Neutralizing antibody to gB2 human cytomegalovirus does not prevent reactivation in patients with human immunodeficiency virus infection

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The incidence of human cytomegalovirus (CMV) genotype gB2 (UL55) is high in patients with human immunodeficiency virus (HIV) infection in the San Francisco Bay area of California. Virus neutralizing antibody (NAb) to human CMV strain Ad169, a gB2 laboratory strain, was measured prospectively in HIV-infected patients, with CD4 T-lymphocyte counts < 200, who were at risk for CMV-associated disease. Patients were grouped according to CMV DNA copy number, as quantified by PCR, and presence or absence of CMV-induced retinitis. Mean NAb titres were similar in all patient groups and unrelated to either virus load or outcome of CMV infection. Both gB2 and mixtures of gB2 with other gB genotypes were represented in isolates from blood and/or urine, even in the presence of high titres of antibody to the gB2 genotype challenge virus.

Patients for study (n = 39) were selected from a larger group of prospectively studied HIV patients who were at risk for CMV-induced retinitis because they had CD4 T-lymphocyte counts < 200 (Rasmussen et al., 1997). Patients were selected by the availability of data on the genotype of CMV, either as an infectious isolate or DNA detected in body fluids by PCR. Patient samples were collected prior to the generalized use of protease inhibitors and none of the patients was being treated with CMV antiviral drugs during their time in the study. Details of prospective patient monitoring, quantification of CMV DNA by PCR and recovery of infectious virus from either leukocytes and urine samples have been described (Rasmussen et al., 1997). Virus load was defined in our previous study as CMV copy number μg⁻¹ of DNA that was extracted from leukocytes. A copy number of 30 was calculated to be the number that distinguished a positive from a negative virus load. Methods for genotyping UL55 (gB) and UL75 have been described (Zipeto et al., 1995). NAbs were quantified by CMV microfocus plaque reduction assay (Rasmussen et al., 1984) and titres are expressed as the log₂ of the last dilution giving a 50 % reduction in CMV f.f.u. All sera were heat-inactivated at 56 °C for 30 min to inactivate both HIV virus and endogenous complement. In brief, twofold dilutions of heat-inactivated sera were incubated with duplicate 50 f.f.u. of CMV Ad169 for 1 h at 37 °C, after which 100 μl of heat-inactivated, serum-containing wells were then added to duplicate microtiter wells containing 500 μl of cell suspension. After incubation, plaques were enumerated.

The immune mechanisms involved in protection from human cytomegalovirus (CMV) reinfection or reactivation are complex. Many laboratories have reported the simultaneous detection of multiple strains of human CMV within immunocompromised patients (Chou, 1986; Collier et al., 1989; Drew et al., 1984). In studies of containment of CMV infection, most have emphasized the importance of cellular immune responses, but high levels of virus neutralizing antibody (NAb) to human CMV strain Ad169, a gB2 laboratory strain, was measured prospectively in HIV-infected patients, with CD4 T-lymphocyte counts < 200, who were at risk for CMV-associated disease. Patients were grouped according to CMV DNA copy number, as quantified by PCR, and presence or absence of CMV-induced retinitis. Mean NAb titres were similar in all patient groups and unrelated to either virus load or outcome of CMV infection. Both gB2 and mixtures of gB2 with other gB genotypes were represented in isolates from blood and/or urine, even in the presence of high titres of antibody to the gB2 genotype challenge virus.
dilutions of serum were incubated with approximately 200 f.f.u. CMV strain Ad169 and incubated for 1 h at 37 °C. Each neutralization mixture was supplemented with 2 % guinea pig complement (Gibco). Of each dilution of post-neutralization mixture, 200 µl was adsorbed to triplicate 24-h-old monolayer cultures of low passage human embryonic lung cells (passage 5–12) that were prepared originally in our laboratory from fresh embryonic lung tissue and preserved in liquid nitrogen for subsequent passage in culture. The inoculum was removed post-adsorption and cultures were overlaid with 1 % agarose supplemented with MEM and 10 % FCS. At 7 days later, the semi-solid overlay was removed and monolayers were stained with May-Grunewald-Giemsa to visualize and count the CMV microfoci microscopically. The per cent reduction in CMV microfoci as compared to controls for each dilution of a single serum sample was plotted manually on Probit paper to obtain the 50 % reduction point. Intertest assay variation was monitored by including at least three aliquots of a neutralization mixture with 10 µg 15D8 complement-dependent monoclonal antibody (mAb) (Rasmussen et al., 1985), which routinely gave between 30 and 60 % end-point neutralization of challenge CMV Ad169. Only assays with 15D8 mAb control values within this range were considered to be valid. In addition, a positive human serum control was frozen in aliquots and a single tube was thawed for titration for each of the 11 experiments that were done to obtain the data in this study. The log2 mean ± SD of the positive control serum sample included in each assay was 4.9 ± 0.5. Intratest variability was monitored by counts of f.f.u. in control cultures. When the variability in counts of microfoci was greater than 30 % among the 12 wells inoculated with CMV control mixtures, the test was invalid.

Patients were grouped according to status of CMV infection (no disease, n = 24; retinitis, n = 12) and maximum CMV DNA copy number ([<30, n = 6; 30–1000, n = 9; >1000, n = 9] copies of CMV DNA from 1 µg buffy coat DNA) attained during a monitoring period of at least 4 months and a minimum of two observations. The stratification of patients by CMV DNA copy number was justified by the statistically significant differences that resulted when virus loads were compared during the monitoring period (P < 0.05, Student’s t-test with Welch’s correction), according to the grouping of <30, 30–1000 or >1000. However, the mean copy numbers between patients who attained more than 1000 copy numbers during monitoring and patients who progressed to retinitis did not differ significantly. Fig. 1 shows the results of the prospective monitoring of NAb titres (a), CMV copy numbers (b) and CD4 T-lymphocytes (c) in each patient group identified by DNA copy number. The titres of NAb in all patient groups were highly variable from patient to patient, across all of the groups. Some patients had NAb titres higher than 24 in the <30 CMV DNA copy number group (patients 325 and 369), while others in the group had titres that were comparable to those detectable in the remaining three patient categories. The lack of correlation of NAb with CMV DNA copy number became more apparent as the virus load increased. In patients with a maximum of between 30 and 1000 copies during monitoring, some patients appeared to be at risk because of low NAb titres (patients 374 and 411), while others in the group maintained high virus loads despite the presence of relatively high titres of NAb. In particular, patient 838, with the highest overall levels of NAb during the entire monitoring period showed no tendency to a reduction in CMV DNA virus load during monitoring. In patients who progressed to 1000 or more copies during monitoring, both with and without retinitis, substantial levels of NAb were detectable throughout the period in the face of increasing virus load.

As is expected in HIV-infected patients with increasing risk for CMV disease, CD4 levels declined significantly as virus load increased (Fig. 1c). However, NAb levels remained relatively unchanged. NAb in patients who progressed to retinitis were not significantly different from those in any of the other three patient groups and had no impact on reduction in virus load. In some of the patients who progressed to retinitis, NAb levels were highest with progression to disease (patients 227, 370, 452 and 456). Mean levels of NAb, when compared among the patient groups with different levels of virus load, were not significantly different (Student’s t-test with unpaired samples and Welch’s correction; data not shown). This is in contrast to the statistically significant increase in virus load among the groups as risk for retinitis increased. In addition, mean titres of NAb in the retinitis groups did not differ significantly from the mean titre of those with comparable virus load but who remained disease free.

Data (not shown) were also analysed according to CMV DNA copy number at time of entry into the study. Twenty patients entered monitoring with <30 copies of CMV DNA, while 16 entered with >30 copies. Three patients with retinitis were excluded from analysis because there was only a single monitoring time. There were no significant differences in the mean titres of NAb between the two groups. When patients entering the study with >30 copies of CMV DNA and who remained disease free (n = 5) were compared to those who progressed to retinitis (n = 11), no differences were detected (Student’s t-test with Welch’s correction).

Finally, none of the mean titres of NAb in the HIV-infected patient groups, stratified on the basis of copy number, were significantly different from the mean titre in a group of 18 normal, CMV seropositive patients selected for study based on a positive ELISA test for CMV (mean ± SE = 5.6 ± 0.2).

The recovery of infectious CMV, either as single gB2 genotype (Fig. 2, grey bars) or as mixtures of gB2 and other genotypes (Fig. 2, black bars) in the presence of NAb, is shown. A gB2 virus was recovered during monitoring, either alone or in mixtures from 18 of the 39 patients in this study. A single patient (650) excreted gB4 as a single genotype. No virus isolations were made among the
patients with <30 copies of CMV DNA; therefore, this panel is omitted from Fig. 2. There were no obvious associations between levels of NAb and either recovery or gB genotype of CMV. Patient 352 (no disease, CMV copy number >1000) had rising NAb titres despite recovery of mixtures of gB2, -3 and -4 at various times during monitoring. Patient 4113 (no disease, CMV copy number 30–1000) had high levels of NAb, but gB2, both as single isolates and mixtures of genotypes, was recovered regularly during monitoring. Thus, in our patients who were shedding gB2 CMV strains, CMV NAb titres measured in vitro to the gB2 genotype Ad169 laboratory strain of CMV had no obvious impact on either CMV DNA copy number, the gB genotype that was shed, the shedding of gB genotype mixtures or outcome of disease.

Our study does not allow us to determine whether pre-existing antibody to gB2 could prevent de novo infection with a non-gB2 genotype. Since HIV-infected patients are likely to be exposed to multiple strains of CMV, we could not know whether mixed infections with multiple gB types represented the acquisition of a new gB strain or reactivation

**Fig. 1.** NAbs to human CMV strain Ad169 gB2 (a), CMV virus load (b) and CD4 T-lymphocyte counts (c) in HIV-infected patients during a 12 month monitoring period. Patients were divided into categories (<30, 30–1000, >1000 and retinitis) based on both CMV load, as determined by quantitative PCR for viral DNA and progression to retinitis. The 12 month monitoring period for each individual patient is shown as an inverted bracket. Each set of coloured bars represents the monitoring of an individual patient. The time of monitoring, using 2 month intervals, is shown by the position of the bar on the bracket.
of a gB type acquired previously. There is ample evidence for the importance of the humoral antibody response in protective immunity to CMV, particularly in maternal–foetal transmission (Boppana & Britt, 1995) and transplant recipients (Snydman, 2001). In HIV patients, high virus NAb titres are reported to slow progression of diagnosed retinitis (Boppana et al., 1995) and, in some studies, CMV NAds have been inversely related to presence or absence of systemic virus load (Alberola et al., 1998). It is possible that the CMV NAb response is less effective in the face of the severe immunocompromise exhibited by the patients in our study who were in the late stages of HIV infection.

Neutralization targets, in addition to the gB glycoprotein, need to be studied for correlation with the consequences of CMV infection. For example, gH is associated with a NAb response in humans, bears strain-specific epitopes and could play a role in virus containment (Urban et al., 1992). Techniques that allow the separation of biologically active NAds that are specific for antigens associated with virus neutralization need to be simplified and applied to prospective studies of high-risk patients to evaluate both the relative importance of NAds to individual glycoproteins and the contribution of a strain-specific NAb response to protection.

Also, we cannot exclude the possibility that quantification of NAds against a patient’s own isolate may show a better correlation with protective immunity than our studies with a prototype gB virus strain of laboratory virus. However, there are formidable technical limitations for culturing CMV clinical isolates to obtain sufficient titres for use in neutralization assays, as well as the potential problem of mixed infections in the immunocompromised host. Such a study of NAds to a patient’s own isolate might be done best in a population of allograft recipients, who are more likely to have only a single genotype than patients with HIV infection.

The search for an in vitro correlate of protective immunity in CMV infection is assuming increasing importance as efforts to produce a CMV vaccine gain impetus. The absence of an animal model necessitates the evaluation of a protective response in humans. The study of immunologically normal patients, such as children who transmit virus to their parents with pre-existing immunity to CMV (Adler et al., 1995), may provide useful information about the importance of a strain-specific NAb response in protection from reinfection with new strains. Such information will help to design the best vectors for the delivery of a CMV vaccine that induces broad-spectrum immunity as well as to improve immunotherapy for active CMV infection.

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