between IF and neutralisation antibody titres (both PN and NA) feasible, the viral input in the IF experiments was adjusted to achieve a degree of cell monolayer infection similar to that observed in the control wells of virus neutralisation experiments with HFF cells. Furthermore, pilot experiments with a number of sera indicated that the use of glioblastoma cells instead of HFF cells for neutralisation assays exerted no influence on the NA and PN activities of sera (data not shown). A typical HCMV-induced syncytium developed in the presence of an HCMV seronegative serum is shown in Fig. 1A. The effect of serum displaying a strong anti-HCMV-induced fusion activity (complete abrogation of syncytia development) is also shown (Fig. 1B). A significant correlation was found between IF and NA antibody titres (overall correlation, \( r = 0.4413; 95\%\ CI: 0.2234–0.6172; p = 0.0001 \)). Sera from six control individuals, four asymptomatic HIV-1-infected subjects and three AIDS patients exhibited identical IF and NA antibody levels. The remaining sera displayed IF antibody titres 2–16-fold lower than NA antibody titres. Median IF antibody levels were substantially lower than median NA antibody levels in sera from immunocompetent individuals (\( p = 0.08 \)), asymptomatic HIV-1-infected subjects (\( p < 0.0001 \)) and AIDS patients (\( p < 0.0001 \)). Sera from HIV-1-infected patients – particularly those with AIDS – contained greater amounts of IF antibodies than sera from healthy immunocompetent controls (Tables 1 and 2). The contribution of IF antibodies to the global neutralising activity of sera appeared to be less substantial in HIV-1-infected patients than in immunocompetent individuals (compare mean IF and NA titres in Table 1).

A positive correlation was found between IF and PN antibody titres in sera from all three groups (overall correlation, \( r = 0.4350; 95\%\ CI: 0.2160–0.6123; p = 0.0002 \)). Median IF and PN antibody titres did not differ significantly (\( p > 0.05 \)) in sera from healthy individuals, asymptomatic HIV-1-infected subjects or AIDS patients. In fact, when individual IF and PN antibody levels were compared in a pair-wise fashion, antibody titres did not vary by more than one dilution in any serum from any study group.

Quantification of antibodies that inhibit HCMV cell-to-cell spread

The next set of experiments examined the ability of the sera to preclude HCMV spread from infected to uninfected adjacent cells (IP). An earlier study demonstrated that plaque development in this assay depends on intracellular transmission of virus from cell-to-cell,
rather than on infection of contiguous cells via free virus released from infected cells [8]. Fig. 2 shows the effect on plaque size exerted by a high titre serum (Fig. 2A) and a typical plaque developed in the presence of an HCMV seronegative serum (Fig. 2B). To establish comparisons between NA and IP antibody titres, an identical viral input was employed in both assays. IP and NA antibody titres in sera from both immunocompetent and HIV-1-infected individuals were found to correlate (overall correlation, $r = 0.4641$; 95% CI: 0.2505–0.6346; $p < 0.0001$). However, median IP antibody titres were lower than median NA titres in sera from immunocompetent individuals ($p = 0.0120$), asymptomatic HIV-1-infected subjects ($p = 0.0127$) and AIDS patients ($p = 0.0713$; not significant). HIV-1-infected patients – particularly those with AIDS – had significantly higher median serum IP antibody titres than immunocompetent individuals (Tables 1 and 2).

**Correlation of gB-specific antibody titres to those of functional antibodies with defined antiviral activities**

To determine whether there was a specific association between the levels of antibodies with functional anti-HCMV activities and those of antibodies to gB, statistical correlation between these parameters was examined in a pair-wise fashion. Median anti-gB antibody titres of sera from AIDS patients, HIV-1-infected asymptomatic individuals and immunocompetent persons were 320, 160 and 80, respectively. The overall correlation between gB and neutralising (NA)
antibody titres was highly significant \((r = 0.601; p < 0.0001)\) as reported previously [6]. In the present study, a significant correlation was observed between the overall median gB antibody titres and those of PN, IF and IP antibodies (Table 3). Correlation was stronger between gB and IP antibodies than between gB and either PN or IF antibodies.

**Discussion**

The data demonstrate that a subset of antibodies in sera from all immunocompetent individuals subject to analysis neutralised cell surface pre-bound HCMV virions, thereby supporting previously reported data. The data also indicated a significant correlation between the overall median gB antibody titres and those of antibodies mediating post-attachment neutralisation, inhibition of cell fusion and blocking virus cell-to-cell spread (Table 3). Correlation was stronger between gB and IP antibodies than between gB and either PN or IF antibodies.

**Table 3.** Correlation between levels of gB antibodies and those of antibodies mediating post-attachment neutralisation, inhibition of cell fusion and blocking virus cell-to-cell spread

<table>
<thead>
<tr>
<th>Parameters correlated</th>
<th>Regression coefficient ((r))</th>
<th>95% CI</th>
<th>(p) value(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gB versus PN antibodies</td>
<td>0.3617</td>
<td>0.1314–0.5540</td>
<td>0.0021</td>
</tr>
<tr>
<td>gB versus IF antibodies</td>
<td>0.3918</td>
<td>0.1658–0.5787</td>
<td>0.0008</td>
</tr>
<tr>
<td>gB versus IP antibodies</td>
<td>0.4332</td>
<td>0.2138–0.6109</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

gB, glycoprotein B; PN, post-attachment neutralising; IF, inhibiting HCMV-induced cell fusion; IP, inhibiting HCMV cell-to-cell spread.

\(^{1}\)Spearman rank correlation test.

\(^{1}\)Statistical significance for \(p < 0.05\).
on competition experiments between murine MAbs and human sera [13]. In most sera, PN antibodies accounted for <50% (c. 12.5–50%) of their total neutralising activity (NA). Antibodies measured by the PN assay most likely neutralise HCMV infection either by inhibiting the fusion process between the viral envelope and the cell membrane, or by blocking an early post-entry event. The present study took advantage of a previously described fusion assay [8–11] to specifically quantify antibodies inhibiting virus–cell fusion. The data suggested that IF antibodies accounted for a minor fraction of the global neutralising activity of these sera. Correlation between IF and PN antibody levels was found to be significant but not optimal – suggesting that antibodies measured by these procedures may not be entirely overlapping in their mode of action. Taken together, data from PN and IF assays indicated that overall, antibodies inhibiting virus penetration into cells contributed to a lesser extent to the total neutralising activity of sera from immuno-competent individuals than antibodies blocking infection at an earlier step (presumably by preventing virus binding to target cells or causing virus aggregation).

Protection against viral infection afforded by neutralising antibodies in vivo may ultimately depend on their mode of action. Data substantiating this contention have been provided recently: protection against bovine respiratory syncytial virus upon experimental infection in gnotobiotic calves correlated with serum levels of antibodies high risk of developing HCMV-related end-organ infection of cell-mediated immunity inherent in HIV-1 infected individuals with advanced AIDS are at high risk of developing HCMV-related end-organ disease. In spite of the presence of high levels of serum neutralising antibodies [5, 6, 14], AIDS patients are frequently unable to control episodes of persistent viraemia which often precede HCMV disease by a variable period of time. Given the functional impairment of cell-mediated immunity inherent in HIV-1 infection, this study investigated whether a qualitatively abnormal neutralising antibody response could be demonstrated in these patients. The results showed PN and IF antibodies to be present to a greater magnitude in sera from HIV-1-infected individuals (particularly AIDS patients), than in those from immunocompetent controls, although their contribution to the total neutralising activity of sera (NA) appeared to be lower in the former group than in the latter. The increasing deletion of the T-cell repertoire that accompanies progression of HIV-1 infection [18], and the subsequent loss of immune system capability to generate antibodies with certain epitope specificities, might account for these data. Whether this finding relates to the apparent inability of HIV-1-infected patients to efficiently control dissemination of active HCMV infection requires further investigation.

The relevance of antibodies blocking virus cell-to-cell spread (IP) in the control of virus infections has been demonstrated in several viral systems. For example, the best correlation of protection against reovirus type 3 was shown to be a reduction in plaque size rather than in plaque number (neutralisation) [19]. HCMV spread over tissues is mostly dependent upon intercellular transmission of virus rather than on infection of contiguous cells by free virions. Thus, IP antibodies might play a significant role in limiting HCMV tissue infection. The present study showed that all sera analysed contained IP antibodies, thus confirming the data from a previous study [13]. Overall, the levels of IP antibodies significantly correlated with those of NA antibodies, although the median titre of the former was substantially lower than that of the latter in all study groups – indicating that the neutralising activity of sera as determined by standard assays does not accurately reflect the capability of sera to interfere with cell-to-cell viral transmission. On the other hand, the present study failed to demonstrate any deficit in the IP antibody response in HIV-1-infected individuals. In fact, as previously reported for NA antibodies [6], higher IP antibody titres were found in these individuals (particularly in AIDS patients) when compared with immunocompetent controls.

HCMV gB is a highly immunogenic virus envelope glycoprotein [12, 13, 20–23] that has been shown to play an important role in virus–cell fusion and in virus cell-to-cell spread [8]. Also, gB is known to elicit a potent neutralising antibody response in man [24–28]. As an indirect approach to estimate the contribution of antibodies to gB to the different antiviral activities analysed, the levels of gB antibodies were correlated with those of PN, IF and IP antibodies. A statistically significant but not optimal correlation was noted between gB antibody titres and those of the different functional antibodies, suggesting that in addition to those against gB, human serum anti-HCMV antibodies with other specificities are capable of mediating both inhibition of virus–cell fusion and interference with intercellular transmission of virus infection. Antibodies against gH/gL – a glycoprotein complex known to elicit a relevant neutralising response [29] in man, and found to be implicated in the promotion of virus–cell fusion [30] – are the most likely candidates to perform such antiviral activities in vivo. Nevertheless, in the absence of data on serum adsorption experiments with individual virus proteins, knowledge of the relative contribution of each antibody type to a given antiviral serum activity seems elusive. Studies addressing this issue are currently underway.

We are grateful to Professor L. Pereira (UCSF, USA) for providing the gB-cell line U373-gB. This work was funded by the FIS (Fondo de Investigaciones Sanitarias), Spain, grant reference 1462/96.
References