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Identification of Elite Neutralizers With Broad and Potent Neutralizing Activity Against Human Cytomegalovirus (HCMV) in a Population of HCMV-Seropositive Blood Donors

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To improve the potency of anti–human cytomegalovirus (HCMV) immunoglobulin preparations, we intended to find elite neutralizers among 9000 HCMV-seropositive blood donors. We identified the top 2.6% neutralizers by use of high-throughput screening and further analyzed the 80 neutralizers with the most effective plasma for strain-independent activity. Of those, 58 had broad neutralizing activity against various HCMV strains and hence were regarded as elite neutralizers. All elite neutralizers were then analyzed to determine their effect on individual virus particles during entry. Most had plasma specimens that preferentially inhibited viral penetration, whereas 2 had exceptional plasma specimens that prevented adsorption of virus to cells. Furthermore, the neutralizing capacity of plasma samples from 3 randomly chosen elite neutralizers was up to 10-fold higher than that for commercial immunoglobulins. In a retrospective analysis of 6 selected donors, anti-HCMV neutralization titers in repeated donations were constantly high over 5 years. In conclusion, plasma samples from elite-neutralizing donors can be considered to improve antibody-based treatment of HCMV infections.

Keywords. Cytomegalovirus; hyperimmunoglobulins; neutralizing antibodies; elite neutralizers; blood donors.

Human cytomegalovirus (HCMV) is a ubiquitously distributed herpesvirus. After primary infection, it causes lifelong latency, and reactivation can result in episodes of viral replication. Primary infection of immunocompetent individuals can cause mild symptoms or mononucleosis-like disease. Reactivations and reinfections are usually asymptomatic. In contrast, immunocompromised individuals, such as patients with AIDS and transplant recipients, can experience severe complications due to primary or reactivated infection. The risk for HCMV infection after transplantation of solid organs or hematopoietic stem cells is about 50%, and adverse outcomes may include graft failure, graft-versus-host disease, and increased susceptibility to other infections [1–4]. Intrauterine HCMV infection occurs at a prevalence of 0.3%–1.5% and is a leading cause of congenital disabilities [5, 6], including hearing loss and other neurological impairments [7, 8]. It was generally assumed that primary infection during pregnancy is more hazardous than reactivation or reinfection, owing to protective effects of preexisting maternal antibodies, but this assumption has been questioned recently after synopsis of the available information [9].

It is obvious that high levels of HCMV-specific antibodies in diagnostic assays are not sufficient for protective immunity [9–11]. In line with this, reports on the impact of HCMV hyperimmunoglobulins in pregnant women with primary HCMV infection [12–14] and in recipients of transplants [1, 15–17] are controversial. In principle, anti-HCMV immunoglobulins were found to be beneficial in transplant recipients, but the effects on morbidity and mortality were only moderate. Direct-acting antivirals such as ganciclovir or foscarnet are effective by targeting the viral polymerase, but their use may be complicated by myelotoxic or nephrotoxic side effects. The recently approved terminase inhibitor letermovir is apparently less toxic but may also be limited by the development of resistance [18]. Therefore, a potent passive immunization strategy is still a desirable alternative. In a recent clinical trial, a combination of 2 neutralizing anti-HCMV antibodies reduced the frequency of HCMV viremia [19, 20]. Interestingly, these antibodies were shown to act mainly via neutralization but not via antibody-dependent cellular cytotoxicity or complement.

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As an approach to improve the quality and efficacy of anti-HCMV immunoglobulin preparations, we screened a large population of blood donors for their neutralizing capacities [21]. On the basis of our findings, we identified elite neutralizers, defined as individuals whose plasma is more potently neutralizing than currently available HCMV hyperimmunoglobulins. The concept of elite neutralization was developed in the field of human immunodeficiency virus (HIV) research, where it describes a small subset of HIV-1–infected individuals who generate high titers of broadly neutralizing antibodies [22, 23]. Elite neutralizers are not identical with elite controllers, who limit viral replication, often in the absence of broadly neutralizing antibodies [24].

Broad and potent antibodies were cloned from elite neutralizers to improve passive immunization strategies against HIV [25], and the same concept was applied to other viruses [26]. Regarding HCMV, the breadth of neutralizing antibodies concerns not only the inhibition of various HCMV strains but also the efficacy against cell type–specific entry routes mediated by different viral glycoprotein complexes. While antibodies of seropositive individuals usually have a high neutralization capacity against infection of endothelial and epithelial cells via the pentamer gH/gL/pUL128/pUL130/pUL131A, infection of fibroblasts via the trimer gH/gL/gO is less efficiently neutralized [21, 27]. This may also be relevant in the context of intrauterine infection, as trophoblast progenitor cells were protected against HCMV infection by antibodies against gB but not by antipentamer antibodies [28].

We therefore investigated plasma specimens from blood donors to identify elite neutralizers with exceptional strain-independent neutralizing activity against infection of both cell types. This could provide the basis for an improved passive immunization strategy for patients endangered by HCMV, including both direct use of plasma preparations and the isolation of monoclonal antibodies from such donors.

MATERIALS AND METHODS

Cells, Viruses, Plasma Samples, and Antibodies

Human foreskin fibroblasts (HFFs) were grown in minimal essential medium with 5% fetal bovine serum (MEM5; GlutaMAX, Life Technologies) and 100 µg/mL gentamicin supplemented with 0.5 ng/mL basic fibroblast growth factor (Life Technologies). Conditionally immortalized human endothelial cells (HEC-LTTs) [29, 30] were cultured in vessels and microplates coated with 0.1% gelatin (Sigma-Aldrich) in endothelial growth medium (EGM BulletKit, Lonza), supplemented with 2 µg/ml doxycycline. During experiments, HFFs and HEC-LTTs were both kept in MEM5.

HCMV strains AD169 [31], Towne [32], Toledo [33], Merlin [34], VR1814 [35], VHL/E [36], and TB40/E [37] and derivatives thereof [38] were used in this study. This panel was chosen as it covers almost all of the established genotypes of HCMV envelope glycoproteins [21]. Viral stocks were generated by centrifuging supernatants of infected cell cultures at 3220 ×*g* for 10 minutes to remove cell debris.

Plasma samples from HCMV-seropositive blood donors were provided by the German Red Cross Blood-Transfusion Service, Baden-Württemberg and Hessen, with informed consent according to human experimentation guidelines (Ethical Board of Ulm University vote number 53/14). Remnant clotting factors were removed by recalcification prior to use in neutralization tests [21].

The immunoglobulin G (IgG) concentration in plasma, in grams/liter, was determined by a turbidimetric assay (Cobas 8000 c502; Roche). Anti-HCMV IgG concentrations were determined as arbitrary units (AU) per milliliter (CMV IgG enzyme-linked immunosorbent assay [ELISA] with a pipetting-control system; Medac, Hamburg).

Antibodies (IgGs) were purified from plasma samples by using protein A chromatography and were dialyzed against phosphate-buffered saline (PBS) as previously described [39].

Gaussia Luciferase-Based Screening for High Neutralizers of Fibroblast Infection

HFFs were seeded on 96-well plates at 1.5×10^4 cells/well. Plasma samples were mixed with the Gaussia luciferase-expressing reporter virus TB40-BAC4-IE-GLuc to obtain a final plasma dilution of 1:400 and a multiplicity of infection (MOI) of 1. The mixture was preincubated for 2 hours at 37°C and added to cells to initiate infection. After cells were incubated for 2 hours at 37°C, the mixture was replaced by MEM5. Cell cultures were then incubated overnight, 20 µL of supernatant of each infected cell culture was mixed with coelenterazine (0.2 µg/mL in PBS supplemented with 5 mmol/L NaCl; PJK), and luminescence signals (measured as relative light units [RLU]) were measured with a microplate reader (Chameleon; Hidex). The neutralizing performance was determined as the ratio of the RLU for reference plasma to the RLU for test plasma.

Determination of Half-Maximal Neutralization Titer (NT_{50}) by an ELISA-Based Neutralization Assay

HFFs or HEC-LTTs were seeded in 96-well plates at 1.5×10^4 cells/well. Plasma samples were serially diluted in duplicates, mixed with TB40/E (MOI = 1), and preincubated for 2 hours at 37°C. The virus-plasma mixtures were incubated with cells overnight at 37°C. The cells were then fixed with 80% acetone (Sigma Aldrich) and stained with a monoclonal antibody against HCMV immediate-early antigen (E13; Argene Biosoft) [40] and a secondary antibody (goat anti-mouse IgG) conjugated with horseradish peroxidase (Santa Cruz Biotechnology). Substrate (ortho-phenylenediamine; Thermo Fisher Scientific) was added for 30 minutes, the reaction was stopped with 1 M sulfuric acid, and the OD_{492} was measured. Mock values (ie,

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signals for plasma without virus) were subtracted from mean values of duplicates, and the background-corrected values (ie, the signal for virus plus plasma) were divided by the maximal signal (ie, signal for virus only) to determine the relative infection rates. Neutralization rates were calculated as 1 − [(signal for virus plus plasma)/(signal for virus only)], and the corresponding dose-response curves were analyzed by nonlinear regression. The plasma concentration at which the neutralization rate equals 0.5 is given as the half-maximal neutralization concentration, and the reciprocal value is the NT_{50}.

Analysis of the Mode of Action of Neutralizing Antibodies

To screen plasma samples from elite neutralizers for their mode of action, their effect on adsorption or penetration of virions was discriminated using the dual-fluorescent HCMV strain TB40-BAC_{KL7}-UL32EGFP-UL100mCherry [41], including HCMV-negative plasma as a negative control. HFFs were seeded at 7.5×10^3 cells/well on a 96-well plate (µClear [black]; Greiner Bio-One). Freshly produced virus was preincubated with plasma samples at 10 times the NT_{50} for 2 hours at 37°C and added to HFFs for 2 hours. Cultures were then fixed with 4% paraformaldehyde in PHEM buffer (25 mmol/L HEPES, 10 mmol/L EGTA, 60 mmol/L PIPES, and 2 mmol/L $MgCl_2$) and permeabilized with ice cold methanol and permeabilization solution (10% sucrose, 1% fetal calf serum in PHEM buffer, and 0.5% Nonidet P40). Subsequently, the cells were stained with anti–α-tubulin monoclonal mouse antibody (Life Technologies) and AlexaFluor350-conjugated goat anti-mouse IgG $F(ab')_2$ (Life Technologies), each diluted in PHEM buffer. Five pictures of each condition were taken at 640-fold magnification with a fluorescence microscope (Axioobserver D1; Zeiss) and evaluated for green and red dot–like fluorescence signals indicative of virions. Virions with green and red fluorescence represent intact enveloped virions attached to cells, whereas virions without the red signal have penetrated cells.

Purified IgGs were analyzed by the same assay with the following modifications: HFFs were seeded at 1×10^4 cells/well. The mCherry signal was enhanced by immunofluorescence staining with rabbit anti-DsRed polyclonal antibodies (Clontech) and Cy3-conjugated goat anti-rabbit IgG $F(ab')_2$ (Jackson ImmunoResearch). Instead of tubulin staining, nuclei were counterstained with DAPI (Sigma-Aldrich). In each of 3 experiments, 6–10 pictures were randomly taken for each condition, and the numbers of particles with green and red fluorescence or green without red fluorescence were counted.

Statistical Analysis

Datasets were analyzed by Kruskal-Wallis 1-way analysis of variance on ranks with the Dunn post hoc test, using the build-in data analyses function of Sigmaplot. If analysis of variance indicated significant differences between groups, P values were determined using unpaired 2-sided t tests. Differences were considered marginally significant when P values were $<.05$, significant when P values were $<.01$, and highly significant when P values were $<.001$.

RESULTS

Identification of Top Neutralizers Among HCMV-Seropositive Blood Donors

To identify routine blood donors with exceptionally high neutralizing capacities, retained plasma samples from 9000 HCMV-seropositive donors were screened for efficient inhibition of HCMV strain TB40/E infection in fibroblast cultures. Using a recently developed Gaussia luciferase–based neutralization assay, plasma samples were tested at a 1:400 dilution in comparison with a highly neutralizing preselected reference plasma to discriminate the 2.5% top-neutralizing samples [21]. If the neutralizing effect exceeded that of the reference (ie, if the ratio of the RLU for reference plasma to the RLU for test plasma was ≥1.1), the respective plasma sample was regarded as a top-neutralizing specimen (Figure 1A). A total of 236 samples (2.6%) fulfilled this criterion, indicating that the strategy of the screening approach worked as intended.

The 80 plasma samples with the highest score were then selected for a subsequent quantitative analysis. Serial dilutions of each plasma specimen were tested in an ELISA-based assay for their effect against strain TB40/E in fibroblasts, and the NT_{50} for HFFs was calculated. The NT_{50} values ranged between 250 and 2950. The majority of samples (64 of 80) had an NT_{50} of >500, and 33 of 80 had an NT_{50} of >1000 (Figure 1B). When a plasma unit (300 mL) is administered to a patient, it will be diluted about 100-fold in the interstitial fluid compartment [42]. Hence, plasma units from these top neutralizers are expected to achieve titers in vivo that are effective against HCMV, based on our in vitro assays. In summary, we successfully identified top-neutralizing plasma samples for further characterization of the breadth of their neutralizing effect in different cell types and against a panel of HCMV strains.

Identification of Elite Neutralizers With Broad Reactivity

Our previous work suggested that plasma with a high neutralization capacity against fibroblast infection usually has at least an equally high neutralization capacity against endothelial cell infection [21]. To test whether this assumption holds true for our 80 top neutralizers, we determined the neutralization titers of their plasma specimens against endothelial cell infection. The ratio of the NT_{50} for HECs to that for HFFs was calculated as a readout for the relative efficiency in HECs versus HFFs, and the log of this ratio was plotted in the order of size for all plasma samples (Figure 2A). A ratio of the log NT_{50} for HECs to the log NT_{50} for HFFs of 0 indicates equal neutralization capacity in both cell types, whereas a log value >0 indicates higher efficiency against infection of endothelial cells. As expected, almost all plasma samples (77 of 80) were more potent against endothelial cell infection. This confirmed our strategy to screen with

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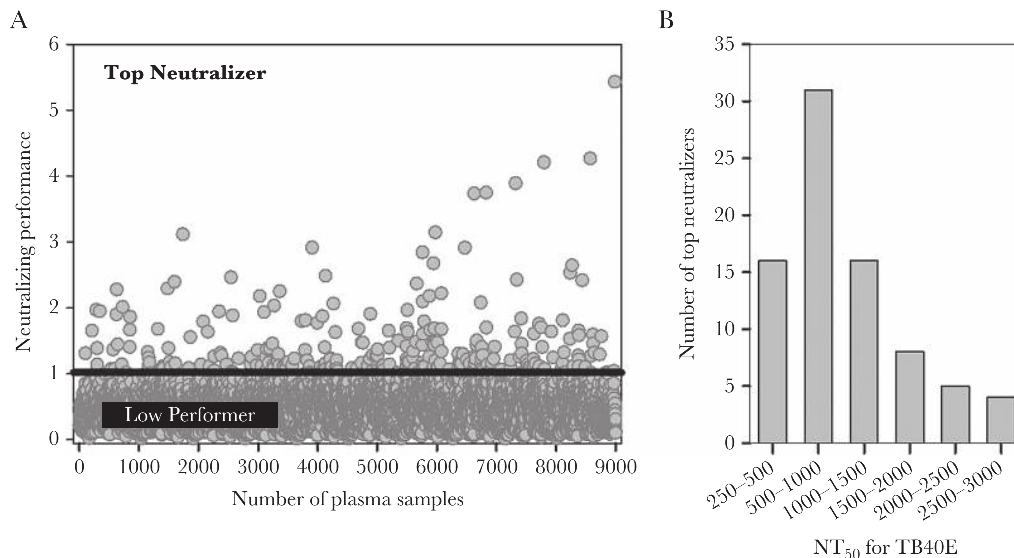

Figure 1. Identification of top-neutralizing plasma samples. *A*, A total of 9000 human cytomegalovirus (HCMV)–seropositive plasma samples were screened for exceptionally high neutralization on human foreskin fibroblasts (HFFs) with a high-throughput assay, using a TB40-BAC4–derived luciferase reporter virus. Their neutralizing activity was compared to reference plasma representing the 97.5th percentile as determined previously [21]. Plasma samples that outperformed the reference (indicated by the black line) were regarded as top-neutralizing samples. *B*, Of these, the 80 with the highest performance scores were quantitatively analyzed by an enzyme-linked immunosorbent assay–based neutralization assay for their half-maximal neutralization titers (NT_{50} values) against HCMV-TB40E on HFFs. The histogram shows that the majority of these selected plasma samples had NT_{50} values of ≥500.

a simplified assay only in fibroblasts and then validate the top scorers also on endothelial cells.

The next step was to identify neutralizers with broad activity against 7 HCMV strains covering most genotypes of HCMV envelope glycoproteins [21]. Plasma samples with NT_{50} values of >100 against all strains were considered broadly neutralizing (Figure 2B). This cutoff was chosen because clinical application of a plasma unit will result in a dilution of about 1/100 in the interstitial fluid, and the same value has been used to define broadly neutralizing plasma in the HIV field [23]. According to this definition, 58 of the 80 tested top neutralizers (71%) showed broadly neutralizing activity against all strains (Supplementary Materials).

In summary, 58 plasma specimens (0.6%) had exceptionally high and broad effective neutralizing ability and were hence designated as elite-neutralizing samples.

Plasma Specimens From Elite Neutralizers Are More Potent Than Currently Available Anti-HCMV Immunoglobulins

Next we tested whether plasma specimens from elite neutralizers are more effective at neutralizing HCMV than commercial hyperimmunoglobulins generated from sera from donors who were selected on the basis of HCMV antibody titers determined by ELISA. Therefore, the neutralizing capacity of 3 elite-neutralizing plasma samples were compared to that of a commercially available HCMV hyperimmunoglobulin and standard immunoglobulin. The latter was included as a baseline control.

The neutralizing capacity of all preparations was measured by the ELISA-based neutralization assay and plotted against the total IgG concentration or the CMV-specific antibody concentration. Comparison of the total IgG concentration reflects the overall neutralizing capacity of the antibodies (Figure 3A), whereas comparison regarding the virus-specific anti-HCMV IgG reflects the specific neutralizing quality of the HCMV-specific antibodies in the sample (Figure 3B). When standardized to the total IgG content, standard immunoglobulin showed the lowest neutralizing capacity, followed by hyperimmunoglobulin, and all 3 elite-neutralizing plasma specimens had higher neutralizing capacities. When standardized to anti-HCMV IgG, it became apparent that the anti-CMV antibodies of standard immunoglobulins and hyperimmunoglobulins had similar neutralizing abilities. In contrast, the preselection of elite-neutralizing donors for exceptionally high neutralization titers was reflected in an approximately 10-fold higher specific quality of their anti-CMV antibodies.

Blocking of HCMV Adsorption and Penetration by Elite-Neutralizing Plasma Specimens

Blocking of various steps of viral entry into the host cell is the most likely mechanism used by antibodies to neutralize viral infection. Therefore, we investigated the mode of action of HCMV-neutralizing antibodies in elite-neutralizing plasma specimens with regard to inhibition of HCMV adsorption and penetration into the host cell. To investigate more precisely which of these entry steps is blocked by plasma specimens from elite neutralizers, we used a dual fluorescent HCMV assay (HCMV-TB40-BAC_{KL7}-UL32EGFP-UL100mCherry), in which

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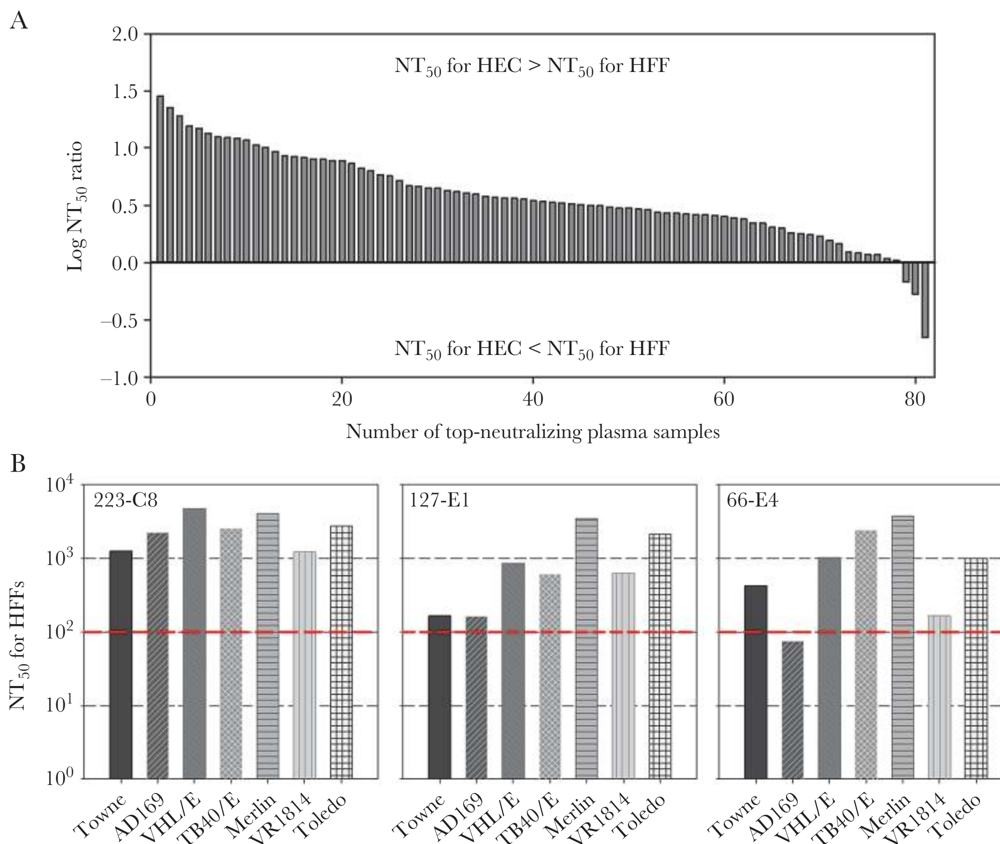

Figure 2. Breadth of neutralization as determined in different cell types and against different human cytomegalovirus (HCMV) strains. *A*, All 80 plasma samples that were previously selected for their prevention of human foreskin fibroblast (HFF) infection were additionally analyzed by an enzyme-linked immunosorbent assay–based neutralization assay for their half-maximal neutralization titers (NT_{50} values) against HCMV-TB40E in human endothelial cells (HECs), and the neutralization capacities in both cell types were compared. The vast majority (96%) of these plasma specimens were at least equally effective against endothelial cell infection, as demonstrated by a log ratio of the NT_{50} for HECs to that for HFFs of ≥0. *B*, The 80 top-neutralizing plasma specimens were then tested for their activity against 7 different HCMV strains. Three different categories were distinguished. The majority (70%; represented by plasma specimens 223-C8 and 127-E1) achieved NT_{50} values of ≥100 (red line) against all strains and were therefore regarded as broadly neutralizing. Of these, 2 (represented by plasma specimen 223-C8) were exceptionally potent because they neutralized all strains at a NT_{50} of ≥1000. A minority (30%; represented by plasma specimen 66-E4) failed to reach an NT_{50} of ≥100 against all strains and were therefore not regarded as broadly neutralizing .

capsid-associated and envelope-associated proteins are labeled with green and red fluorescent tags [41]. This allowed to distinguish complete virus particles that have attached to the cell from virus particles that have successfully penetrated into the cytoplasm and thereby lost their envelope.

Each of the 58 elite-neutralizing plasma samples was adjusted to 10 times the NT_{50} for HFFs and preincubated with the dual fluorescent HCMV for 2 h. The mixtures were then added to fibroblasts for 2 hours to allow adsorption and penetration of the virus. Subsequently, the plasma-treated cultures were fixed and analyzed for green and red fluorescent signals among individual virus particles and compared to cultures treated with HCMV-negative plasma. Enveloped particles displayed fluorescence in both the green and the red channels, whereas naked particles (ie, virions without an envelope) were fluorescent only in the green channel. Naked particles were assumed to have penetrated into the cytoplasm. Three different effects on HCMV particle entry could be discriminated (Figure 4). First, 42 of 58 plasma specimens showed inhibition of penetration (ie, the total particle number was unaffected, but most particles retained their envelope). Second, 2 of 58 specimens inhibited the adsorption (ie, the total number of particles was greatly reduced, but most of them succeeded to penetrate). Third, 6 of 58 specimens caused an aggregation of particles (ie, enlargement and increased intensity of fluorescence signals indicating aggregation of individual particles, which resulted in reduced infection efficiency in the immediate early antigen control staining). A combined effect was observed in 8 of 58 samples.

To confirm that these effects were actually due to the antibodies within the plasma samples, the respective blood donors were revisited, additional plasma samples were obtained, and IgGs were isolated and analyzed with the same assay. Purified IgGs from 4 donors with penetration-inhibiting specimens had a significantly reduced number of penetrated particles, as compared to IgG from an HCMV-seronegative donor (Figure 4B), whereas the IgG preparations from 2 donors with adsorption-inhibiting

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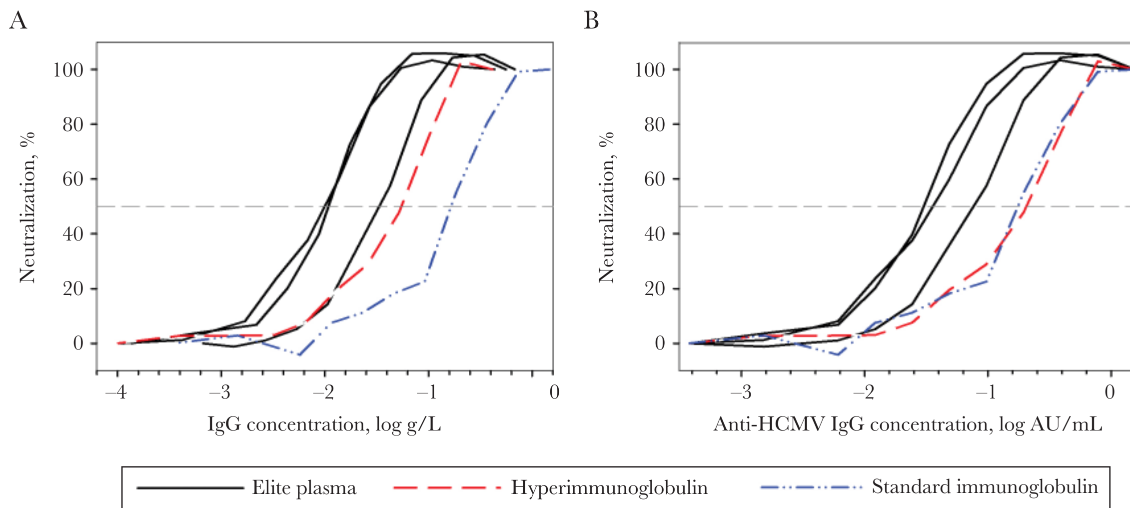

Figure 3. Comparison of 3 elite-neutralizing plasma samples to commercially available immunoglobulin preparations. *A*, All immunoglobulin preparations were standardized to their total immunoglobulin G (IgG) concentration and compared regarding their neutralizing capacity against TB40E, as measured by an enzyme-linked immunosorbent assay–based neutralization assay, on fibroblasts. The elite-neutralizing plasma specimens showed higher neutralizing capacities than standard immunoglobulin and hyperimmunoglobulins. *B*, When the immunoglobulin preparations were standardized to their anti–human cytomegalovirus (HCMV) IgG concentration, the elite-neutralizing plasma specimens were also superior to the commercially available immunoglobulin preparations. Note that hyperimmunoglobulin has a neutralization capacity similar to that of standard immunoglobulin when normalized to anti-HCMV IgG levels, reflecting that the respective donors were not specifically selected for high neutralizing performance. The dashed lines represent 50% neutralization.

specimens significantly reduced the total number of attached particles (Figure 4C). In all cases, the mode of action of the complete plasma was reflected in the activity of purified IgGs from the respective donor.

Taken together, antibodies from the elite neutralizers with the greatest neutralization capacity primarily blocked penetration of virus particles, but 2 elite neutralizers with an exceptional neutralization capacity produced antibodies that blocked HCMV at the level of virus attachment.

Neutralizing Capacities of Elite Neutralizers Remain Stable Over Several Years

Finally, we tested whether neutralization titers of elite neutralizers are stable over time. Therefore, retained plasma samples from 6 donors who had donated repeatedly for 5 years were investigated to determine neutralizing capacities against both cell types, and total anti-HCMV-IgG levels were also determined (Figure 5).

In concordance with data shown in Figure 2A, the NT_{50} values for HECs exceeded those for HFFs almost 10-fold, with the exception of donor 2, who had similar neutralization capacities for both cell types. This donor was also remarkable because he displayed a high NT_{50} for HFFs despite a low anti-HCMV IgG level, indicating a particularly high functionality of these antibodies. In contrast to the clear interdonor differences, there was remarkably little intradonor variation over time. The overall anti-HCMV levels and the NT_{50} values for both cell types remained stable over 5 years, with only occasional deviation (<3-fold). This indicates that stable production of highly neutralizing plasma can be expected from donors who are identified as elite neutralizers.

DISCUSSION

Using a systematic screening approach, we identified blood donors with exceptionally potent and broadly neutralizing antibodies against HCMV. Several lines of evidence suggest that anti-HCMV antibodies can in principle reduce the risk of vertical HCMV transmission during pregnancy and the extent of viremia and HCMV-associated morbidity in immunocompromised patients [13, 14, 43, 44]. At present, however, neither natural humoral immune responses nor active or passive immunization are sufficient to provide a reliable protection [9, 12, 45], and the extent to which neutralization or more-indirect mechanisms, such as antibody-dependent cellular cytotoxicity or complement activation, contribute to protection by antibodies is unclear. It was suggested that the efficacy of passive immunization could be increased by improving the quantity and/or quality of the neutralizing antibodies as compared to that of currently available immunoglobulin preparations [46–48]. A combination of 2 neutralizing monoclonal antibodies recently yielded encouraging results in kidney transplant recipients [20]. Therefore, we aimed to identify elite-neutralizing blood donors with exceptionally broad and potent antibody responses against HCMV.

The concept of elite neutralization was developed in the HIV field: although most HIV-infected individuals produce neutralizing antibodies, escape mutants often develop, and the humoral immune response hence fails to control virus replication. A minority of HIV-infected individuals, however, will develop

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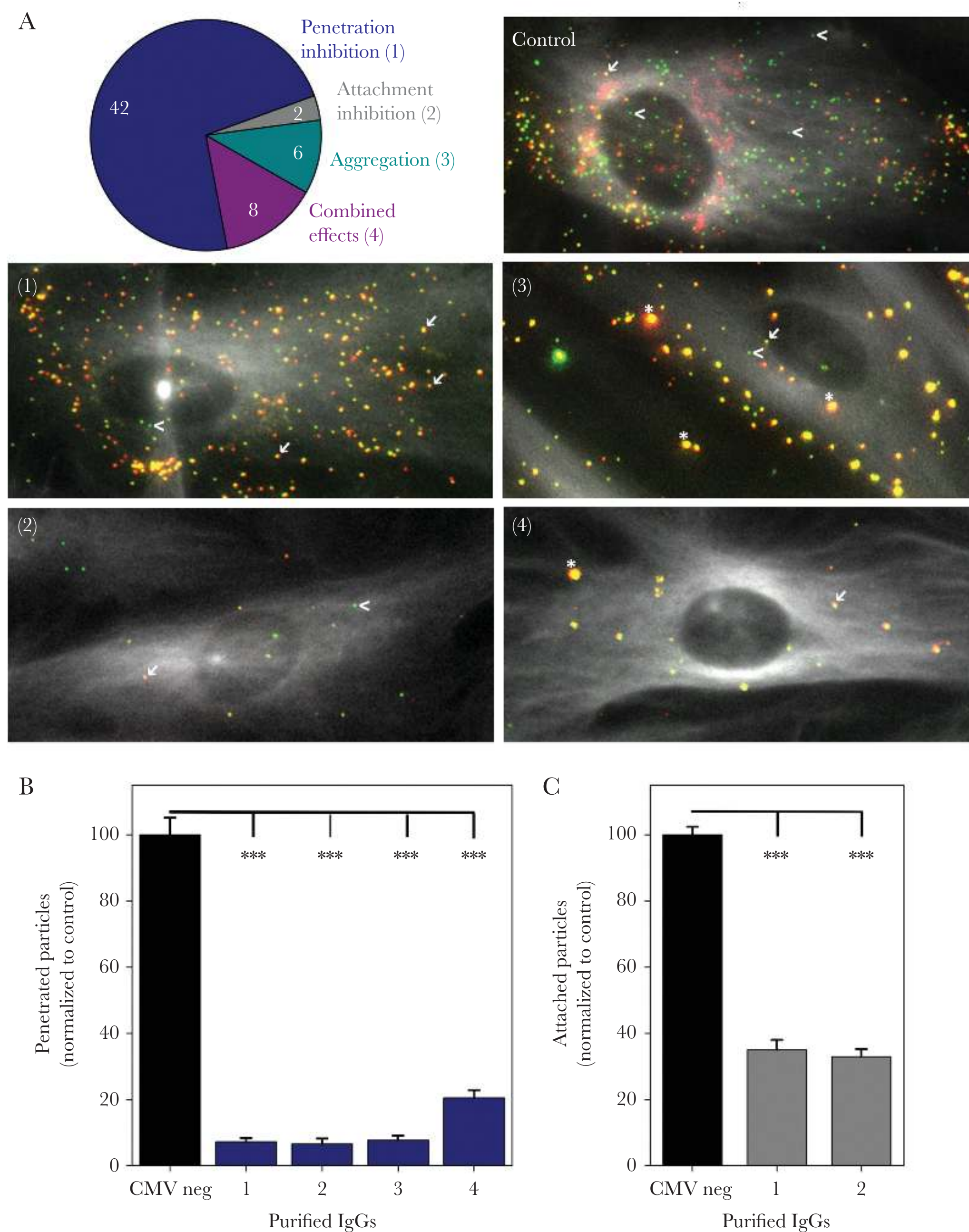

Figure 4. Mode of action of the elite-neutralizing plasma samples. *A*, The effect of all 58 plasma samples with broad and potent neutralization capability (obtained from elite neutralizers) on adsorption and penetration was investigated with a dual-fluorescent derivative of human cytomegalovirus (HCMV), TB40/E, in which the envelope glycoprotein gM is tagged with mCherry and the capsid-associated tegument protein pp150 is tagged with enhanced green fluorescent protein. Dual-labeled virus was preincubated for 2 hours with each plasma sample at a concentration reflecting 10 times the half-maximal neutralization titer. The mixture was then added to fibroblast cultures and, after 2 hours of infection, the effects of the respective plasma on adsorption and penetration were analyzed. Yellow dot-like signals represent enveloped (ie, nonpenetrated) viral particles (indicated by arrows), whereas green dot-like signals represent penetrated particles (capsids) that have already lost their envelope (indicated by open arrows). Red dots represent viral defective particles (dense bodies). Red patchy signals in the perinuclear region of the control represent gM accumulations occurring after capsid penetration. Exceptionally, larger signals were found that represent aggregation of several particles (indicated by white asterisks). Cells were counterstained for α-tubulin, which is displayed in white. Events falling in the different categories were counted and compared to values obtained with cultures infected in the absence of antibodies (control). The pie chart shows that 42 plasma samples inhibited penetration, whereas only 2 plasma samples specifically inhibited adsorption, 6 plasma samples caused aggregation, and 8 plasma samples had combined effects. *B*, To test whether the effects on penetration were actually due to antibodies, purified immunoglobulin G (IgG) from 4 donors with penetration-inhibiting plasma were retested with the dual fluorescent virus. IgG from an HCMV-seronegative donor was used as reference, and the particle numbers were separately normalized to the reference values for each experiment. Each bar represents one donor, indicated as numbers on the *x*-axis. All 4 preparations significantly reduced the number of penetrated particles. *C*, To test whether the effects on adsorption were actually due to antibodies, purified IgG from the 2 adsorption-inhibiting plasma samples were retested with the dual fluorescent virus. IgG from an HCMV-seronegative donor was used as a reference, and the particle numbers were separately normalized to the reference values for each experiment. Each bar represents 1 donor, indicated as numbers on the *x*-axis. Error bars represent standard errors of the mean. neg, negative. ***$P < .001$.

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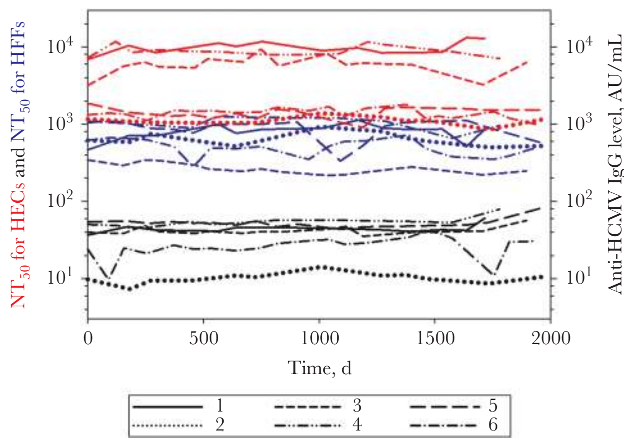

Figure 5. Stability of human cytomegalovirus (HCMV)–neutralizing capacities. The cell type–specific neutralizing capacities of plasma samples from 6 elite-neutralizing donors were analyzed retrospectively over approximately 5 years (donors 1–6 are represented by different line types). In parallel, corresponding anti-HCMV immunoglobulin G (IgG) concentrations were measured at each donation time point by a commercially available enzyme-linked immunosorbent assay (ELISA; by black lines). Human foreskin fibroblast–specific (blue lines) and human endothelial cell–specific (red lines) half-maximal neutralization titers (NT_{50} values) of each plasma donation were determined by an ELISA-based neutralization assay and plotted on a logarithmic scale.

exceptional antibodies that are highly potent and broadly active against most genetic variants of HIV [23]. Such antibodies are now evaluated in clinical trials to improve antibody-based interventions for the treatment of HIV infection [49]. The situation with HCMV is similar, since most HCMV-infected individuals develop a neutralizing antibody response that does not reliably limit viral replication or transmission [9]. It is tempting to assume that—like in the HIV field—elite HCMV neutralizers with exceptionally potent and broad antibodies exist and may serve as a source to improve passive immunization.

Ideally, plasma with an elite capacity for HCMV neutralization should be highly efficient against a variety of virus strains and against both the pentamer-dependent and the trimer-dependent HCMV entry pathway. Fifty-eight of 9000 HCMV-seropositive blood donors (0.6%) had plasma specimens that fulfilled these criteria, which resembles the frequency of donors (1.7%) demonstrating elite anti-HIV neutralization capacity when similar criteria were used (ie, neutralization titers of >100 against various virus strains). These elite-neutralizing donors had remarkable qualitative features. First, their antibodies exhibit more-balanced activity against the 2 entry routes. With elite-neutralizing donors, the ratio of the NT_{50} for HECs to that for HFFs was about 2, whereas it is usually about 10. This could indicate that their antibodies are particularly active against the trimer-dependent route. Second, the neutralizing capacity of their anti-HCMV antibodies appears to be up to 10-fold higher than that of normal immunoglobulin and HCMV hyperimmunoglobulin. Third, 2 elite neutralizers inhibited HCMV infection at the level of virus attachment. B cells producing anti-HCMV antibodies have been successfully cloned from seropositive individuals [50], and it is therefore tempting to speculate that cloning B cells from elite neutralizers could yield particularly potent anti-HCMV antibodies. In particular, a targeted combination of penetration inhibitors and attachment inhibitors directed against gB, trimer, and pentamer may be beneficial due to additive effects.

Regarding clinical applications of plasma from elite-neutralizing donors, it is relevant that their neutralization capacities were stable over long periods. They can therefore be revisited repeatedly, either for collection of plasma specimens for direct therapeutic use or for isolation of B cells that produce antibodies with particular qualities.

In conclusion, screening of HCMV-seropositive blood donors with a 2-step selection procedure helped identify elite HCMV-neutralizers with broad and potent anti-HCMV capacities. Plasma specimens of such donors might serve as a basis for improved passive immunization strategies.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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