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Dissection of the antibody response against herpes simplex virus glycoproteins in naturally infected humans

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Abstract

Relatively little is known about the extent of the polyclonal antibody (PAb) repertoire elicited by HSV glycoproteins during natural infection and how these antibodies affect virus neutralization. Here, we examined IgGs from ten HSV-seropositive individuals originally classified as high or low virus shedders. All PAbs neutralized virus to varying extents. We determined which HSV entry glycoproteins these PAbs were directed against: glycoproteins gB, gD and gC were recognized by all sera but fewer reacted against gH/gL. We previously characterized multiple mouse monoclonal antibodies (MAbs) and mapped those with high neutralizing activity to the crystal structures of gD, gB and gH/gL. We used a biosensor competition assay to determine whether there were corresponding human antibodies to those epitopes. All ten samples had neutralizing IgGs to gD epitopes but there were variations in which epitopes were seen in individual samples. Surprisingly, only three samples contained neutralizing IgGs to gB epitopes. To further dissect the nature of these IgGs, we developed a method to select out gD- and gB-specific IgGs from four representative sera via affinity chromatography, allowing us to determine the contribution of antibodies against each glycoprotein to the overall neutralization capacity of the serum. In two cases, gD and gB accounted for all of the neutralizing activity against HSV-2, with a modest amount of HSV-1 neutralization directed against gC. In the other two samples, the dominant response was to gD.

Importance

Antibodies targeting functional epitopes on HSV entry glycoproteins mediate HSV neutralization. Virus neutralizing epitopes have been defined and characterized using murine monoclonal antibodies. However, it is largely unknown whether these same epitopes are targeted by the humoral response to HSV infection in humans. We have shown that during

natural infection, virus-neutralizing antibodies are principally directed against gD and gB and to a lesser extent, gC. While several key HSV neutralizing epitopes within gD and gB are commonly targeted by human serum IgG, others fail to induce consistent responses. These data are particularly relevant to the design of future HSV vaccines.

Introduction

Herpes simplex virus (HSV) infects the mouth, skin, eye, and genital regions. The hallmark of herpes simplex viruses is their ability to establish a life-long latent or persistent infection in sensory neurons with reactivations that cause recurrent disease or viral shedding without evidence of clinical symptoms. Currently, 16% of the United States population is seropositive for HSV-2 and 54% for HSV-1 (1). Although HSV-2 is typically the causative agent of genital lesions, there is an increased incidence of genital HSV-1 infection. Control and clearing of the virus and genital lesions has been ascribed to the generation of cellular immunity (2). Recently, Schiffer et al. (3) suggested that mucosal HSV-2 specific CD8+ T-cells represent containment of prior viral shedding rather than a correlate of future protection. The importance of the humoral response in humans with recurrent shedding is unknown; however, it has recently been shown that antibodies play a key role in HSV infection and protection (4-6). In particular, the essential HSV entry glycoproteins gD, gB, gH/gL, which are the major stimuli of virus neutralizing antibodies (7-12), are crucial to this host response. In addition, we showed that HSV gC is a major inhibitor of the complement cascade and therefore innate immunity (13-15).

In previous studies, we developed panels of polyclonal (PAb) and monoclonal (MAb) antibodies for all five glycoproteins and these antibodies have been characterized for their ability

71 to neutralize virus (8, 10, 16-18). For the gB MAbs, the epitopes have been further localized on
72 via EM imaging of gB-Fab complexes (19). Antibodies against entry glycoproteins use several
73 different mechanisms to inhibit virus infection, namely: 1) blocking the interaction between gD
74 and receptor; 2) blocking gD-gH/gL interaction; 3) blocking gH/gL-gB interaction; and 4)
75 interfering with gB's ability to fuse membranes (12, 16, 20-22). Interestingly, the MAbs that
76 show virus neutralizing activity recognize both linear and conformation-dependent epitopes.

77 Specific antibodies that recognize important epitopes on HSV envelope glycoproteins are
78 poorly defined in the human response to natural infection by virus. However, for either vaccine
79 design or evaluation of sera from vaccines, it is important to identify those epitopes on the entry
80 glycoproteins that stimulate virus neutralizing antibodies. In addition, those antibodies are likely
81 essential in clearing infectious HSV during reactivation events. The overall objective of this
82 study is to determine the magnitude and repertoire of antibodies that recognize HSV viral
83 glycoproteins in sera of patients that are naturally infected with HSV, and then relate this
84 information to the mechanisms of virus neutralization and virus shedding.

85 Here, we studied ten sera of people who are naturally infected with HSV-2 and in whom
86 the rate of genital shedding was previously assessed for frequency and magnitude (23). Six
87 persons were infected with both HSV-1 and HSV-2, while four were infected only with HSV-2.
88 We found that in every case, the patients developed type-common neutralizing sera to these
89 viruses. In order to learn more about what constitutes the humoral response to HSV as it occurs
90 in humans, we used four methods to further examine the IgGs isolated from serum: 1) testing the
91 purified IgGs against Western blots of purified glycoproteins of both serotypes; 2) epitope
92 analyses of these ten IgGs by biosensor competition assays with neutralizing mouse MAbs
93 against specific epitopes of gD and gB; 3) fractionation of four antibodies into gD specific and

gB specific IgGs using tandem affinity chromatography; and 4) determination of virus neutralization by these four anti-gD and anti-gB specific IgGs.

Materials and Methods

Cells and soluble proteins. African green monkey kidney (Vero) cells were grown in Dulbecco's modified Eagle medium (DMEM) with 5% FBS. All soluble HSV glycoproteins were produced from baculovirus-infected insect (Sf9) cells. gD1(306t) and gD2(285t) were purified using a DL6 immunosorbant column (24-26). gC1(457t) and gC2(426t) were purified as previously described (27). gH1(803t)/gL1 and gH2(803t)/gL2 were purified via a C-terminal six-His tag by use of Ni-nitrilotriacetic acid resin and elution with imidazole (28). gB1(803t), gB2(720t)+His and gB2(727t) were purified by use of a DL16 immunosorbent column (29-31).

Antibodies. gD-specific MAbs 1D3, DL11, MC2, MC5, MC14, and MC23 (16, 17, 32) and gB-specific MAbs C226, DL16, H1817, H1695, SS10, SS55, SS63, and SS144 (8, 33-36) were characterized previously. PAbs used in this study were as follows: rabbit (R) serum R7 was raised against purified gD2 (and cross-reacts with gD1) (37), R176 was prepared against purified gH2(803t)/gL2 (38), R137 was raised against purified gH1(803t)/gL1 (39), R68 was raised against purified, denatured gB1 (8, 40), and R90 was raised against purified, native gB2 from HSV-2 (333) infected cell lysates; R47 and R64 were raised against native gC1 and gC2, respectively (41).

Human samples. Sera samples were obtained from HSV-2 infected persons enrolled in natural history studies at the University of Washington Virology Research Clinic in Seattle, WA. From each participant daily swabs of genital secretions were collected to characterize their viral shedding rate as described in Tronstein et al. (23). The samples were purposefully chosen for this study from 5 patients with high shedding rates, defined as detection of HSV on >10% of days, and 5 patients with low shedding rates, defined as <10% of days. All laboratory assays were performed by investigators who were masked to the shedding phenotype. IgG was purified from human sera by protein G chromatography (HiTrap; GE Healthcare) according to the manufacturer's instructions. Following purification, IgGs were dialyzed against phosphate-buffered saline (PBS). IgG yields ranged from 2.6 mg/mL to 7.2 mg/mL. IRB #815813 (Human sera with HSV2 antibodies, approval date: April, 2014).

Western blotting. Purified glycoproteins were separated by electrophoresis on a 10% sodium dodecyl sulfate-polyacrylamide gel under denaturing or "native" (32) conditions. Separated proteins were transferred to nitrocellulose and probed with IgG purified from human sera. A control blot was cut into strips and probed with rabbit PAbs specific for each glycoprotein.

Biosensor/surface plasmon resonance (SPR) experiments. Experiments were performed using a Biacore 3000 optical biosensor (GE Healthcare, Biacore Life Sciences) at 25°C. Filtered and degassed HBS-EP buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20) was used in all experiments. An anti-His antibody (QIAGEN, Inc.) was covalently coupled to a CM5 sensor chip (Biacore) following our previous protocol (42). Next, 200 resonance units (RU) of purified gD2(285t) or gB2(720t) were captured by the anti-His

antibody via their C-terminal His tags. Purified human IgGs were then injected for 240 s, followed by IgGs from mouse MAbs (75-150 μg/ml), also for 240 s. After each experiment the chip surface was treated with brief pulses of 0.2 M Na_2CO_3 (pH 11) until the RU signal returned to baseline, then a new cycle was started. All injections were performed at a flow rate of 5 μL/min.

Virus neutralization assays. Serial dilutions of IgG were mixed with HSV-1 (KOS) or HSV-2 (333) and the mixture was incubated at 37^{o}C for 1 h. Monolayers of Vero cells grown in 48-well plates were then incubated with the IgG-virus mixture for 24 h. Cells were fixed with methanol-acetone (2:1) and plaques were visualized by the black plaque assay (27).

Selection of gD- and gB-specific IgGs with glycoprotein antigen-sorbent columns. Briefly, 1.1 g activated CNBr Sephorose 4B (Pharmacia) was swollen in 0.001 N HCl at room temperature (RT) for 1 h. The gel was washed with 100 ml of 0.001 N HCl on a glass filter. Soluble, purified gB2(727t) or gD2(285t) (10mg) in 2.8 mL PBS plus 2.8 mL of freshly made 0.1M carbonate buffer (0.2 M sodium bicarbonate, 1M NaCl, pH 8.3) was mixed with the gel overnight at 4^{o}C. Next, the mixtures were washed with 1M ethanolamine (pH 8.0), then with 0.1 M sodium acetate 1M NaCl (pH 4.0), and finally with 0.1 M sodium borate 1M NaCl (pH 8.0), all at RT. The protein-gel mixtures were then equilibrated with TS washing buffer (10mM Tris-HCl pH 7.2, 0.5M NaCl) at 4^{o}C and loaded into individual columns. Both columns were washed extensively with TS buffer before loading IgGs from human sera. The flow through was collected and recycled through each column 5X followed by washing with TS buffer. The gD- or gB-specific IgGs were eluted with 3 mL of 0.1M ethanolamine (pH 11.5). The selection of gD

IgG was done first, followed by passing the gD-depleted IgG sample through the gB column. The eluted samples were further dialyzed with PBS and concentrated with centrifugal filter units (Millipore). For sample 6, we passed the gD- and gB-depleted IgG over a gC1(346t) column (43) using the same procedure.

Results

We obtained 10 blinded serum samples from a study of people naturally infected with either HSV-2 alone (samples 2, 4, 5, 8) or both HSV-1 and HSV-2 (samples 1, 3, 6, 7, 9, 10) (Table 1) (23). Serum samples 1-5 were from people who shed virus infrequently (low shedders), while samples 6-10 were from people who shed virus frequently (high shedders). Purified IgG was used in all analyses.

Virus neutralization. The human antibodies were screened for virus neutralizing activity against either HSV-2 (333) (Fig. 1A) or HSV-1 (KOS) (Fig. 1B) via a standard plaque reduction assay. Virus neutralization activity was classified as weak if it required >30 µg/mL IgG to elicit 50% plaque reduction (Table 1). Of the five IgGs from low shedders, three (samples 1-3) neutralized HSV-2 poorly while the remaining two (samples 4 and 5) neutralized HSV-2 well (Fig. 1A). For the high shedders, four of five samples (samples 6, 7, 9, and 10) showed strong neutralizing activity against both HSV serotypes. While the numbers are small, it does not appear that high neutralizing activity effectively controls shedding.

IgG binding to purified HSV glycoproteins. Purified gD, gB, gH/gL, and gC (for both HSV-1 and HSV-2) were run on SDS-PAGE gels under "native" (conformationally correct) (32) conditions. Western blots were then probed with each of the ten sample IgGs. As a control,

we probed another blot with a rabbit PAb generated specifically to each purified glycoprotein (Fig. 2A). Each of the ten human IgGs reacted against gD and gB of both HSV serotypes (Fig. 2B and 2C). All sample IgGs recognized HSV-2 gC. However, only two samples (numbers 6 and 10) reacted strongly against HSV-1 gC or gH/gL of either serotype. It is possible that the lack of detection of gH/gL may be due to improper conformation of the heterodimer, as even "native" Western blots contain a small amount of detergent (32).

Thus, the Western blot data indicate that all ten sera had a significant response to type-2 gD, gB, and gC, as well as type-1 gD and gB. It was surprising that so few samples recognized gH/gL of either serotype, either under native (Fig. 2) or denaturing (data not shown) conditions. As the most consistent responses were directed at gD and gB, we focused our next series of experiments on them.

Competition with murine anti-gD MAbs. Antibodies against entry glycoproteins use several different mechanisms to inhibit virus infection (12, 16, 20-22). The six anti-gD MAbs used in this study (Fig. 3A) all affect gD function, but in different ways. Three MAbs block gD-receptor binding (1D3, MC23, Dl11) and two block the gD-gH interaction (MC2 and MC5) (16). We also included the non-neutralizing MAb MC14, which significantly enhances neutralization by MAb MC2 (16). The locations of the epitopes of these MAbs are shown mapped to the gD crystal structure (Fig. 3B).

To characterize the responses to these six gD epitopes in naturally-infected individuals, we used a protein-binding competition assay using surface plasmon resonance/biosensor (8, 44, 45). The goal was to determine whether and to what extent the binding of these MAbs to gD is diminished by pre-incubation of gD with human IgG. Reduced MAb binding would indicate the

presence of human antibodies to an epitope that binds to or overlaps that of the neutralizing MAb. We first captured soluble gD2(285t) (to accommodate the type-2 specificity of MAb MC2) (16) on the surface of a biosensor chip via its 6-His tag. Each human IgG was then flowed across the chip surface to allow binding of gD-specific antibodies (Fig. 4A). A separate chip surface containing gD was exposed, in parallel, to buffer alone. Individual mouse MAb IgGs were flowed across the test and control surfaces. The difference in MAb binding to the two surfaces upon completion of the injection was used to calculate the MAb blocking activity of each human IgG. For example, if the human IgG contained antibodies that bound the same (or nearby) epitope as that of the mouse MAb, the mouse MAb would be unable to bind gD. However, if these antibodies were absent from the human IgG, the mouse MAb should have free access to the epitope and be able to bind to gD. Curves for all samples are shown in Fig. S1 and blocking of each MAb is summarized in bar graphs (Fig. 4B). We used an arbitrary cutoff point of 20% in scoring samples positive for blocking (45).

Surprisingly, none of the ten IgGs blocked MAbs 1D3 or MC14 (Fig. 4B, Table 2), both of which bind to linear epitopes (Fig. 3A). However, all ten IgGs blocked at least one of the six MAbs and the overall level of binding correlated with the extent of neutralization. Thus, four of the weak virus-neutralizing IgGs (samples 1, 2, 3, 8) blocked MC2, MC5, and MC23 at or below 30% (Fig. 4). Five IgGs with high virus-neutralizing activity (samples 4, 5, 6, 9, 10) significantly blocked MC5, MC23, and DL11 from binding to gD but had less effect on MC2. Consequently, these IgGs targeted both receptor binding and a post-receptor binding step. In contrast, sample 7 was unique. It blocked predominantly MC2 (78% blocking) and only weakly blocked DL11 (27%) (Fig. 4B, Table 2), suggesting that IgG from sample 7 may neutralize primarily by blocking a post-receptor binding step (based on the mechanism of action of MAb

MC2). Thus for gD, there were a variety of responses among the ten human sera and both receptor binding and post-receptor binding functions were targets.

Competition with murine anti-gB MAbs. A subset of our panel of anti-gB MAbs (Fig. 5A) (8) were chosen for this study. We grouped the antibodies into four functional regions (FR, as defined by Bender et al. (8)) involved in virus neutralization (36, 46) and mapped them to the crystal structure of post-fusion gB (Fig. 5B). FR1 is located at the base of gB and contains the fusion loops; it is the site of gB-lipid association (28, 46, 47). FR1 is made up of two distinct structural domains, and different MAbs bind to each of these (SS55 to domain I, blue, and SS144 to domain V, red) (Fig. 5B). FR2 is in the middle lobe of gB and is postulated to associate with gH/gL during the fusion process (20, 48); it is represented by MAb C226. FR3 is within the gB crown, which is a site for gB-cell interaction (29, 33); MAbs SS10 and SS63 bind here. Lastly, FR4 is located within the gB N-terminus (8) and its crystal structure is unknown (dashed lines, Fig. 5B). MAb H1817 binds within FR4 (residues 31-43) (8, 35).

We next screened within the ten human IgGs, whose binding to gB shown in Fig. 6A, for the ability to block the binding of these six gB-specific antibodies using the biosensor competition assay (Fig. S2). We used gB2(720t) in this assay, as all of the anti-gB MAbs bind to both type-1 and type-2 gB. Remarkably, seven out of the ten IgGs (samples 1, 2, 3, 4, 5, 7, 8) did not block any of the MAbs from binding to gB (Fig. 6B, Table 2), suggesting that none of these individuals raised antibody to these important neutralizing sites. This much more limited response is in contrast to what we saw for gD. Of note, all ten samples reacted strongly with gB via native Western blot (Fig. 2B and 2C), suggesting that the majority of people raised antibody to non-neutralizing epitopes of gB.

Three IgG samples competed with at least one neutralizing gB MAb. IgG from these samples blocked the binding of the MAbs SS55 and SS144 (Fig. 6B), two Mabs to FR1. Sample 10 also blocked the binding of FR2 MAb C226 (Fig. 6B). It is noteworthy that none of the IgGs competed with MAbs with epitopes in the gB crown/FR3 (SS10, SS63) or the gB N-terminus/FR4 (H1817) (Fig. 6B, Table 2). We suggest that these IgGs neutralize virus by binding to FR1, thereby inhibiting fusion loop insertion. For sample 10, gB-specific IgG likely neutralize virus by inhibiting the gB-gH/gL interaction as well (20, 49).

Purification of gD- and gB-specific IgGs. The following experiments were designed to dissect within the human IgGs for glycoprotein-specific neutralization activity. We developed antigen-sorbent columns to capture gD-specific and gB-specific IgGs (Fig. 7A). We selected four highly neutralizing human IgGs (samples 4, 6, 7, and 10) that were predicted by our biosensor competition assay to have different mixes of antibodies directed against gD- and gB-neutralizing epitopes (Figs. 4B and 6B, Table 2).

Total IgGs were passed over a gD2(306t) column to which the soluble gD was covalently linked to Sepharose 4B. The flow-through for each sample was collected and saved for the next step; IgGs bound to the column gD were eluted under acidic conditions (Fig. 7A). The eluate and flow-through were then tested by ELISA against soluble gD2. The flow-through was found to be devoid of anti-gD activity (Fig. 7B). This flow-through fraction was then passed over a gB2(727t) antigen-sorbent column (Fig. 7A). The eluate contained anti-gB activity and none remained in the column flow-through (Fig. 7C). The gD- and gB-specific pools of IgGs represented approximately 2% each of the total starting IgG. The final flow-through contained all of the remaining anti-HSV IgGs (“other”, e.g. anti-gC, anti-gH/gL). Since three of the IgGs subjected were from people dual-infected with HSV-1 and HSV-2 (samples 6, 7, 10; Table 1),

we tested each IgG pool for their ability to neutralize both HSV-1 and HSV-2 (Fig. 8). For clarity, we report our analysis of each sample separately.

Sample 10. Both anti-gD specific IgG (white circles) and anti-gB specific IgG (black circles) neutralized HSV-2 (Fig. 8A) and HSV-1 (Fig. 8B) equally well. Interestingly, the remaining IgG (gray triangles), devoid of anti-gD and anti-gB specific antibodies, failed to neutralize HSV-2 (Fig. 8A) yet neutralized 36% of HSV-1 at the highest concentration tested (50 μg/mL) (Fig. 8B). The data suggest that for sample 10, all of its neutralizing activity against HSV-2 comes from anti-gD and anti-gB IgGs, but other antibodies (such as anti-gC or anti-gH/gL) play some role in neutralizing HSV-1.

Sample 6. gD- and gB-specific IgGs prepared from sample 6 neutralized HSV-2, although the gD-specific IgG was more potent (Fig. 8A). Against HSV-1, the difference in neutralizing activity was quite striking. Whereas the gD-specific IgG was just as potent against HSV-1 as against HSV-2, the gB-specific IgG failed to reach 50% neutralization at the highest concentration tested (12.5 μg/mL) (Fig. 8B). Sample 6 did not compete with a key anti-gB neutralizing MAb (C226 in FR2, Fig. 8A) and this may be the reason why its gB-specific neutralization activity is lower than that of sample 10. Similar to sample 10, the remaining IgG ("other") for sample 6 exhibited anti-HSV-1 but not anti-HSV-2 neutralizing activity (Fig. 8). To determine what might be in the "other" fraction, we passed the flow-through of sample 6 (after passage over the gD and gB columns) over a column that contained gC1. The IgG in the eluate had virus neutralizing activity against HSV-1 but not HSV-2 (data not shown). No detectable virus neutralizing activity remaining in the flow-through (data not shown). There was no detectable virus neutralization activity remaining in the flow-through (data not shown). Thus,

sample 6 contains modest neutralizing activity targeting gC1 but antibodies against other glycoproteins, including gH/gL, were insufficient to neutralize virus.

Sample 7. This sample competed well against the anti-gD MAb MC2 but to a much lesser extent against the other anti-gD MAbs (Fig. 4). Interestingly, sample 7 failed to compete with any gB-specific MAbs (Fig. 6). After separation, gD-specific IgG neutralized HSV-2 and HSV-1. However, as expected, the gB-specific IgG and the remaining flow-through IgG did not (Fig. 8). We conclude that for sample 7, all of its virus neutralizing activity comes from gD-specific IgG and within that IgG, an "MC2-like" antibody accounts for the majority of the response to gD.

Sample 4. IgG from sample 4 blocked several anti-gD MAbs with a pattern similar to sample 10 (Fig. 4). Additionally, sample 4 exhibited poor blocking of gB-specific MAbs (Fig. 6). All neutralizing activity against both HSV-1 and HSV-2 was contained within the gD-specific IgG fraction (Fig. 8). We saw no difference in neutralizing activity between HSV-1 and HSV-2, even though sample 4 IgG came from an individual infected with HSV-2 only (Table 1). The anti-gD neutralizing activity of sample 4 was weaker than that of samples 6 and 10, which is also what we observed for virus neutralization by the samples' total IgG (Table 1). In addition, gD binding activity of the total IgG was much lower for sample 4 than for samples 6 or 10 (Fig. S1A).

For the samples fractionated using our tandem-capture system, most of the virus neutralizing activity was traced to either anti-gD IgGs (samples 4 and 7) or a combination of anti-gD and anti-gB antibodies (samples 6 and 10). For samples 6 and 10, there appeared to be some HSV-1 specific neutralization activity within the "other" IgG fraction at the highest

317 concentration tested (50 μg/mL) (Fig. 8B). In the case of sample 6, this is largely due to
318 antibody directed at gC1 (data not shown). Both of these samples were obtained from patients
319 infected with both HSV-1 and HSV-2 (Table 1).

320

321 **Discussion**

322 Little is known about the antibody repertoire in humans elicited by HSV glycoproteins during
323 infection, although neutralizing activity against gD and gB has been documented in human sera
324 (50, 51). We examined the sera from ten HSV seropositive individuals who had been previously
325 tested and classified according to their frequency of viral shedding (23). When we began this
326 study, we started with what could be regarded as two relatively simple questions: 1) what
327 comprises a neutralizing antibody response to HSV in naturally infected humans; and 2) is there
328 any relationship between the extent of virus shedding and neutralization? Our results highlight
329 the complexity of the answers even in this small set of samples. While all ten samples contained
330 neutralizing IgG, six were identified as containing strong neutralizing antibodies (Table 1).
331 However, we could find no association between IgG neutralization activity and the rate of viral
332 shedding. Furthermore, all ten samples contained IgGs strongly reactive against HSV gB, gD,
333 and gC. We were surprised by how few samples contained antibodies that react against purified
334 gH/gL of either serotype.

335 **Competition with neutralizing MAbs**. Here we showed that we could delve more
336 deeply into specific epitopes of gD and gB that were recognized by the ten samples using
337 competition analysis. However, it should be noted that blocking of the neutralizing mouse MAb
338 by the human PAb does not mean the epitopes are identical; rather we can say a similar

neutralizing antibody is likely to be in the polyclonal mix. What did all of the IgGs have in common? All ten contained IgG that blocked MAbs which interfere with gD-receptor binding (MC23, DL11). Thus, this interaction is a prime target of an effective immune response. We plotted the percent MAb blocking of these 10 samples against their neutralization scores and calculated R^2 values for each plot (data not shown). The level of competition against DL11 and MC23 correlated quite well with total IgG neutralization rank (DL11: R^2=0.83; MC23: R^2=0.73). MAb MC5 blocking activity was observed in all samples, but was not as consistently predictive of neutralizing activity (R^2=0.36).

Although most IgGs blocked MC2, which targets a post-receptor binding function of gD (16, 49), by less than 33%, sample 7 blocked this MAb by 78%. Fractionation of antigen-specific IgG showed that this MC2-like IgG component likely accounts for the majority of this sample's HSV neutralizing activity. Thus, antibodies that target activation of gH/gL by gD can be induced in naturally-infected humans and provide potent virus neutralizing activity.

None of the human IgGs competed with the anti-gD MAbs 1D3 and MC14, both of which bind to linear epitopes. In a study just completed using samples from the recent GlaxoSmithKline vaccine trial with gD as the immunogen, we also noted a lack of competition between antibodies raised against gD and anti-gD MAbs to linear epitopes (45). Thus, these regions of gD are poorly immunogenic in the natural host regardless of whether the protein is presented to the human immune system as a subunit vaccine or as one of many antigens produced during infection. One wonders if future HSV vaccines (52, 53) should be designed to induce PAbs in humans against these important linear gD epitopes.

In contrast to the rich response to neutralizing epitopes of gD, we found only three individuals with IgG that blocked anti-gB neutralizing MAbs. FR1 epitopes were targeted by all three of these samples while an FR2 epitope was targeted by only one sample. FR1 is located at the base of gB and contains the fusion loops (FL) (8, 46, 47). One mechanism of neutralization by FR1 MAbs is by blocking FL insertion into target membranes. A previous study of the humoral response to gB in HSV infected humans found that antibodies targeting FR1 epitopes were well represented (50). This same study found that sera from HSV-1 infected individuals exhibited more effective blocking of FR1 epitopes than sera from HSV-2 infected patients. Consistent with this, the 3 samples from the current study that strongly blocked FR1 epitopes also came from patients infected with both HSV-2 and HSV-1. FR2 MAbs block infection by blocking an essential interaction between gH/gL and gB. Although several other gB epitopes contained within FR3 and FR4 were screened, none were strongly blocked by the serum IgG from infected individuals. This was particularly surprising in light of the strong reactivity of all samples to gB by Western blot (Fig. 2). Thus, it appears that these individuals targeted epitopes (perhaps non-neutralizing) that were not represented within our panel of MAbs.

Human anti-HSV neutralizing IgG is predominantly against gD and gB. Using affinity chromatography, we isolated gD and gB specific IgGs from four representative sera with strong or very strong neutralizing activity. Consistent with the well-documented role of gD as a dominant immunogen and inducer of neutralizing antibodies (51), virus neutralizing activity was observed in the gD-specific IgG fraction from each sample. In the two samples shown to possess gB MAb-blocking antibodies, the gB-specific IgG fraction also neutralized. Interestingly, the gB-specific IgG from sample 6 exhibited greater potency against HSV-2. This was somewhat unexpected since the ectodomains of gB1 and gB2 (except for the extreme N-termini) exhibit

high sequence identity (54, 55) and very few neutralizing gB MAbs are type-specific. In particular, the gB MAbs blocked by sample 6 IgG (SS55 and SS144) are type common.

For samples 6 and 10, there was a small amount of HSV1-specific neutralizing activity in the IgG depleted of gD- and gB-specific antibodies. Further analysis of sample 6 showed that this activity was directed against HSV-1 gC. Despite this, the relatively weak neutralizing activity directed against this protein was somewhat surprising. In addition to its role in immune-evasion (13-15), gC1 has been shown to generate neutralizing antibodies in animal models (56-59). We found no additional neutralizing activity in the flow-through after removal of gD2-gB2- and gC1-specific fractions from sample 6 IgG. This was unexpected since sample 6 IgG was one of only a few that recognized gH/gL by Western blot. gH/gL is highly immunogenic in rabbits and mice (39) and generates high-titer neutralizing antibodies directed against gH (10, 11, 60-62). Whether the poor response to gH/gL of either serotype is limited to the samples tested here or is more generally true is worth examining on additional samples.

We believe we have demonstrated that we have the tools to dissect the human immune response to HSV glycoproteins. The methods we applied to this small set of samples represent a template for examining samples taken from other cohorts of infected individuals and from subjects who are vaccinated against HSV using protein-based vaccines.

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626 **Figure Legends**

627 **Fig. 1.** Virus neutralization. Sample IgGs were tested for their ability to neutralize HSV-2 (A)
628 or HSV-1 (B). Plaque numbers were determined for each sample and plotted as a percentage of
629 plaques obtained in the absence of human IgG (y-axis). IgG concentration is indicated on the x-
630 axis. Sample numbers are indicated at the top of part (A); those from dual-infected (HSV-1/2)
631 individuals are indicated with an asterisk (*).

632

633 **Fig. 2.** Western blots. Purified glycoprotein D, gB, gH/gL, and gC were blotted onto
634 nitrocellulose via non-denaturing Western blot. (A) Control blots were probed with a mixture of
635 rabbit polyclonal IgG against each protein. For HSV-2 proteins (top panel), rabbit PAbs R90
636 (anti-gB), R176 (anti-gH/gL), and R64 (anti-gC) were used. For HSV-1 proteins (bottom panel),
637 PAbs R68 (anti-gB), R47 (anti-gC) and and R137 (anti-gH/gL) were used. Anti-gD PAb R7 is
638 type-common and was used for both sets of blots. Molecular weight markers are shown to the
639 left in kilodaultons. For the human samples, blots probed with IgG from individuals classified as
640 low virus shedders are shown in (B) and high shedders are shown in (C). Samples from dual-
641 infected (HSV-1/2) individuals are indicated with an asterisk (*).

642

643 **Fig. 3.** HSV gD MAb tree. (A) Diagram showing gD antigenic sites and associated MAbs used
644 in this study. The defining properties of MAbs within each branch of the tree are indicated.
645 Antigenic sites are defined by "Groups" of MAbs that compete with each other for gD binding.
646 MAb Group designations are shown in boxes. gD residues implicated in MAb binding are in
647 parentheses below group names. Representative MAbs are shown in large font below group

names, with known residues of *mar* (MAb-resistant) mutants indicated below. Virus-neutralizing MAbs are shown in bold type. Receptor-blocking MAbs are indicated by an asterisk. (B**)** Two views of the gD structure rotated 180 degrees. The locations of the epitopes of MAbs listed above are indicated.

Fig. 4. (A) Binding of subject IgG to gD2 via biosensor. A fixed amount of each IgG was injected across a chip surface coated with purified protein. The levels of IgG binding (response units, RU) for human sample IgG are shown. The samples with the strongest HSV-2 neutralization activity are represented by black bars and the weaker neutralizers by white bars. Error bars indicate standard error for at least two experiments. (B) Biosensor analysis: blocking of neutralizing gD MAbs via human subject IgG. Each bar indicates the percent blocking activity (as compared to no antibody control) against the anti-gD MAbs tested (1D3, MC14, MC2, MC5, MC23, DL11). Samples classified as strong HSV-2 neutralizers are highlighted in black along the bottom row. A representative experiment is shown.

Fig. 5. HSV gB MAb tree. (A) Diagram showing gB antigenic sites and associated MAbs used in this study. Antigenic sites are defined first within gB functional regions (FR) as defined by Bender et al. (8), and secondly by "Groups" of MAbs that compete with each other for gB binding. MAb Group designations are shown in boxes, color-coded to their epitope binding region as shown on the gB crystal structure in (B). gB residues implicated in MAb binding are in parentheses below group names. Representative MAbs are shown in large font below group names, with known residues of *mar* mutants indicated below.

670

671 **Fig. 6.** (A) Binding of subject IgG to gB2 via biosensor. (B) Biosensor analysis: blocking of
672 neutralizing gB MAbs via human subject IgG. Data for both (A) and (B) presented as in Fig. 4.

673

674 **Fig. 7.** Purification of gD- and gB-specific IgGs. (A) Total human IgGs were passed over a
675 gD2(306t) column where soluble gD is covalently linked to Sepharose 4B. The elute (white
676 circles) and flow-through (white triangles) for each sample were tested by ELISA for binding to
677 soluble gD (B); optical density (OD 405 nm) is shown on the y-axis, and IgG concentration on
678 the x-axis of each ELISA graph. The flow-through was next passed over a gB2(724t) antigen-
679 sorbent column, separating out the gB-specific IgGs (eluate, black circles) from the flow-through
680 which contained anti-gC, anti-gH/gL, and other anti-HSV IgGs (gray triangles). Once again, the
681 elute and flow-through for each sample were tested by ELISA, this time for binding to soluble
682 gB (C).

683

684 **Fig. 8.** Virus neutralization of antigen-sorbent column-selected IgGs. Anti-gD, anti-gB, and
685 flowthough ("other") IgGs were obtained as outlined in Fig. 7. IgGs were tested for their ability
686 to neutralize HSV-2 (A) or HSV-1 (B). Samples 6, 7, and 10 were from HSV-1 and HSV-2
687 dual-seropositive individuals, while sample 4 was from a person infected with HSV-2 only.
688 Plaque numbers were determined for each sample and plotted as a percentage of plaques
689 obtained in the absence of human IgG (y-axis). IgG concentration is indicated on the x-axis.

690

691

692

693 Table 1. Characteristics of IgG from each human sera sample

sample	HSV status	shedding[a]	neutralization[b]		reacts with glycoprotein[c]							
			HSV-1	HSV-2	gB-1	gB-2	gC-1	gC-2	gD-1	gD-2	gHgL-1	gHgL-2
1	1,2	low	23	90	+++	+++	+/-	+	+	+++	+	-
2	2	low	125	90	+++	+++	-	+++	+	+++	+/-	-
3	1,2	low	125	190	+++	+++	-	+	+	+++	+	-
4	2	low	23	18	+++	+++	+/-	+++	+++	+++	+	-
5	2	low	30	12	++	+++	-	+++	+	+++	-	+
6	1,2	high	8	5	+++	+++	+++	+++	+++	+++	++	-
7	1,2	high	7	12	+++	+++	-	+++	+	+++	-	-
8	2	high	55	38	+++	+++	-	+++	++	+++	-	-
9	1,2	high	3	10	+++	+++	+/-	+	++	+++	+	+
10	1,2	high	9	7	+++	+++	+++	+++	+++	+++	++	+

694 [a] As determined by Tronstein et al. (23). "High" shedding rates are defined as detection of HSV

695 on >10% of days, while "low" shedding rates are defined as <10% of days.

696 [b] Values represent a 50% reduction in plaques as compared to control (no IgG) and are shown as

697 μg/mL IgG.

698 [c] Determined by IgG reactivity to purified proteins via Western blot (Fig. 2).

699

700 Table 2. Percent blocking of mouse MAbs from human IgG samples

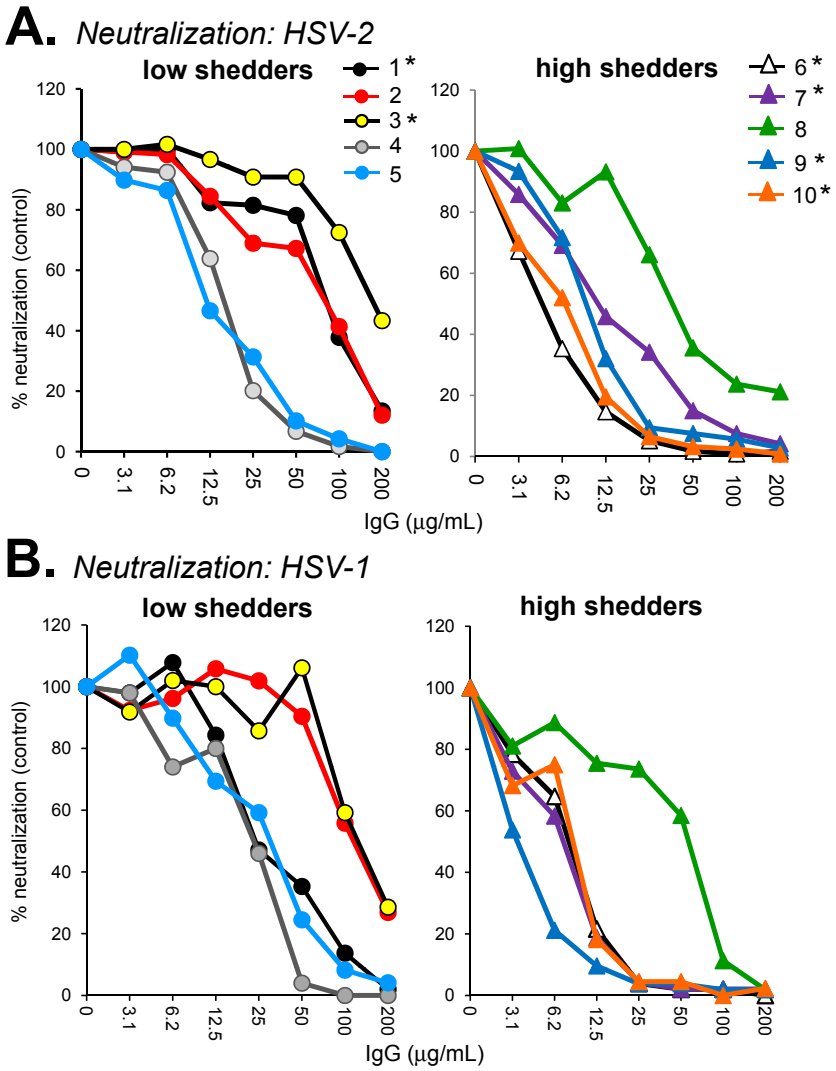
sample	**% blocking**[a] (αgD MAbs)						**% blocking** (αgB MAbs)					
	1D3	MC14	MC2	MC5	MC23	DL11	SS55	SS144	C226	SS10	SS63	H1817
1	2	-	0	28	20	15	5	-	1	-	-	-
2	0	3	7	15	26	19	-	-	-	-	4	-
3	6	8	22	30	23	5	-	10	-	-	6	-
4	10	-	13	42	55	58	1	13	0	-	-	-
5	16	-	21	42	50	47	-	10	3	-	8	-
6	10	-	26	62	90	99	43	46	-	-	-	-
7	-	-	78	16	16	27	-	-	-	-	1	-
8	0	0	30	19	25	16	-	3	-	-	1	-
9	-	-	33	42	61	59	24	29	13	14	15	-
10	-	-	7	30	76	68	57	36	46	1	13	-

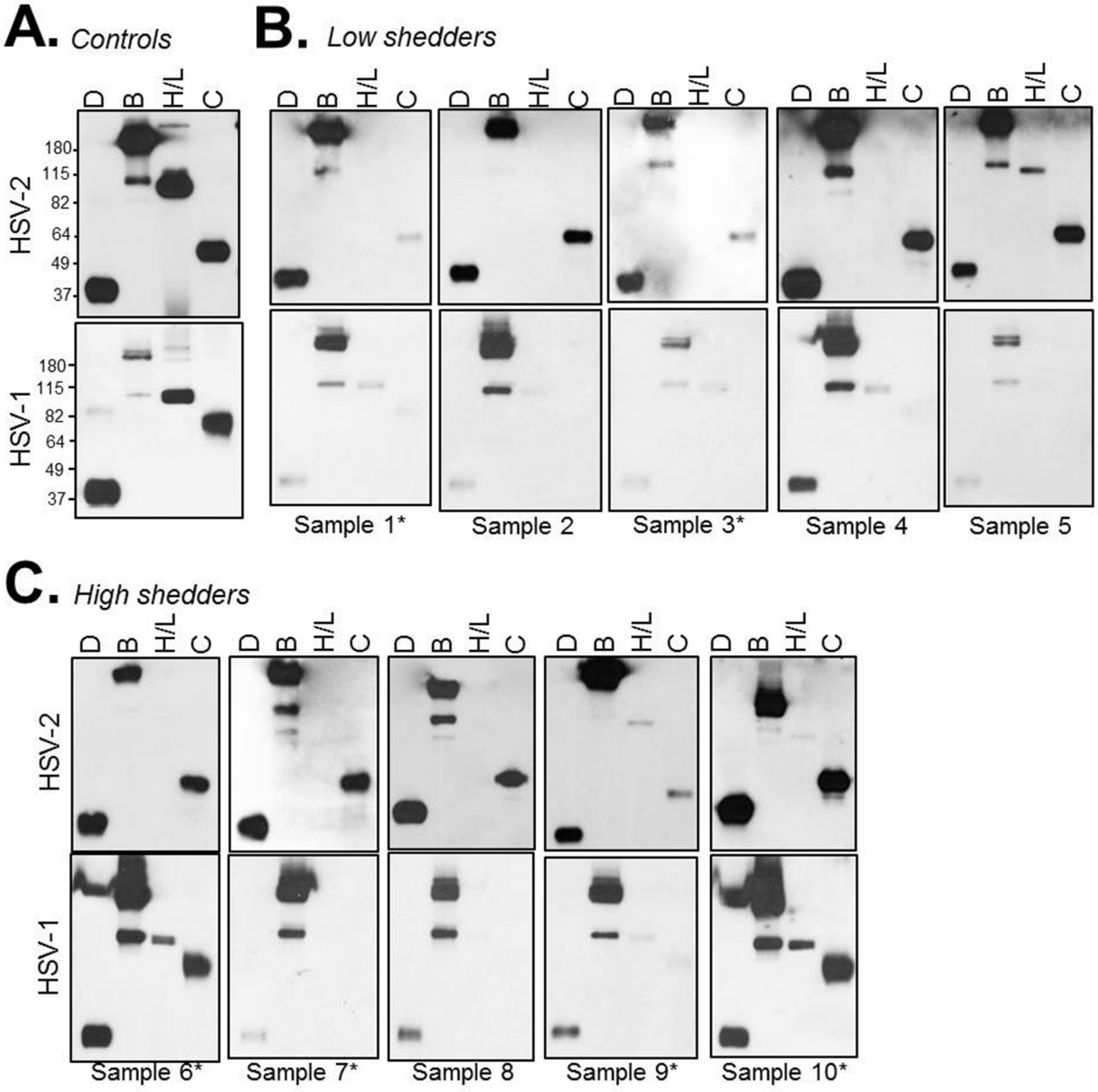
701 [a] Percent blocking activity (as compared to no antibody control). A dash represents numbers

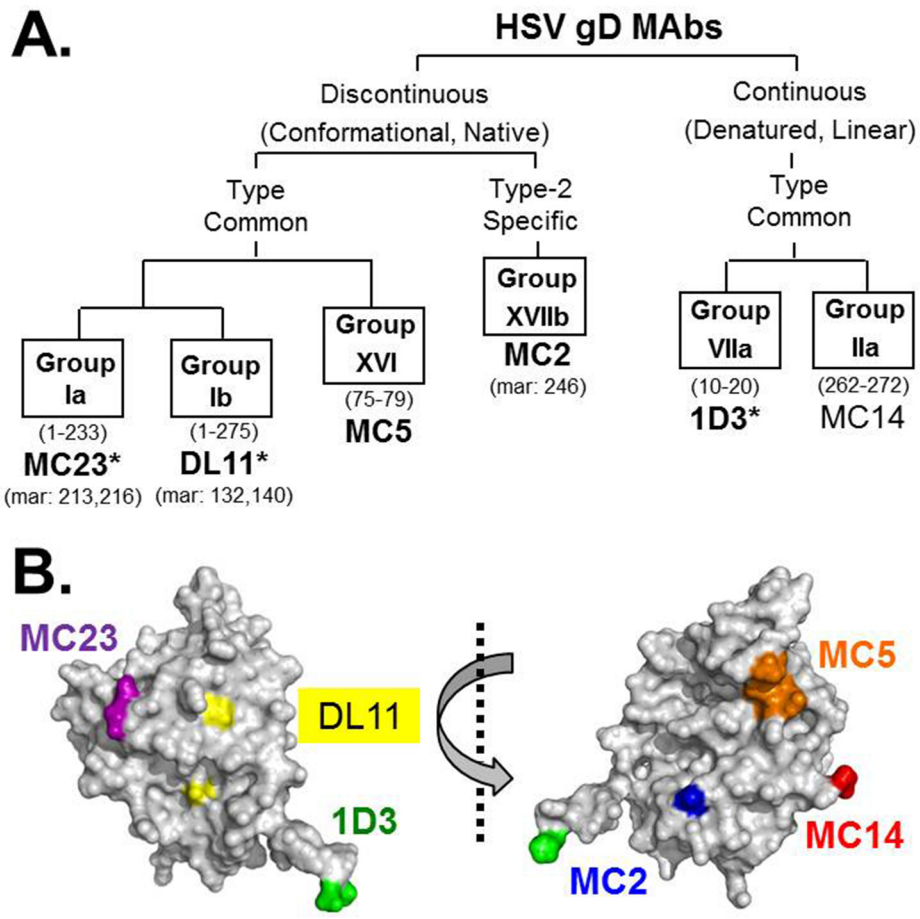
702 below 0% (no blocking).

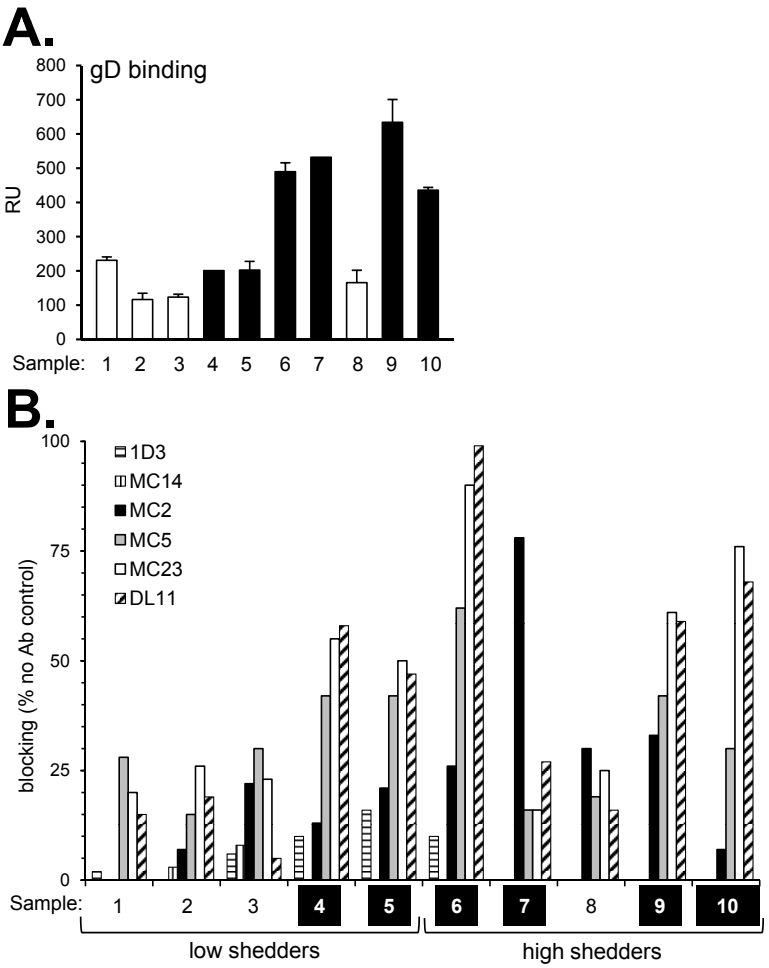
703

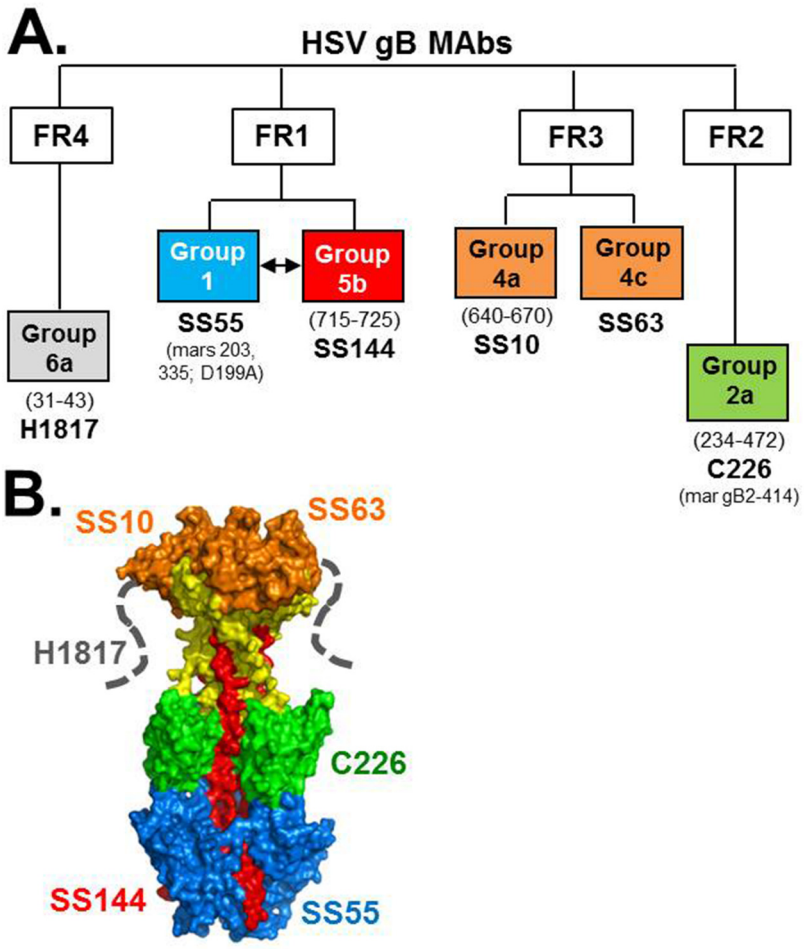
704

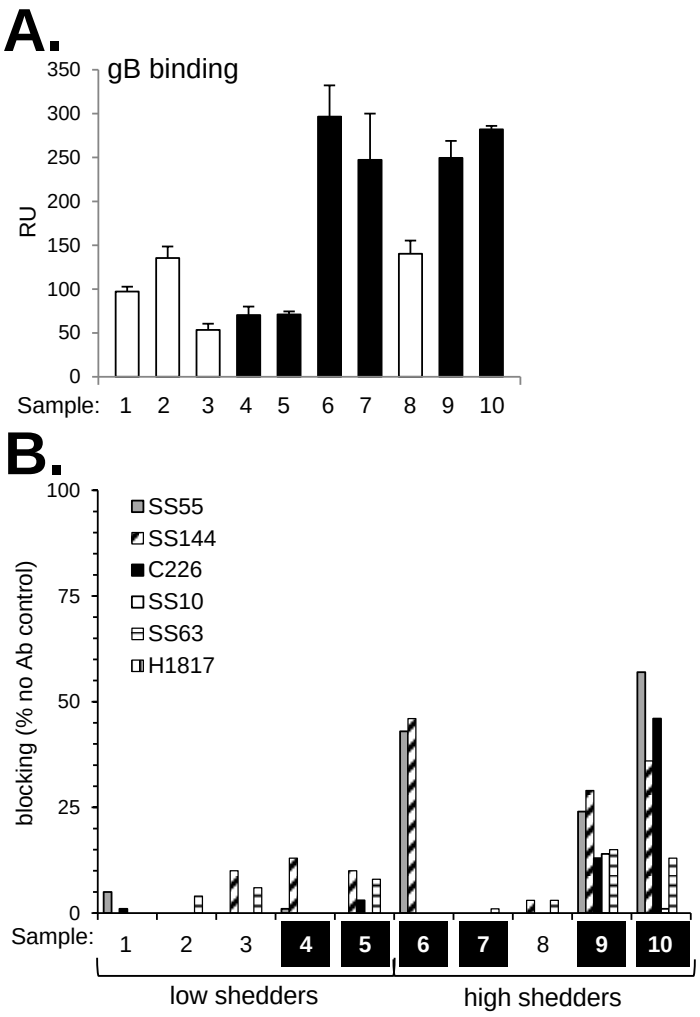


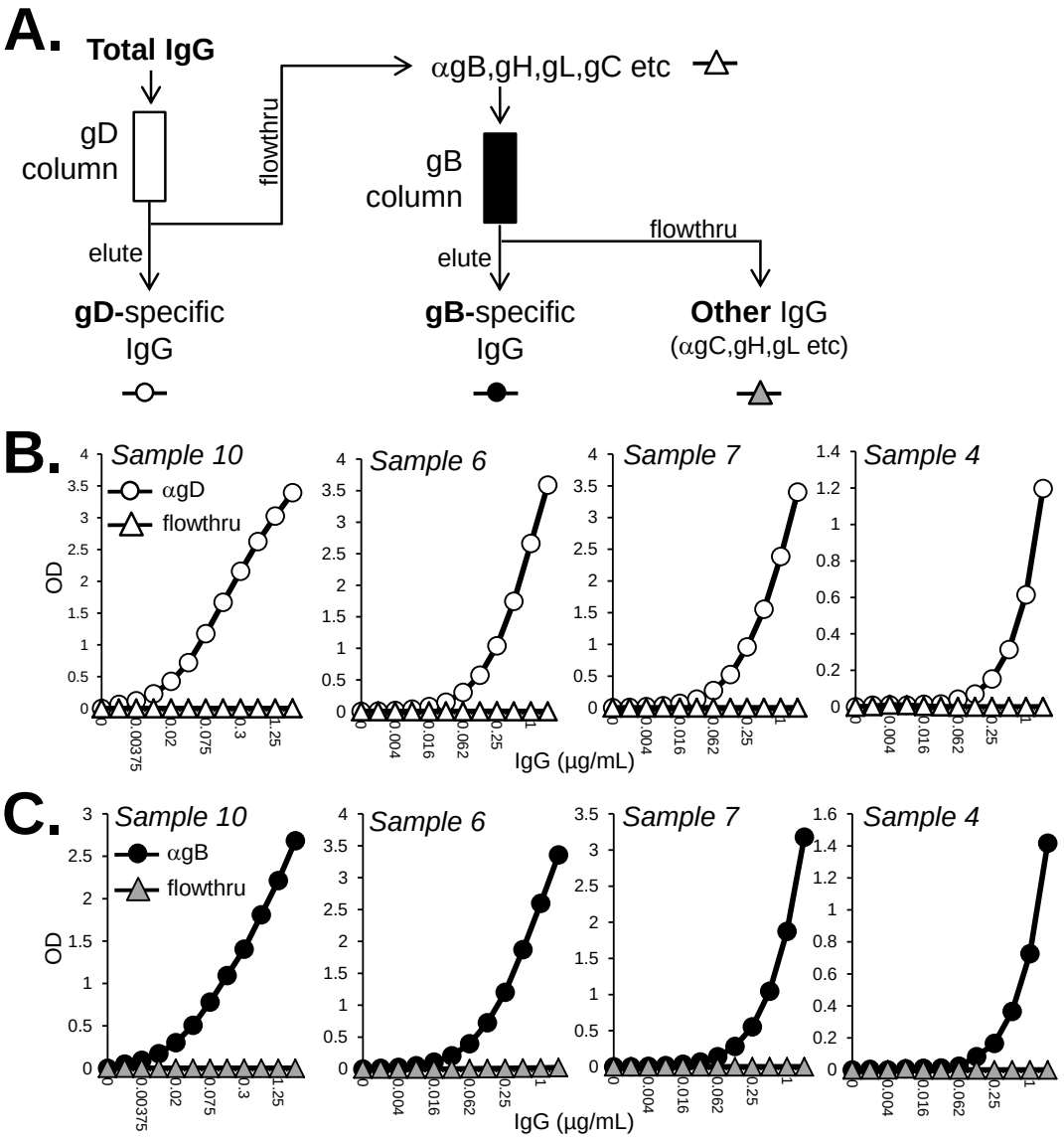




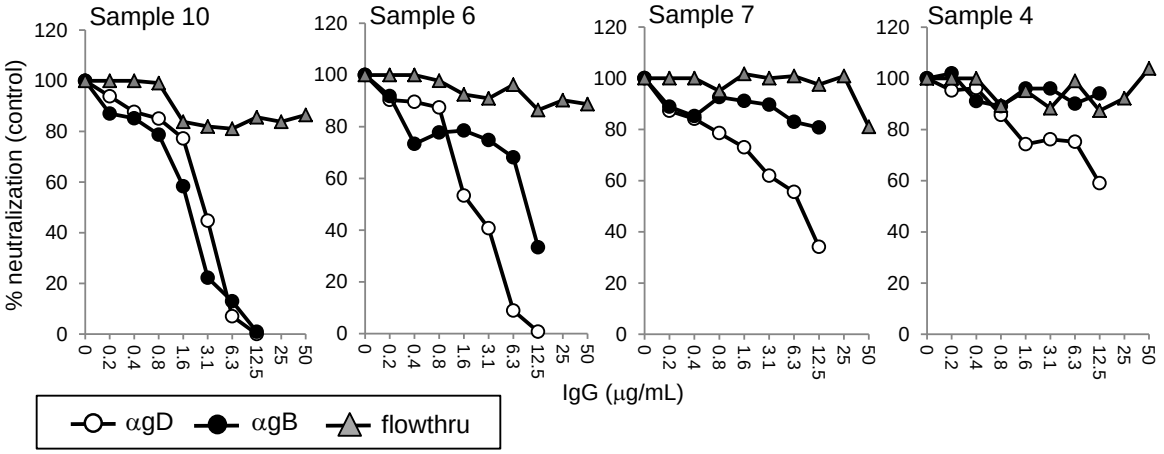








A. *Neutralization: HSV-2*



B. *Neutralization: HSV-1*