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Purification and Functional Characterization of Antibody Subclasses Produced in Response to HSV-2 gC Immunization in Mice

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Purification and Functional Characterization of Antibody Subclasses Produced in Response to HSV-2 gC Immunization in Mice

by

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ABSTRACT

Previous experimental vaccines for herpes simplex virus type 2 containing glycoprotein D (gD-2) singly or in combination with glycoprotein B have shown to elicit high levels of neutralizing antibodies, but failed to provide protection in clinical studies, suggesting the need for alternative strategies to improve subunit vaccine efficacy. Animal studies have shown that inclusion of HSV-1 glycoprotein C enhances neutralizing activity of a gD-1 vaccine, and so it is currently under consideration for inclusion in a prophylactic HSV subunit vaccine. It is thought that the enhancement of neutralizing activity by anti-gD-1 antibodies is due to the production of antibodies that block complement C3b binding to gC. To support the development of a more efficacious vaccine containing gC, we asked the question, "What is the relative contribution of the immunoglobulin subclasses that function in blocking gC activities?" To this end, BALB/c mice were immunized with soluble gC-2 adjuvanted with Th2-inducing Merck Aluminum Adjuvant and Th1-inducing synthetic CpG oligonucleotide. A quantitative antibody isotype ELISA of the pooled sera from gC-immunized mice showed significant 4-fold and 6-fold increases of IgG2a and IgG2b antibody subclasses, respectively, as compared to mock-immunized pooled sera. Because IgG1 showed a 1.5-fold increase in gC-immunized mice compared to mock-immunize mice, it indicated that the CpG oligonucleotide-induced Th1 response dominated over the MAA-induced Th2 response. The IgG1, IgG2a, IgG2b, IgG3 and IgM antibody subclasses were purified from the gC-immunized and mock-immunized mice sera via Protein A, size exclusion, and hydroxyapatite chromatographies. The relative neutralizing activity against HSV-2 infection and the C3b blocking activity of each purified antibody subclass product were

compared. The results of neutralization tests showed that IgG2a, IgG2b, and IgG3 had 10-fold higher neutralizing activity against HSV-2 than IgG1, and 100-fold higher neutralizing activity than IgM. In blocking C3b binding to gC-2, the antibody subclass activity ranked as IgG2b>IgG2a>>IgG3>IgG1. An endpoint ELISA assay demonstrated ~4-fold higher anti-gC antibody titers in the IgG2 subclasses, as compared to IgG1 and IgG3 subclasses, when total antibody concentration was normalized. This suggests that the enhanced activity of the IgG2 subclasses may be due to increased presence of IgG2 antibodies specific to gC-2 epitopes that function in neutralization or C3b binding. Together, the results support the notion that inclusion of a Th1-inducing adjuvant with gC-2 in a prophylactic HSV vaccine should enhance the efficacy of gD-containing vaccine.

INTRODUCTION

Infection with herpes simplex virus (HSV) can lead to a lifelong latent infection which can become reactivated in response to environmental cues. Reactivated HSV can present as painful infections of the mucous membranes such as gingivostomatritis, herpes labialis and genital HSV infections. Keratoconjunctivitis, visceral HSV infections, encephalitis, Kaposi varicella-like eruption, and erytheme multiforme are also serious complications which can arise in newborns or immune compromised hosts.[1] Oral herpes caused by HSV type 1 (HSV-1) is the most common type of infection, with a disease prevalence of 40-63% of people in the United States in 2010.[2] The second most common type of HSV infection is genital herpes caused by HSV type 2 (HSV-2), with a disease prevalence of 16-19 % of people in the United States in 2010.[2] Current treatments include a general-purpose antiviral drug to interfere with viral replication, which reduces the frequency and physical severity of outbreak-associated lesions. These current treatment options for lifelong infections are inadequate, and do not prevent the shedding of virus, which can lead to transmission of the infection. This necessitates in part the need to develop a successful prophylactic HSV vaccine to prevent viral infection.

Development of an effective vaccine has proven difficult. Current strategies generally use either attenuated live viruses, or a recombinant protein subunit strategy.[1] The latter approach uses select HSV envelope glycoproteins to amplify the immune response and to induce virus-neutralizing antibodies. HSV is an enveloped double-stranded DNA virus, encoding at least 10 such glycoproteins. Of these, four HSV-2 glycoproteins are required for viral infection to occur. Both HSV-2 heparan sulfate (HS) – binding glycoproteins B (gB) and C (gC) are known to bind target cell surface HS,

although gB is responsible for the majority of HSV-2 viral attachment and gC binding is not essential for infection to occur.[3] The currently accepted mechanism of HSV-2 viral fusion begins with several gC and gB proteins binding to cell-surface HS, such that viruses become concentrated on the cell surface. The binding of gC and gB to HS is reversible, but ultimately bring viral gD in close proximity to one of its less abundant host cell surface receptors.[3,4] It is thought that gD undergoes a conformational change upon binding its cognate receptor, and that this results in the subsequent recruitment of gB homotrimers and heterodimers made of glycoprotein H and glycoprotein L (gH/gL). Together, gD, gB, gH, and gL form the multi-glycoprotein complex required for membrane-fusion activity. Through a poorly understood mechanism, this complex then forms a fusion pore between the viral membrane and the host cellular membrane, permitting the release of viral nucleocapsid and tegument proteins into the host cytoplasm, and completing the final step of viral infection.[4] This leads to initial replication in host epithelial cells, followed by invasion of sensory neurons and finally the establishment of latency and HSV disease pathology.[4]

Of the HSV-2 glycoproteins that function in viral fusion, gB, gD, and gH/gL have all shown promising protection results in animal models, however similar lasting protection in humans has not been proven.[5,6] In a phase II clinical trial in 2002, a gD-2 vaccine developed by GlaxoSmithKline (GSK) showed significant protection (>70% efficacy) from HSV disease in women who were seronegative to both HSV-1 and HSV-2. However, there was no significant difference in the development of genital lesions between vaccine and placebo recipients in men and seropositive women.[7] In a follow up large phase III clinical trial of HSV-1 and HSV-2 seronegative women in 2010, the gD-2

vaccine provided no protection from herpetic disease. Another large human clinical study, conducted by Chiron Corporation in 1998, evaluated the efficacy of a vaccine composed of both gB-2 and gD-2 subunits. Results showed that the rates of HSV-2 infection, the duration of the infections, and the frequency of reactivation were not significantly different between vaccine and placebo recipients.[5] The results of these clinical studies suggest that a vaccine composed solely of the glycoproteins required for viral entry may not be sufficient for protection in humans.

Due to these disappointing responses in clinical studies, the necessity for alternative vaccine strategies and the inclusion of other potential vaccine components, such as gC, have recently received more attention. In addition to the minor function of gC-2 in mediating viral binding to the target cell surface HS, it also functions to block activation of the host complement cascade via elimination of a major innate effector component of the host immune system.[8] Specifically, gC has been shown to bind complement C3b, preventing interaction of C3b with C5 and properdin, and inhibiting the activation and amplification of both the classical and alternative complement cascades, thus blocking complement-mediated viral neutralization.[9] *In vitro*, viral neutralization experiments were conducted which compared wild-type HSV-1 to an HSV-1 gC mutant virus lacking the ability to bind C3b. The mutant virus was more susceptible to antibody- and complement- mediated neutralization, suggesting that gC binding to C3b suppresses the neutralizing activity of complement and antibody.[10] The importance of antibody- and complement-mediated neutralization was verified *in vivo* in a mouse flank model of HSV infection. In this model, HSV-1 gCnull virus exhibited 100-fold reduced virulence, compared to wild-type HSV-1.[11] Moreover, HSV-1 gCnull virus produced significantly

more disease in C3 knock-out mice than in wild-type complement-intact mice. A separate mouse study showed that immunization in complement-intact mice with a soluble form of gC-1 lacking the transmembrane and cytoplasmic domains protected the mice from herpetic disease post-challenge by wild-type virus. By contrast, there was no difference in the severity of herpetic disease between gC- and mock-immunized mice challenged with an HSV-1 gC mutant virus lacking the ability to bind C3b.[10] Passive transfer studies with purified immunoglobulin G (IgG) from the gC-1 immunized mice conferred protection of recipient naïve mice from HSV-1 challenge, supporting the role of gC antibodies in mediating protection from herpetic disease. Indeed, similar results were observed when naïve mice were passively immunized with a monoclonal antibody that blocks C3b-gC interactions yet exhibits no neutralization activity *in vitro* (M. Brown and J. Balliet, unpublished observations). Together, these studies show that antibody-mediated blocking of the gC immune inhibition function provides protection against HSV-1 disease, and that gC is an important virulence factor.

The studies with gC led to the hypothesis that inclusion of gC in a gD vaccine may show improved results over a gD-only vaccine which, by itself, does not provide protection against the HSV inhibition of complement-mediated immunity. To test the hypothesis, a gC-1/gD-1 vaccine efficacy study was conducted in mice.[12] The results showed that gC-1, when included in combination with a soluble gD-1 subunit vaccine, enhanced efficacy as compared to immunization with either antigen alone. Specifically, when evaluating protection against a challenge with HSV-1, strain-NS, there was 60% survival in mice immunized with 10 ng gD-1 alone, and 100% survival in mice immunized with 10 ng gD-1 + 10 μg gC-1. Furthermore, IgG from mice immunized with

gC-1, gD-1, or combination gC-1/gD-1 vaccination were purified, and the study compared the relative ability of the purified IgG to neutralize HSV-1 *in vitro.* This showed that anti-gC-1 IgG synergistically enhanced complement-dependent neutralization by anti-gD-1 IgG 87-fold compared to anti-gD-1 IgG alone, and 20-fold compared to anti-gC-1 IgG alone.[12] This result supports the hypothesis that anti-gC antibodies are able to prevent the immune inhibition function, leading to higher serum neutralizing activity and enhanced vaccine efficacy. Although previous gD or gD/gB HSV vaccines induced high neutralizing antibody titers and potent T-cell responses after immunization, the vaccine efficacy may have been severely limited without inclusion of a gC component in the vaccine to block the complement inhibition function.[12] Further understanding of the generation of functional anti-gC antibodies that block gC/C3b binding and the immune inhibition function is therefore required in order to assess their contribution to enhancing the neutralizing activity of anti-gD antibodies.

Several studies have looked at the functional benefits of anti-gC antibodies and their accessibility to gC, expressed either on the viral envelope or on the infected cell surface. One study determined the gC/C3b blocking ability of purified IgG antibodies from HSV-1 infected humans, HSV-1 infected mice, and gC-1 immunized mice.[13] In both an ELISA-based gC/C3b blocking assay and a rosetting assay performed with C3b-coated erythrocytes, it was shown that the human anti-gC antibodies were not efficient at blocking the binding of gC to C3b. Therefore, human antibodies produced during natural HSV-1 infection are not effective at binding gC within the domains required for inhibition of C3b binding. Notably, upon comparison of the gC/C3b blocking activity of purified antibodies from the HSV-1 infected mice and the gC-immunized mice, they

showed that gC-immunization induced higher levels of antibodies to the C3b binding domain of gC and improved gC/C3b blocking activity of the antibodies in both *in vitro* assays. Because gC-1 immunization in mice is therefore more effective than natural HSV-1 infection at eliciting gC/C3b blocking antibodies, by extension, it is predicted that gC-immunization of humans will also elicit higher levels of gC/C3b blocking antibodies than those produced from natural infection. Therefore, the results support further investigation for the inclusion of gC in an HSV subunit vaccine, due to the ability of anti-gC antibodies to block the gC/C3b interactions, thus enhancing the neutralizing activity of anti-HSV subunit antibodies.

Interestingly, previous work studying the function of anti-HSV antibodies has shown differences in the neutralizing and complement-binding capabilities of the immunoglobulin subclasses produced in mice.[14,15] Four subclasses of IgG have been identified in mice; IgG1, IgG2a, IgG2b and IgG3, which have varying biochemical, immunological, and physiological properties.[16,17] Due to this, the mechanism and extent of viral neutralization should vary between subclasses, as will the binding affinity to their substrate, making it important to fully characterize the antibody subclasses produced in response to viral infection or vaccine immunization. Both the nature of the antigen and the adjuvant used in immunization can influence the subclass bias through differential cytokine expression.[18] The induction of Th2 cytokines, such as interleukin-4, result in IgG class switching to IgG1, and the induction of Th1 cytokines, such as transforming growth factor-ß and interferon-γ, result in IgG class switching to IgG2a, IgG2b, and IgG3.[19] In order to begin exploring the individual functional contributions of anti-HSV antibody subclasses, McKendall *et al.* immunized BALB/c mice with HSV-1 and then

purified the IgG subclasses from the serum. Compared to non-immune serum, immunized mice had 10-, 5-, and 2-fold increases in IgG1, IgG2a, and IgG2b, respectively.[15] The purified antibodies were then tested in a neutralization assay, which showed that IgG1 antibodies had the weakest neutralizing activity, while the IgG2a and IgG2b antibodies had 4- and 2-fold higher levels of neutralizing activity, respectively.

In light of the recent interest in gC as an antigen to induce neutralizing antibodies capable of blocking an HSV immune inhibition function, it would be helpful to expand upon the McKendall *et al.* study by specifically comparing the functional activity of purified anti-gC antibodies. This will also help to fully evaluate HSV vaccine candidates and optimize the adjuvant employed. In the current study, BALB/c mice were immunized with HSV gC-2 adjuvanted with Merck Aluminum Adjuvant (MAA) and a synthetic CpG oligonucleotide. The IgM and IgG subclass responses were quantified in gC-immunized and mock-immunized sera, and then the individual subclasses were purified from the sera of gC-immunized mice. The functional differences between the purified antibody subclass products IgM, IgG1, IgG2a, IgG2b, and IgG3 were then characterized; looking specifically at their varying capacities to bind gC-2, to prevent complement C3b binding to gC-2, and to neutralize HSV-2 infection.

MATERIALS AND METHODS

Immunization schema

Laboratory animals were handled in accordance with The Guide for the Care and Use of Laboratory Animals (NIH Publication no. 85-23, revised 1996) and the guidelines of the Institutional Animal Care and Use Committee of Merck and Co., Inc at West Point, PA. Six week old BALB/c female mice were obtained from Taconic Farms, Inc. Mice were placed into two groups of 90 animals each. Mice in group I were mock-immunized with MAA and a synthetic CpG oligonucleotide (50 μg/dose) on days 0, 7, and 21. Animals were anesthetized and terminally bled on day 35 to obtain mock-immunized mice sera. Individual serum samples from four mice were retained. Mock-immunized mice sera were pooled from the remaining 86 mice. Mice in group II were immunized with gC-2 445 (recombinant gC construct containing the first 445 amino acids of the ectodomain) adjuvanted with MAA and a synthetic CpG oligonucleotide (50 μg/dose) on days 0, 7, and 21. Animals were anesthetized and terminally bled on day 35 to obtain gC-immunized mice sera. Individual serum samples from ten mice were retained. gC-immunized mice sera were pooled from the remaining 80 mice.

Chromatographic purification of mouse IgG subclasses

Antibody subclasses were purified from mice sera using chromatographic techniques (Figure 1). First, Ig subclasses were separated by HPLC (AKTA^{TM} Purifier, GE Healthcare). Pooled sera from group II was diluted 1:10 with 100 mM sodium phosphate dibasic, and pH adjusted to 7.5. Diluted sera were filtered (Corning® cellulose acetate filter, 0.45 μm) and applied to a Pierce® Protein A cartridge (Thermo

Scientific). The non-bound fraction was saved for subsequent IgM purification. Elution of IgG subclasses was accomplished with a step-wise pH gradient using mixtures of 100 mM sodium phosphate, pH 7.5 (Buffer A) and 100 mM sodium citrate, pH 3.0 (Buffer B). The first fraction was collected at pH 6.0 (79% Buffer A, 21% Buffer B), the second fraction was collected at pH 4.5 (59% Buffer A, 41% Buffer B), and the third fraction was collected at pH 3.0 (100% Buffer B). Fractions were neutralized by collection in tubes containing 1 M sodium phosphate, pH 7.5. SDS-PAGE, Western blot, and quantitative ELISA were used to identify IgG subclasses and determine purity in the non-bound fraction and in the three elution fractions. Fractions 1 and 3 were separately concentrated with 10k MWCO Amicon® centrifugal filters (Millipore) and applied to a Superdex200® size exclusion chromatography column (Pharmacia Biotech) to prepare purified IgG1 and IgG2b, respectively. Fraction 2 was applied to an anti-IgG3 affinity chromatography column prepared with AminoLink® Plus Immobilization Kit (Thermo Scientific) and goat anti-mouse IgG3 antibody (Southern Biotechnology Associates). The non-bound fraction from the anti-IgG3 affinity chromatography column was concentrated with 10k MWCO Amicon® centrifugal filters, and applied to Superdex200® size exclusion chromatography column to prepare purified IgG2a. Purified IgG3 was eluted from the anti-IgG3 affinity chromatography column with 0.2 M glycine, pH 3.0, and was neutralized immediately by the addition of 1 M sodium phosphate, pH 7.5. All purified antibodies were dialyzed into phosphate-buffered saline (PBS) with 10k MWCO Slide-a-Lyzer® dialysis cassettes (Thermo Scientific).

A Lambda 45 spectrometer (Perkin Elmer) was used to measure the absorbance at 280 nm of each purified IgG subclass product. An A_{280} extinction coefficient (1 mg/ml) of 1.36 AU was used to calculate the final antibody concentrations.

Chromatographic purification of mouse IgM

The non-bound fraction from the Protein A column was loaded onto a Superdex200® size exclusion chromatography column. SDS-PAGE and Western blot were used to identify fractions containing IgM. IgM-containing fractions were pooled and dialyzed into 10 mM sodium phosphate, pH 7.5 and 100 mM NaCl with a 10k MWCO Slide-a-Lyzer® dialysis cassette. Dialyzed material was then applied to a ceramic hydroxyapatite type II column (BioRad BioScale^{TM} Mini CHT Type II). A ten column volume gradient to 100 mM sodium phosphate, pH 7.5 and 100 mM NaCl was run, and fractions were collected to separate IgM from serum proteins. IgM-containing fractions were pooled and dialyzed into PBS with a 10k MWCO Slide-a-Lyzer® dialysis cassette.

A Lambda 45 spectrometer was used to measure the absorbance at 280 nm of the hydroxyapatite product. An A_{280} extinction coefficient (1 mg/ml) of 1.0 AU was used to estimate the total protein concentration in the sample.

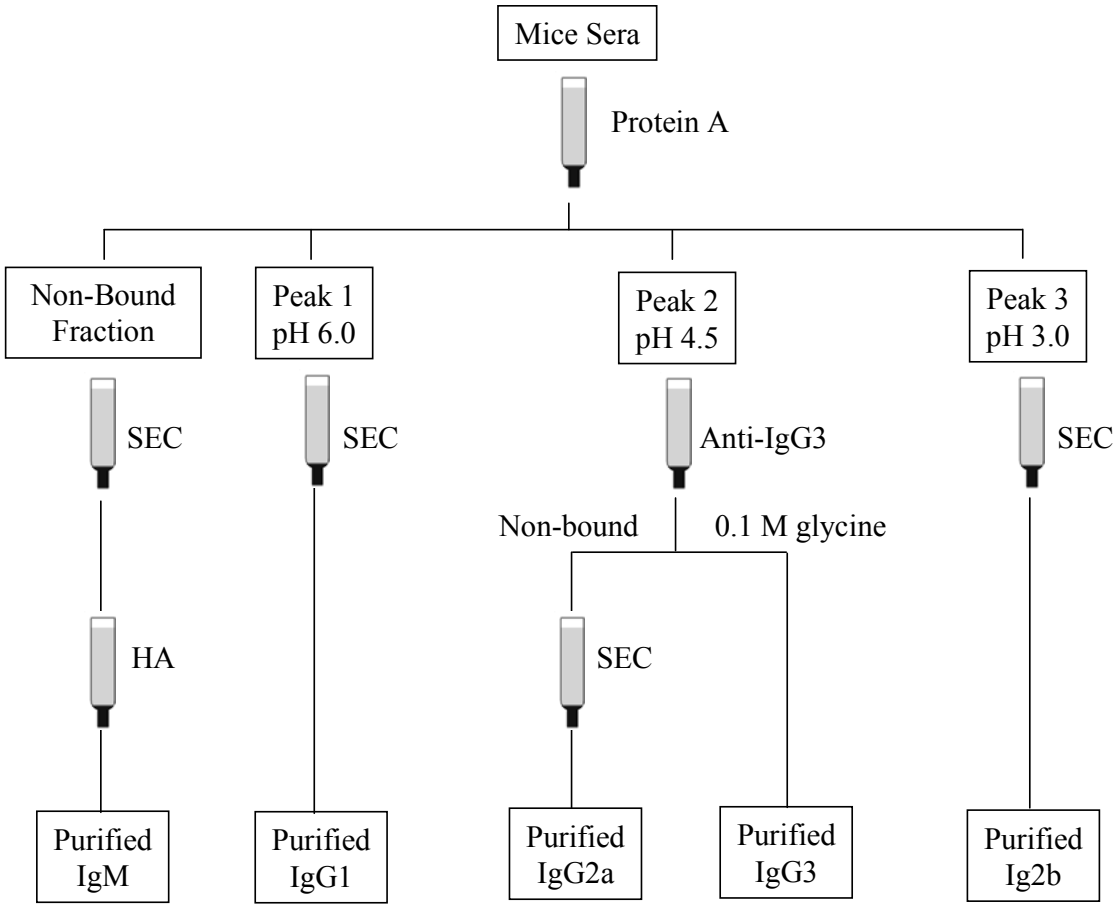


Figure 1. Outline of chromatographic purification of antibody subclasses from mice sera. Mice sera were first separated over a Protein A column. IgM was purified from the Protein A non-bound fraction with Superdex200® size exclusion chromatography (SEC) and Hydroxyapatite Type II Chromatography (HA). IgG1 was purified from Peak 1 (pH 6.0 step elution) of the Protein A column by SEC. IgG2a was purified from Peak 2 (pH 4.5 step elution) of the Protein A column by applying the non-bound fraction of the anti-IgG3 affinity column to SEC. IgG3 was purified from Peak 2 (pH 4.5 step elution) of the Protein A column by applying it to an anti-IgG3 affinity column and a step elution in 0.1 M glycine pH 3.0. IgG2b was purified from Peak 3 (pH 3.0 step elution) of the Protein A column by SEC.

SDS-PAGE and Western blot analysis

Mice sera, intermediate fractions from the antibody purifications, and final purified antibody subclass products were analyzed by SDS-PAGE and Western Blot to support the purification. Samples were mixed with sample buffer containing reducing

agent (Invitrogen NuPAGE® LDS 4x sample buffer with 4% ß-mercaptoethanol), heated for 9 minutes at 90ºC, and then loaded on 15-well NuPAGE® 4 to 12% BisTris gels (Invitrogen). Electrophoresis was carried out at 200 V constant voltage for 35 minutes. Gels were either stained with SimplyBlue™ SafeStain (Invitrogen), or transferred to nitrocellulose membranes (iBlot® Kit Reagents, Invitrogen). Western blotting was performed using the SNAP i.d.™ detection system (Millipore). Membranes were blocked three times with blocking buffer (PBS, 0.5% non-fat dried milk, 0.1% Tween®-20). Membranes were incubated 10 minutes with 3 ml of a 1:500 dilution of alkaline-phosphate conjugated goat-anti mouse IgG1, IgG2a, IgG2b, IgG3, or IgM isotype specific antibody (Southern Biotechnology Associates) in blocking buffer. Membranes were washed three times with 0.1% Tween®-20 in PBS, and developed in 10 ml of 1-Step™ NBT/BCIP substrate (Thermo Scientific) for 5 minutes.

The SDS-PAGE gel, stained with SimplyBlue™ SafeStain, of the IgM antibody subclass product showed the presence of contaminating serum proteins, and so a densitometry analysis was used to calculate the percentage of IgM in the sample. Serial 1:3 dilutions of the IgM antibody subclass product were analyzed by SDS-PAGE as outlined above. The gel was imaged (BioRad GelDoc™ EZ Imager), and the relative band intensity was calculated to estimate IgM purity.

Quantification of mouse IgG subclasses and IgM by isotype ELISA

Ninety-six well polystyrene microtiter plates (Corning Costar® EIA/RIA medium-binding plates) were coated at 4ºC overnight with 5 µg/well affinity-purified unlabelled goat anti-mouse Ig(H+L), human absorbed antibody (Southern Biotechnology

Associates) in PBS, pH 7.5. The next day, the plates were washed four times in ELISA wash buffer (PBS, 0.05 % Tween®-20) and then blocked for 2 hours at room temperature using 100 µl of ELISA blocking buffer (PBS, 1% bovine serum albumin [BSA] and 0.1 % Tween®-20). The plates were washed four times with wash buffer prior to the addition of samples. Samples were prepared as follows. Serum samples were first diluted 1:500 in blocking buffer, while Protein A fractions and purified antibody subclass products were first diluted 1:100 in blocking buffer. This was followed by serial 1:3 dilutions of the 1:500 diluted serum samples and 1:100 diluted protein samples, made in blocking buffer. Calibration curves were prepared using affinity-purified mouse antibody subclass standards (Southern Biotechnology Associates) diluted in blocking buffer and ranged in concentration from 2000 ng/ml to 2 ng/ml. One hundred microliters of all samples and standards was added in duplicate to the 96-well plates. The plates were incubated at room temperature for 1 hour with mixing, and then washed four times with wash buffer. The secondary antibodies were HRP-labeled goat anti-mouse Ig (Southern Biotechnology Associates). Goat anti-mouse IgG1, IgG2a, IgG2b, and IgM were all used at a 1:40,000 dilution in blocking buffer, and goat anti-mouse IgG3 was used at a 1:20,000 dilution in blocking buffer. One hundred microliters of the secondary antibodies was added to the plates, incubated at room temperature for 1 hour with mixing, and then the plates were again washed four times with wash buffer. Following the final wash, 100 µl of 1-Step™ Ultra-TMB-ELISA substrate (Thermo Scientific) was added to each well, incubated for 10 minutes at room temperature and then the reaction was stopped by adding 100 µl of 2 M sulfuric acid. Absorbance was read at 450 nm (Molecular Devices SpectraMax® Plus). The mean absorbance of replicate samples of

antibody subclass standards were fit to four-parameter curves. The concentrations of each subclass in all analyzed samples were then interpolated from the linear portion of the calibration curves.

Anti-gC antibody ELISA

Ninety-six well polystyrene microtiter plates (Immulon® 4-HBX extra-high binding plates, Thermo Scientific) were coated at 4ºC overnight with 1 µg/well gC-2 445 in bicarbonate buffer, pH 9.0. The next day, the plates were washed four times in wash buffer. One hundred microliters of blocking buffer was added to each well, and incubated for 2 hours at room temperature. The plates were washed four times with wash buffer prior to addition of samples. Samples were prepared as follows. gC-immunized and mock-immunized mice sera samples were first diluted 1:500 in blocking buffer, and purified antibody subclass products were normalized to 2 µg/ml in blocking buffer. Serial 1:2 dilutions of the 1:500 dilution serum samples and the 2 µg/ml protein samples were then made in blocking buffer. One hundred microliters of each sample was transferred in triplicate to the 96-well plates. The plates were incubated at room temperature 1 hour with mixing, after which they were washed four times with wash buffer. One hundred microliters of the secondary antibody HRP-labeled goat anti-mouse Ig(H+L) (Southern Biotechnology Associates) diluted to 1:20,000 in blocking buffer was added to each well, and incubated at room temperature for 1 hour with mixing. The plates were again washed four times with wash buffer, and then 100 µl of 1-Step™ Ultra-TMB-ELISA substrate (Thermo Scientific) was added to each well and plates were incubated for 5 minutes at room temperature. The reaction was stopped by the addition

of 100 µl of 2 M sulfuric acid. Absorbance was read at 450 nm (Molecular Devices SpectraMax® Plus). Titers were defined as the dilution factor that produced an OD equal to three times the OD of blank wells.

Viral neutralization assays

In-vitro cell based ELVIS® neutralization assays were performed using 96-well plated ELVIS® cells (Diagnostic Hybrids, Inc.), which are a BHK cell line stably transfected with *lacZ* driven by the HSV-1 UL39 promoter. Upon HSV infection of ELVIS® cells, viral immediate-early genes induce the UL39-LacZ cassette. Cells were received at 95% confluency, and were recovered by removing media and replacing with 100 µl/well fresh media (GIBCO® DMEM without phenol red, 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2mM L-glutamine). Cells were incubated at 37ºC for 3 hours. Serum samples were diluted 1:50 in media. Serial 1:4 dilutions of the 1:50 dilution of serum samples, purified antibody subclass products, and positive control gD antibody DL11[20] were prepared in media in 96-well Nunc plates at a final volume of 60 µl/well. HSV-2 strain MS (4.3×10^6 plaque forming units/ml) was diluted 1:40 in media containing 13.4% Low-Tox-M® rabbit complement (Cedarlane). Sixty microliters of virus/complement mixture was added to the 96-well Nunc plates containing diluted antibodies. Four wells on each plate were left as virus-only controls, and four wells were left as media-only controls. Plates were incubated at 37ºC for 1 hour, and then 100 µl of the virus/antibody mixtures was transferred to ELVIS® cell plates. Following a 20 hour incubation at 37ºC, the ELVIS® cell plates were developed using the Gal-Screen® kit (Applied Biosystems). One hundred microliters of detection

reagent, diluted according to manufacturer guidelines, was added to each well of the assay plates and incubated at room temperature for 1 hour in the dark. Each sample was then transferred to white OptiPlate^{TM}-96 plates (Perkin Elmer), and read on a Dynex Luminometer. The means of the measured relative luminescense units (RLUs) from the virus-only and media-only wells were used to define the maximum and minimum RLU values of the assay so that the 50% neutralization point could be determined. The measured RLUs from the un-fractionated sera and purified antibody subclass products were plotted against the dilution factor or known antibody concentration, respectively, to calculate their 50% neutralization value.

gC-2/C3b blocking ELISA

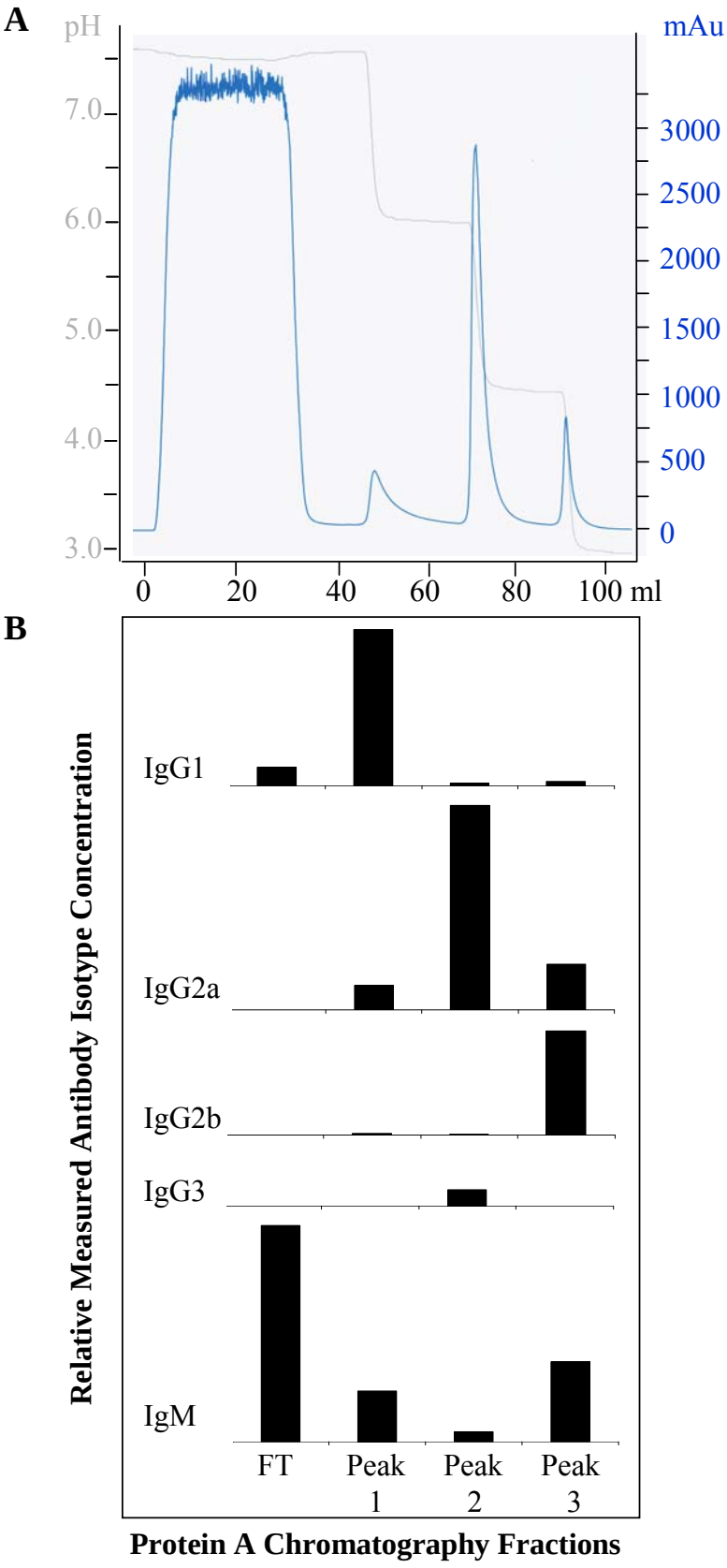
Ninety-six well polystyrene microtiter plates (Immulon® 4-HBX extra-high binding plates) were coated at 4ºC overnight with 2 µg/well human C3b complement (Complement Technology) in bicarbonate buffer, pH 9.0. The next day, plates were washed four times with wash buffer. One hundred microliters of blocking buffer was added to each well, and incubated for 2 hours at room temperature. The C3b coated and blocked plate was washed four times with wash buffer. In a separate low-binding 96-well Nunc plate, serial 1:1.75 dilutions of purified antibody subclass products and a positive control anti-gC monoclonal antibody MP1[21] were made in blocking buffer. Seventy-five microliters of gC-2 445 was added to 75 µl of antibody, and incubated at room temperature for 1 hour with mixing. One hundred microliters of antibody-gC mixture was transferred to wells of the C3b-coated plate, with the exception that four wells contained gC-only and four wells contained blocking buffer-only. Plates were

incubated for 1 hour at room temperature with mixing, to allow binding of gC to C3b. The plates were washed four times in wash buffer. To detect gC bound to C3b, 100 µl of rabbit anti-gC polyclonal antibody #81 (R81, provided by R. Eisenberg and G. Cohen, University of Pennsylvania) at a 1:2000 dilution in blocking buffer was added to the plates. Plates were incubated at room temperature for 1 hour with mixing, and then washed four times with wash buffer to remove any unbound R81. One hundred microliters of the secondary antibody HRP-labeled goat anti-rabbit IgG (H+L) (Southern Biotechnology Associates) diluted 1:10,000 dilution in blocking buffer was added to each well, and the plates were incubated at room temperature 1 hour with mixing. After the plates were washed four times with wash buffer, 100 µl of 1-Step™ Ultra-TMB-ELISA substrate (Thermo Scientific) was added to each well, incubated for 10 minutes at room temperature, and the reaction was stopped by the addition of 100 µl of 2 M sulfuric acid. Absorbance was read at 450 nm (Molecular Devices SpectraMax® Plus). The mean ODs of gC- and blocking buffer-only wells were graphed to define the maximum and minimum of gC-binding to C3b. Based on these values, the 50% gC-2/C3b binding was calculated. The measured ODs from the purified antibody subclass products were then plotted against the known antibody concentration to determine the concentration at which 50% of gC-2/C3b binding was blocked.

RESULTS

Purification of antibody subclasses

The IgG subclass antibodies were purified from the pooled sera of gC- immunized BALB/c mice with a Pierce® Protein A cartridge. Three distinct peaks were detected after step elution from the column at pH 6.0, 4.5, and 3.0 (Figure 2a). The non-bound fraction and fractions containing each elution peak were assayed using the quantitative isotype ELISA to determine the concentration of each antibody subclass in the fractions (Figure 2b). Although a small amount of IgG1 was detected in the non-bound fraction, most of the IgG was captured by the Protein A cartridge. Moreover, IgM was expected only in the Protein A non-bound fraction; however some IgM was also detected in each of the elution fractions, suggesting that some bound to the column. The fraction containing peak 1, which eluted at pH 6.0, was composed of 67% IgG1, as well as 12% IgG2a and 22% IgM. The fraction containing peak 2, which eluted at pH 4.5, was composed of 88% IgG2a, as well as 7% IgG3 and 4% IgM. The fraction containing peak 3, which eluted at pH 3.0, was composed of 45% IgG2b, as well as 20% IgG2a and 35% IgM.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pH
7.0
6.0
5.0
4.0
3.0
mAu
3000
2500
2000
1500
1000
500
0
0
20
40
60
80
100 ml
B
Relative Measured Antibody Isotype Concentration
IgG1
IgG2a
IgG2b
IgG3
IgM
FT
Peak 1
Peak 2
Peak 3
Protein A Chromatography Fractions

Figure 2. (A) AKTA™ chromatogram of the Pierce® Protein A purification. Grey line indicates pH, which ranged from 7.4 to 3.0, indicated on the scale on the left. Blue line indicates the absorbance at 280 nm, which peaked at ~3000 mAu and is indicated on the scale on the right. The first peak on the left corresponds to the non-bound fraction, followed by step elutions of Peak 1 (pH 6.0), Peak 2 (pH 4.5) and Peak 3 (pH 3.0). **(B) Quantitative antibody isotype ELISA of Pierce® Protein A fractions.** The relative concentration of the five antibody subclasses was determined for each of the four Protein A fractions (bottom) using a quantitative antibody isotype ELISA as described in the Materials and Methods section.

Subsequent polishing steps improved the purity of all IgG subclasses (Table I, Figure 3). IgG3 and IgG2a were separated from the Peak 2 fraction with an anti-mouse IgG3 affinity chromatography column. The non-bound fraction from the IgG3 affinity column contained IgG2a and IgM, while the elution product of the IgG3 affinity column contained the final purified IgG3 product (90% pure IgG3). IgM was successfully removed from peaks 1, 3, and the non-bound fraction of the IgG3 affinity column by Superdex200® size exclusion chromatography. This yielded the final purified IgG1 product (85% pure IgG1), the final purified IgG2a product (97% pure IgG2a), and the final purified IgG2b product (82% pure IgG2b).

Table I. Quantitative antibody isotype ELISA of purified antibody subclass products

Sample	IgG1 (µg/ml)	IgG2a (µg/ml)	IgG2b (µg/ml)	IgG3 (µg/ml)	IgM (µg/ml)	Purity (%)
Purified IgG1	429	73	1	0	0	85
Purified IgG2a	12	709	6	0	0	97
Purified IgG2b	12	39	231	0	0	82
Purified IgG3	0	26	0	249	0	90
Purified IgM	0	0	0	0	172	100

**The concentration of each antibody subclass was calculated from the purified antibody subclass products with the quantitative antibody isotype ELISA.

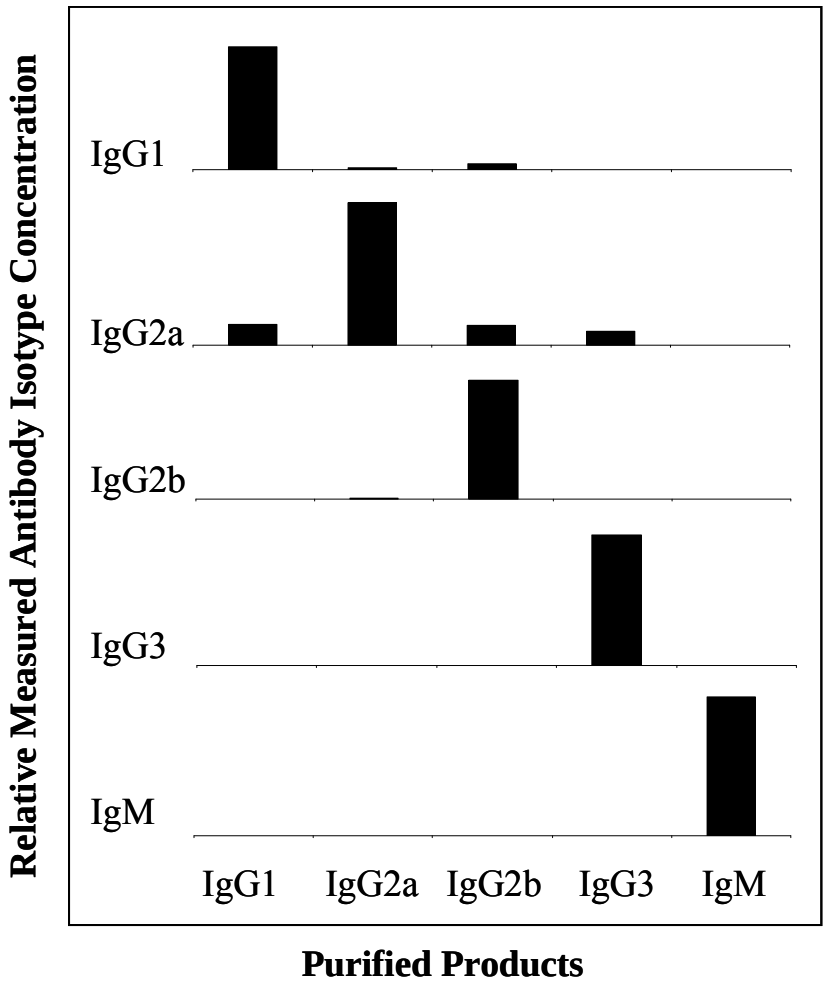

Figure 3. Quantitative antibody isotype ELISA of purified antibody subclass products. The relative concentration of the five antibody subclasses (labeled on left side) was determined with the quantitative antibody isotype ELISA for each of the final purified antibody subclass products (labeled on bottom).

SDS-PAGE and Western blotting with specific alkaline phosphatase conjugated goat anti-mouse IgG1, IgG2a, IgG2b, IgG3 or IgM were used to support purification at all steps in the process (Figures 4 and 5). The results of the Western blot correlate closely with the results of the quantitative antibody isotype ELISA, where the primary

contaminant in the final purified products included IgG2a in the IgG1, IgG2b, and IgG3 products.

Although some IgM was purified by size exclusion chromatography from the elution peaks 1, 2, and 3, the majority of IgM was purified from the Protein A non-bound fraction. The non-bound fraction was applied to a Superdex200® size exclusion chromatography column, and SDS-PAGE and Western blotting identified fractions containing IgM. These fractions were pooled and IgM was polished with ceramic hydroxyapatite type II chromatography and a sodium phosphate pH 7.4 elution gradient. This successfully removed all IgG subclasses detected by the quantitative antibody isotype ELISA and Western blot, although some serum proteins remained. SDS-PAGE and densitometry of the IgM product were used to calculate that ~ 50% of the total protein content was IgM (Figures 4 and 5).

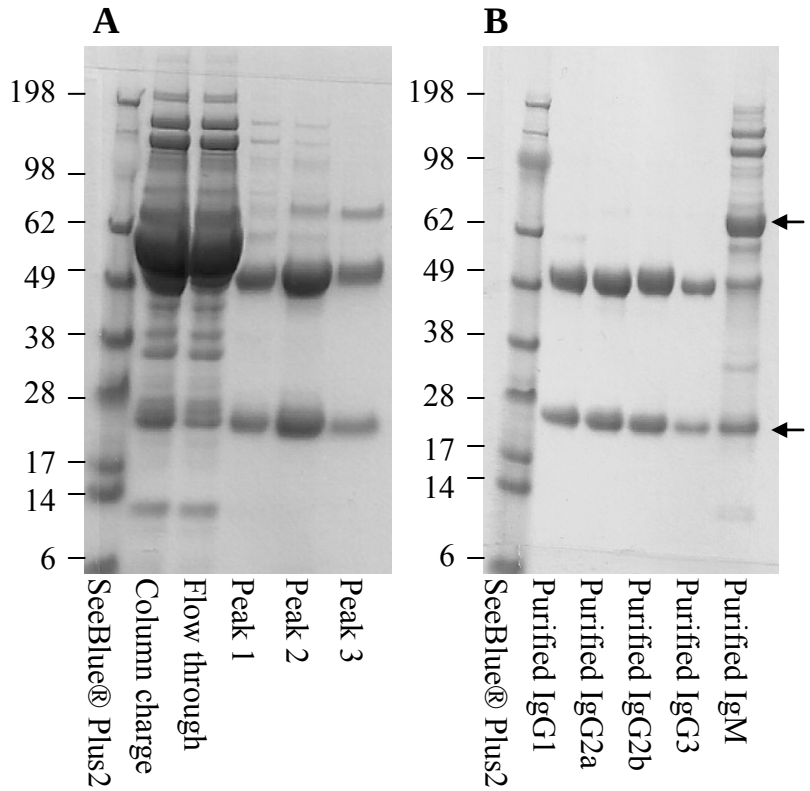

Figure 4. (A) SDS-PAGE of Pierce® Protein A fractions. The pooled gC-immunized mice sera (column charge), Protein A non-bound fraction, elution peak 1 (pH 6.0), elution peak 2 (pH 4.5) and elution peak 3 (pH 3.0) were separated on 4-12% BisTris gels in MES buffer. Proteins were visualized by staining with SimplyBlue™ SafeStain. **(B) SDS-PAGE of purified antibody subclass products.** After subsequent chromatography polishing steps, the final purified IgG1, IgG2a, IgG2b, IgG3, and IgM products were separated on 4-12% BisTris gels in MES buffer. Proteins were visualized by staining with SimplyBlue™ SafeStain. The arrows indicate bands in the final IgM product that corresponds to the IgM heavy and light chains.

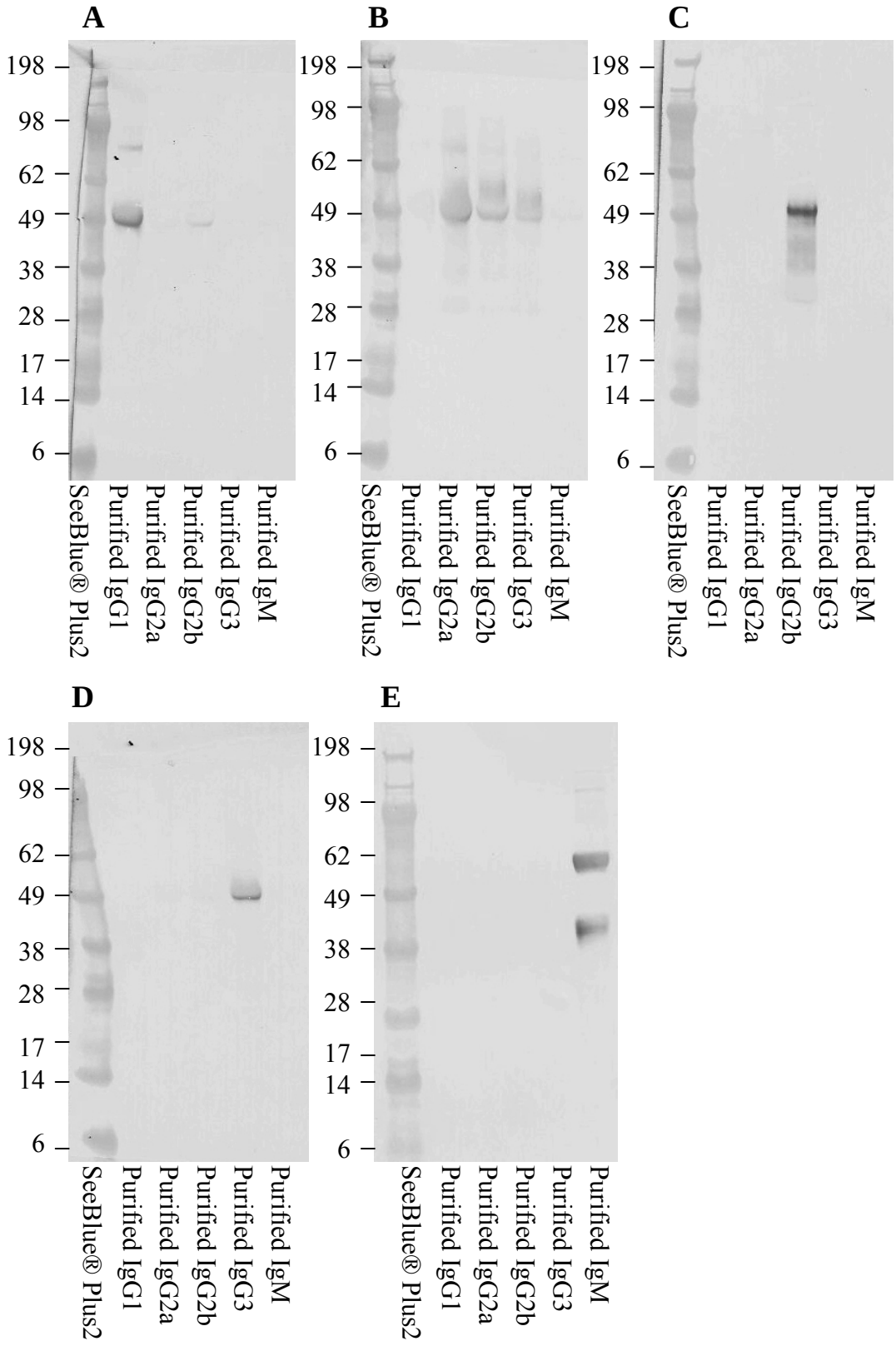
A
B
C
D
E
198
98
62
49
38
28
17
14
6
SeeBlue® Plus2
Purified IgG1
Purified IgG2a
Purified IgG2b
Purified IgG3
Purified IgM

Figure 5. Western blot of purified antibody subclass products. After separation of purified antibody subclass products on 4-12% BisTris gels in MES buffer, proteins were transferred to nitrocellulose membrane. Membranes were incubated with alkaline phosphatase conjugated goat anti-mouse (A) IgG1, (B) IgG2a, (C) IgG2b, (D) IgG3, or (E) IgM, and developed with 1-Step™ NBT/BCIP.

Quantification of antibody subclasses in gC-immunized and mock-immunized mice sera

A quantitative ELISA was used to measure the antibody subclass concentration in the pooled gC-immunized and mock-immunized mice sera (Figure 6). When comparing the total quantities of detected antibodies in the gC-immunized mice as compared to the mock-immunized mice, it was observed that the total antibody concentration increased 2.5-fold after gC-immunization, from 2.4 mg/ml to 6.0 mg/ml. Between the two pooled groups of un-fractionated sera, there was little difference in the amount of IgM. There was a 1.5-fold increase in the total IgG1 concentration, and a 1.5-fold decrease in the total IgG3 concentration of gC-immunized mice, as compared to mock-immunized mice. Notably, a 4-fold increase in IgG2a and a 6-fold increase in the IgG2b concentration in gC-immunized mice were observed, as compared to mock-immunized mice. In comparing the concentration of the antibody subclasses in the pooled gC-immunized mice sera, the IgG2a subclass represented the highest concentration of antibody at 4.4 mg/ml (73% of all antibodies in the serum), followed by IgG1 at 0.84 mg/ml (14%), IgM at 0.41 mg/ml (7%), IgG2b at 0.30 mg/ml (5%), and IgG3 at 0.07 mg/ml (1%).

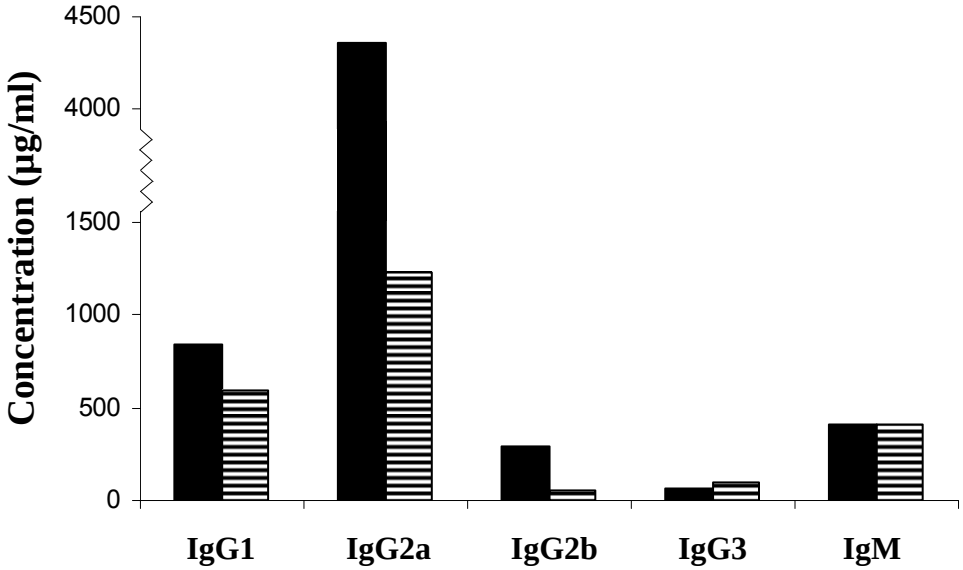

Figure 6. Quantitative antibody isotype ELISA of pooled gC-immunized and mock-immunized mice sera. Plates coated with goat anti-mouse Ig(H+L) were incubated with the diluted sera from pooled gC-immunized (■) and mock-immunized mice (☰), or with purified antibody subclass standards. The secondary HRP-conjugated goat anti-mouse antibody was added to specifically detect the antibody subclasses IgM, IgG1, IgG2a, IgG2b, and IgG3. Plates were read at 450 nm, and the calibration curves of purified antibody subclass standards were fit to a 4-parameter curve. Concentrations of each antibody subclass were interpolated from the calibration curves, and are graphed in µg/ml.

In order to look at the variation in antibody subclass responses between individual gC-immunized and mock-immunized mice, serum from eight gC-immunized mice and four mock-immunized mice were analyzed by the quantitative antibody isotype ELISA (Figure 7). As compared to the mock-immunized mice, there was an increase in the concentrations of IgG1 (P=0.148, Mann-Whitney t-test), and significant increases in the concentrations of IgG2a (P=0.004), and IgG2b (P=0.004) in gC-immunized mice. In contrast, there was a significant decrease in the overall concentration of IgG3 after gC-

immunization (P=0.004), and no change in the concentration of IgM (P=0.214). For all subclasses except mock-immunized IgM, the gC-immunized and mock-immunized mice sera pools fell within the range of measured antibody concentrations for individual mice, suggesting that the pools are representative of the responses measured in individual mice. The results of this quantitative ELISA show that relatively more antibodies underwent class switching to the IgG2 subclasses than to the IgG1 subclass after gC-immunization, indicating that the CpG-induced Th1 response dominated over the MAA-induced Th2 response.

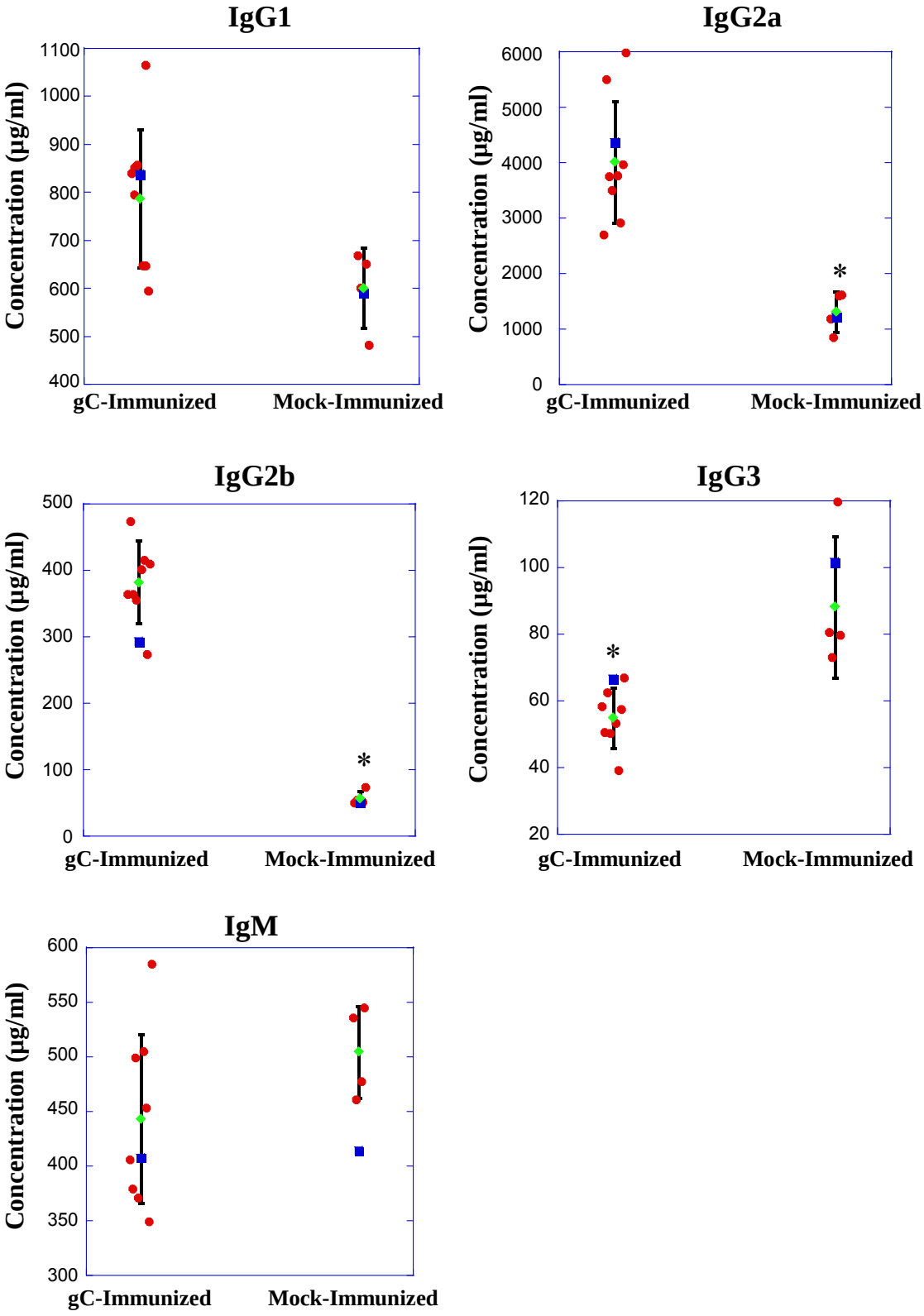
IgG1
Concentration (µg/ml)
1100
1000
900
800
700
600
500
400
gC-Immunized
Mock-Immunized
IgG2a
Concentration (µg/ml)
6000
5000
4000
3000
2000
1000
0
*
gC-Immunized
Mock-Immunized
IgG2b
Concentration (µg/ml)
500
400
300
200
100
0
*
gC-Immunized
Mock-Immunized
IgG3
Concentration (µg/ml)
120
100
80
60
40
20
*
gC-Immunized
Mock-Immunized
IgM
Concentration (µg/ml)
600
550
500
450
400
350
300
gC-Immunized
Mock-Immunized

Figure 7. Quantitative antibody isotype ELISA of individual gC-immunized and mock-immunized mice sera. Plates coated with goat anti-mouse Ig(H+L) were incubated with the diluted serum from individual (●) or pooled (■) gC-immunized and mock-immunized mice, or with purified antibody subclass standards. A secondary HRP-conjugated goat anti-mouse antibody was added to specifically detect the antibody subclasses IgM, IgG1, IgG2a, IgG2b, and IgG3. Plates were read at 450 nm, and the calibration curves of purified antibody subclass standards were fit to a 4-parameter curve. Concentrations of each antibody subclass were interpolated from the calibration curves, and are plotted in μg/ml. The mean antibody concentrations for individual mice are shown (♦) along with error bars indicating standard deviation. Asterisk indicates significant differences in antibody subclass concentration between the individual gC-immunized and mock-immunized mice, as determined using the Mann-Whitney t-test.

gC-specific antibody responses in gC-immunized and mock-immunized mice sera

An endpoint ELISA was used to determine the relative anti-gC antibody titers from individual mice, and from the pooled sera of gC-immunized and mock-immunized mice. No antibodies from the pooled mock-immunized mice sera were found to bind gC-2. The individual gC-immunized mice had titers ranging from 1,500,000 to 3,500,000, with a geometric mean of 2,150,000 (Figure 8). The pooled sera from eighty gC-immunized mice had a titer of 2,200,000, which fell within the range of the individual mice samples. This shows that the gC-immunized mice had anti-gC specific antibodies that were not present in mock-immunized mice, and that the pooled sera of gC-immunized mice is representative of the gC-specific response in individual mice.

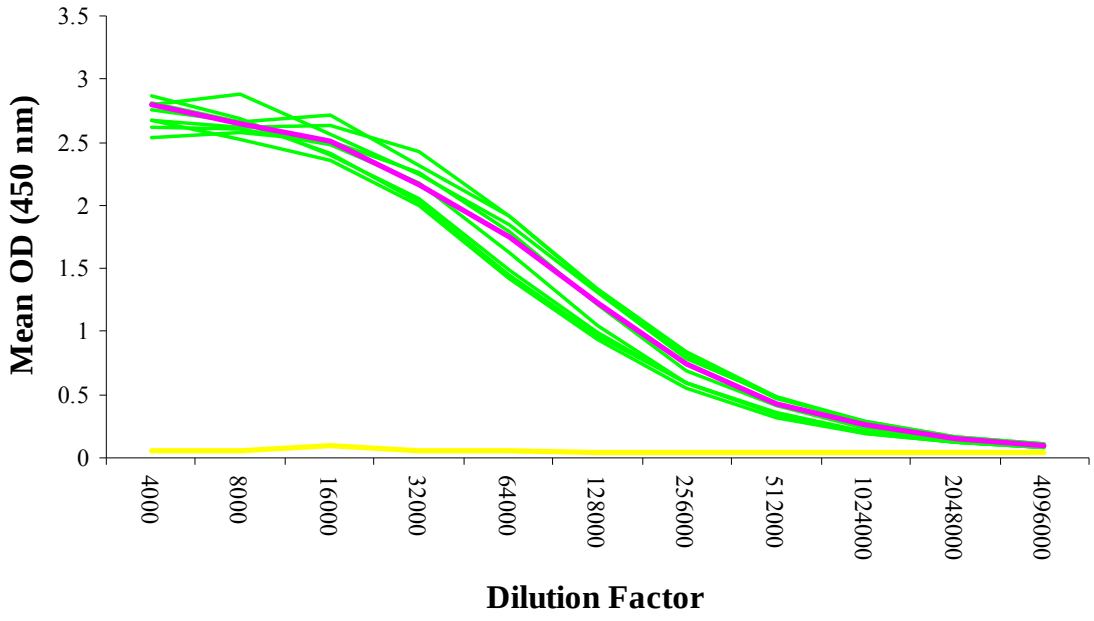

Figure 8. Anti-gC antibody ELISA of gC-immunized mice sera. Mice were vaccinated IM on days 0, 7, and 21 with adjuvanted gC-2 445 or with adjuvant alone. Animals were bled 2 weeks post final vaccination (day 35). Sera IgG titers to gC-2 445 were determined by plates coated with gC-2 445, incubated with the diluted serum of eight individual gC-immunized mice (green), pooled gC-immunized mice sera (pink) or pooled mock-immunized mice sera (yellow). The secondary HRP-conjugated goat anti-mouse antibody was used to detect gC-bound antibodies, and plates were read at 450 nm.

gC-specific antibody responses in purified antibody subclass products

An endpoint ELISA was used to demonstrate the relative anti-gC antibody titers of the purified antibody subclass products (Figure 9). There were no anti-gC IgM antibodies detected in this experiment. The titers of purified IgG2a (450) and IgG2b (450) products were approximately 4-fold higher than the titers of purified IgG1 (150) and IgG3 (100) products. After observing these results, an ELISA was performed to test whether the increase in IgG2 titers could be explained by differential binding affinity of the secondary detection antibody to the individual antibody subclasses (data not shown). A secondary goat anti-mouse Ig (H+L) antibody was used in both the capture and

secondary antibody detection steps, and equal concentrations of the purified antibody subclass products were analyzed. In this experiment, there were no observable differences between the binding affinities of the four purified IgG subclass products to the secondary antibody. This supports the conclusion that the observed increase in anti-gC titers for the IgG2 subclasses are due to either increased affinity or increased concentration of gC-specific antibodies, as compared to IgG1 and IgG3.

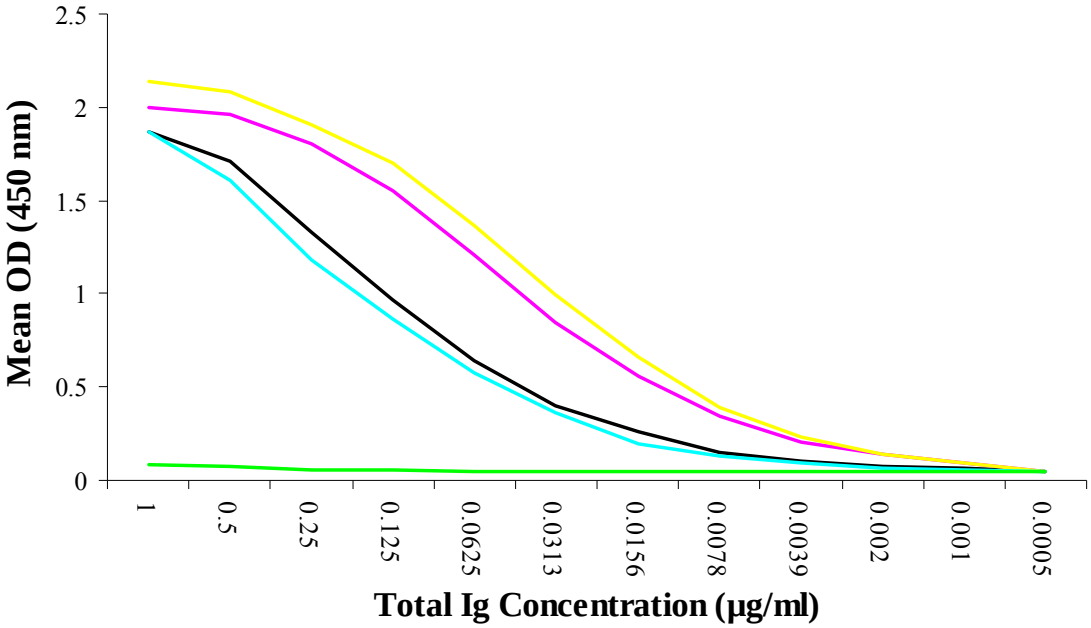

Figure 9. Anti-gC antibody ELISA of purified antibody subclass products. Plates coated with gC-2 445 were incubated with serial dilutions of purified IgG1 (black), IgG2a (pink), IgG2b (yellow), IgG3 (blue) and IgM (green) from gC-immunized mice sera. The secondary HRP-conjugated goat anti-mouse antibody was added to detect gC-bound antibodies, and plates were read at 450 nm.

Neutralization assay of gC-immunized and mock-immunized mice sera

An *in vitro* neutralization assay was used to determine the relative ability of individual mice to neutralize HSV-2 infection (Figure 10). No neutralizing activity was detected in any of the individual or pooled mock-immunized mice sera samples.

Neutralizing activity was observed in individual gC-immunized mice sera, where 50% neutralization titers (NT_{50}) ranged from 1:1400 to 1:6400 and were significantly higher than mock-immunized mice sera (P=0.007). The pooled gC-immunized mice sera showed an NT_{50} at a 1:5000 dilution, which fell within the range of neutralizing activity measured for individual mice. No apparent correlation between an individual mouse's neutralizing activity could be found to either the total level of anti-gC specific antibodies or to the relative amount of a particular antibody subclass in that individual. Although there was individual mouse-to-mouse variation in neutralizing activity, it was important to show that all of the individual mice did develop a neutralizing immune response after gC-2 445 vaccination.

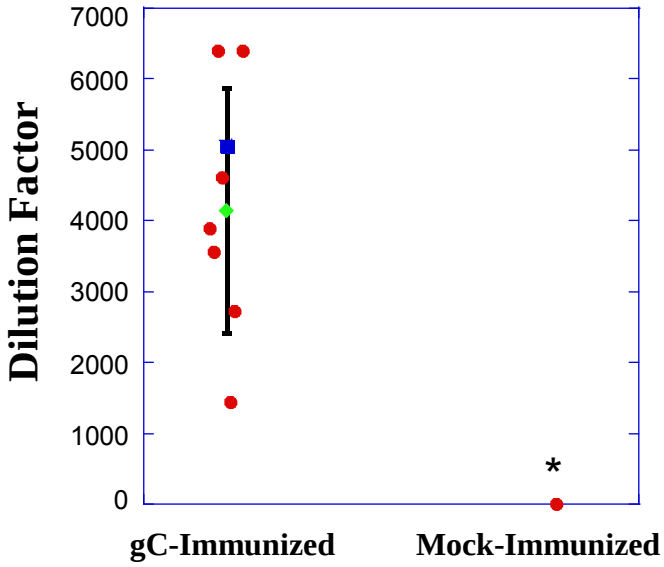

Figure 10. ELVIS® cell HSV-2 neutralization assay of individual gC-immunized and mock-immunized mice sera. Diluted gC-immunized and mock-immunized mice sera samples were mixed with HSV-2, strain MS. The virus-antibody mixtures were then incubated with ELVIS® cells for 20 hours at 37ºC. The level of HSV-2 infection was determined enzymatically using the Gal-Screen® kit. The 50% neutralizing titers (NT_{50}) for individual mice (●) and pooled sera (■) are plotted. The mean NT_{50} (♦) and the standard deviation (error bars) are shown. Asterisk indicates significant difference in the neutralizing activity between gC-immunized and mock-immunized mice as determined using the Mann-Whitney t-test.

Neutralization assay of purified antibody subclass products

The purified antibody subclass products were investigated for their ability to inhibit the infection of ELVIS® cells by the HSV-2, strain MS (Figure 11). The antibody subclass concentration required to neutralize 50 % of HSV-2 infection (NT_{50}) was highest for IgM, which was measured at 350 μg/ml, indicating that it was inefficient at neutralizing HSV-2. IgG1 had improved activity with an NT_{50} of 23 μg/ml. IgG2a, IgG2b, and IgG3 were the most efficient at neutralizing HSV-2, exhibiting NT_{50} concentrations of ~2 μg/ml. This shows that the Th1-response induced IgG2a, IgG2b, and IgG3 antibodies, as compared to the Th2-response induced IgG1, have either more specific antibodies targeting the neutralization domains of gC, or have higher affinity to these domains.

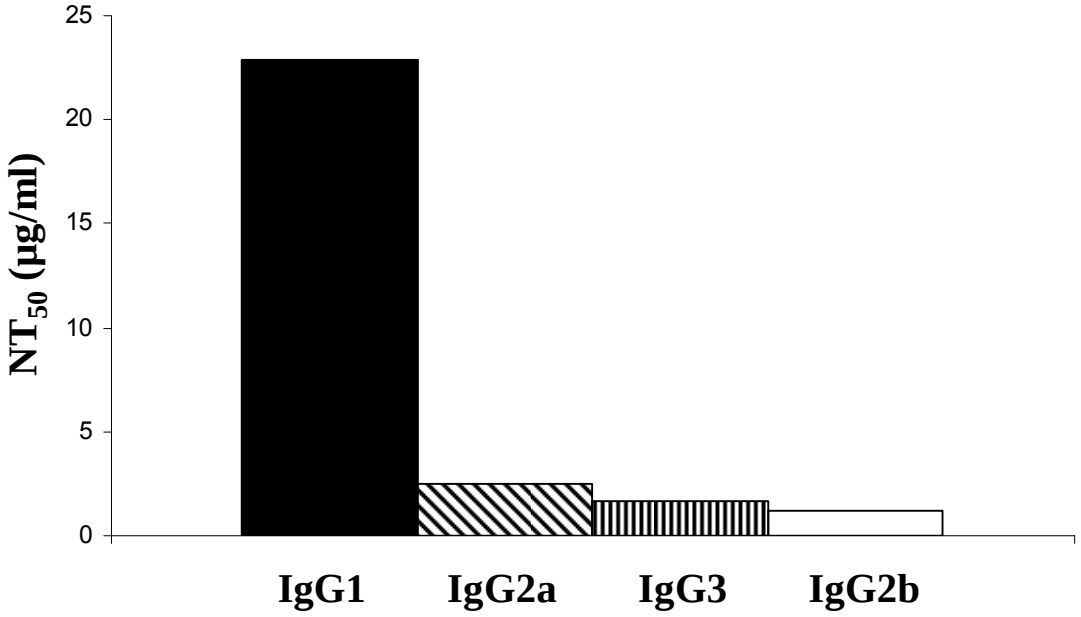

Figure 11. NT_{50} of purified antibody subclass products. Dilutions of purified antibody subclass products were mixed with HSV-2, strain MS. The virus-antibody mixtures were then incubated with ELVIS® cells for 20 hours at 37ºC. The level of HSV-2 infection was determined enzymatically using the Gal-Screen® kit. The NT_{50} for each antibody subclass was determined. In order of their increasing neutralizing activity, NT_{50}s are graphed for IgG1 (■), IgG2a (▨), IgG3 (▥), and IgG2b (□).

gC-2/C3b blocking assay with antibody subclass products

The purified antibody subclass products were examined for their ability to block the binding of gC-2 445 to human complement component C3b in an ELISA assay (Figure 12). The purified IgM did not block gC-2/C3b interactions. Purified IgG1, IgG2a, IgG2b and IgG3 all inhibited binding of gC-2 to C3b. The average concentration of purified Ig required to block 50% of gC-2 binding to C3b (EC_{50}) was 10 μg/ml. In order of decreasing efficiency to block gC-2 binding to C3b, IgG2b was the most efficient (EC_{50} = 7.5 μg/ml), followed by Ig2a (EC_{50} = 9 μg/ml), IgG3 (EC_{50} = 12 μg/ml), and IgG1 (EC_{50} = 13 μg/ml). Therefore, although all of the purified antibody subclass products from gC-immunized mice were capable of blocking this important immune inhibition mechanism, the IgG2a and IgG2b subclasses are the most efficient.

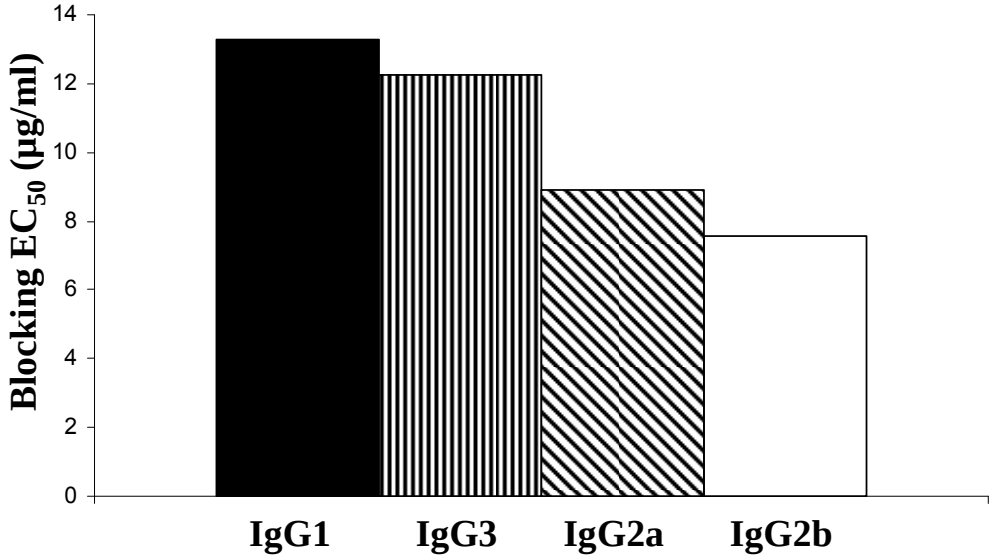

Figure 12. Levels of gC-2/C3b blocking activity of purified antibody subclass products. Dilutions of purified antibody subclass products were mixed with HSV-2 gC protein. The gC/antibody mixtures were then incubated with human C3b coated on an ELISA plate. The amount of gC-2 bound to C3b was quantified using polyclonal anti-gC antibody R81 and anti-rabbit HRP conjugate goat anti-rabbit IgG. The EC_{50} for each antibody subclass was determined. In order of their increasing gC-2/C3b blocking ability, EC_{50}s are graphed for IgG1 (■), IgG3 (▥), IgG2a (▨), and IgG2b (□).

DISCUSSION

The IgG subclass responses from the polyclonal anti-sera of BALB/c mice after HSV-2 gC immunization were evaluated. In the current study, mice were immunized with gC containing both Th2-inducing aluminum adjuvant, MAA, and a Th1-inducing synthetic CpG oligonucleotide, and so the relative IgG subclass expression levels could not be predicted at the initiation of the study. Therefore, it is important to point out the levels of induced antibody subclasses in gC-immunized mice, as compared to mock-immunized mice, to show whether the Th1- or Th2-immune response dominated after immunization with both MAA and synthetic CpG oligonucleotide adjuvants. As compared to the mock-immunized mice sera, there were overall increases in the levels of IgG1, IgG2a, and IgG2b. An increase in antibody class-switching to the IgG1 and IgG2 subclasses was expected, as protein antigens have been shown to induce thymus-dependent responses dominated by these three subclasses.[19] The IgG3 subclass, however, has been shown to be induced by carbohydrate antigens and a thymus-independent response.[19] Accordingly, there was no induction of IgG3 in gC-immunized mice, but instead, a measurable decrease in total IgG3 levels was observed, as compared to mock-immunized mice.

Looking more closely at the three IgG subclasses that showed increased expression after gC-immunization, as compared to the levels measured in mock-immunized mice, the most pronounced increases were seen in the IgG2a (4 fold increase) and IgG2b (6 fold increase) subclasses. Notably, IgG2a was present at 4.4 mg/ml in the pooled gC-immunized mice sera. This constituted 73% of all antibodies present in the gC-immunized mice sera, compared to IgG2a making up 40% of all antibodies present in

the mock-immunized mice sera. The results of these tests suggest that the synthetic CpG oligonucleotide is a more potent adjuvant than MAA, swinging the immune response to one dominated by the Th1-response and induction of the IgG2 subclasses. In designing a subunit HSV-2 vaccine containing a gC component, it will be important to choose the best adjuvant for gC. Proper adjuvant selection could enhance the overall immune response and favor the induction of the antibody subclasses that would best function in HSV-2 neutralization and in the blocking of gC-2/C3b binding. This would allow the complement component of the innate immune system to work synergistically with the viral neutralization, and should enhance vaccine efficacy. From the results of these tests, the IgG2 subclasses appeared to be the most efficient in neutralizing virus and blocking gC-2/C3b interactions *in vitro*, which suggests that gC immunization with a Th1-inducing adjuvant would provide optimal protection from HSV-2 infection. Interestingly, one study looked at the relative concentrations of IgG subclasses in BALB/c mice after HSV-2 infection, and showed that there was a significant dominance of serum antiviral IgG1.[15] In the current study, it was shown that compared to the other IgG subclasses, IgG1 had relatively poor neutralization efficiency and capacity to block gC-2/C3b binding. This may help explain the poor control of natural infection, and further support the selection of a Th1-inducing adjuvant that favors the production of the more effective IgG2 subclasses.

The purified antibody subclass products were analyzed for their ability to neutralize HSV-2 infection, and all IgG subclass products had some degree of neutralizing activity. Purified IgG2a, IgG2b, and IgG3 all exhibited NT_{50}s at concentrations close to 2 μg/ml; whereas IgG1 exhibited an NT_{50} at a ~10-fold higher concentration of 23 μg/ml. Purified IgM was shown to have poor neutralizing activity,

with an NT_{50} of 350 µg/ml. This was not surprising, as IgM antibodies express variable regions that have not yet been modified by somatic mutation, and therefore tend to bind antigens with low affinity.[18] This was further confirmed in the anti-gC endpoint ELISA which did not measure any anti-gC specific IgM antibodies.

It has been previously shown that IgG2a and IgG2b are generally the most potent antibody subclasses for activating effector responses and enhancing ability to initiate the complement cascade.[19] The first step in activating the complement cascade involves the binding of C1q to the antibody's F_c region. For this to occur, the antibody must have the appropriate binding site amino acids that have a high affinity to C1q. Furthermore, antibodies must have sufficient flexibility within the hinge region to allow the F_{ab} arms to move freely and permit C1q to come into proximity of its F_c binding site. Based on these two criteria, it is known that, in mice, IgG3 is the best activator of complement, followed closely by IgG2a and IgG2b. Murine IgG1, on the other hand, has very minimal capacity to bind C1q and thus activate the complement cascade.[22] Although two neutralization epitopes in gC have been mapped, the complement cascade may also contribute to the overall neutralization measured in the assay, given that IgG2 and IgG3 subclasses are most efficient in activating complement.[23] This could be tested by evaluating the antibody subclasses in the neutralization assay in the absence of complement. Under these conditions, it would be interesting to see if relative ranking would remain the same.

The exact mechanism through which anti-gC antibodies neutralize HSV-2 is not currently known. Although gC-2 is known to bind cell surface HS and play a role in HSV-1 viral binding, this function is not essential for HSV-2 infection.[24] Specifically, it was shown that an HSV-2 mutant lacking gC had normal specific infectivity, specific

binding activity, rates of penetration and growth kinetics.[24] It was concluded that gC-2 was not the primary glycoprotein responsible for binding HSV-2 to the target cell, as is the case for HSV-1. Therefore, the mechanism of neutralization by anti-gC antibodies should not involve antibodies that block gC-2/HS binding. Taking the model of HSV-2 entry into cells into consideration, there is a hypothesis that explains how the anti-gC antibodies may neutralize infection even though gC is not required for HSV-2 infection to occur. Anti-gC antibodies may be binding to gC on the viral surface in a manner that causes steric hindrance that interferes with viral fusion. Anti-gC antibodies may sterically inhibit the binding of virus to the cell surface by impairing the binding of gB or gD to their cognate cell surface receptors. Anti-gC antibodies may also sterically hinder the formation of the multi-protein fusion complex composed of gB, gD, gH and gL, by impeding the interaction of the viral glycoproteins.[4] Specifically, because the binding of gD to its cellular receptor and the formation of the multi-glycoprotein complex are both required steps for HSV-2 membrane fusion, it is possible that bulky anti-gC antibodies bound to specific sites on gC may sterically hinder the formation of the viral fusion complex.

Through the mechanism of steric hindrance or by other unknown means, it was clear in the current study that the IgG2a, IgG2b, and IgG3 anti-gC antibodies were very effective in neutralizing HSV-2 infection *in vitro*. These findings were similar to a previous study that looked at the neutralizing capacity of anti-HSV antibodies in BALB/c mice after HSV-2 infection. That study demonstrated that the IgG2a and IgG2b subclasses had significantly greater neutralizing activity, while the IgG1 antibodies had reduced neutralizing activity.[15] In response to both HSV-2 infection and gC-

immunization, it appears that the IgG2 and IgG3 antibodies are either better directed at neutralizing epitopes or have increased avidity to the neutralizing epitopes, compared to IgG1 antibodies.

Although gC-2 in the viral envelope does not play a dominant role in HSV-2 binding, it appears to be important in protecting the virus from the host immune system. Therefore, in addition to comparing the antibody subclass' relative ability to neutralize HSV-2 infection, the current study looked specifically at their relative ability to block gC-2/C3b binding, which would allow normal complement-mediated functions to proceed *in vivo*. Compared to its cell surface receptor, gC-2 has a seven-fold higher affinity for C3b than for HS.[3] This works to the virus' advantage in blocking C3b viral opsonization, thus limiting a major effector arm from the innate immune system. In the current study, it was seen that all four IgG subclasses of anti-gC antibodies were capable of blocking the gC-2/C3b binding in an ELISA assay. Their relative efficiency at inhibiting this interaction was IgG2b>IgG2a>>IgG3>IgG1. IgG2a and IgG2b were more effective at blocking this gC-2/C3b interaction than IgG3 and IgG1. IgM did not block gC-2/C3b binding in this assay, which again, is not surprising due to the reduced antigen affinity of IgM. The results of the gC-2/C3b blocking assay provide further evidence for the advantages of gC-immunization with a Th1-response inducing adjuvant. This would favor class-switching to the IgG2 subclasses, which were shown to function optimally in blocking the HSV-2 mechanism of immune inhibition.

In both the neutralization assay and the gC-2/C3b blocking assay, the increased activity of the IgG2 subclasses could be explained by either the increased presence of total gC-specific antibodies in these subclasses, or increased specificity of the antibodies

to gC-2 epitopes that play roles in neutralization or C3b binding. In support of this conclusion, the anti-gC endpoint ELISA showed 4-fold higher titers for the IgG2a and IgG2b subclasses, as compared to the IgG1 and IgG3 subclasses, when total antibody concentration was held constant.

A large body of work over the last 25 years has shown that gD vaccines induce large amounts of potent neutralizing antibodies, yet fail to provide lasting protection in clinical trials.[5] In all, the current study supports further consideration of a prophylactic HSV-2 vaccine consisting of gC-2 and gD-2 formulated with an adjuvant that induces a Th1 response. The current study showed that gC-immunization leads to production of neutralizing antibodies, and previous work has shown that in the presence of complement, anti-gC and anti-gD IgG has greater neutralizing activity than either alone.[12] In addition, the current study showed that gC-immunization results in the production of antibodies capable of blocking gC-2/C3b binding. In both the neutralizing and gC-2/C3b -blocking functions, the Ig2a and IgG2b subclasses had the highest activity; therefore, a preferential Th1-induced response would provide superior protection. Previous work looking at the efficiency of anti-gD antibody subclasses to neutralize HSV infection *in vitro* and to provide passive protection also showed that IgG2a and IgG2b were more effective than IgG1.[25] Therefore, the induction of both anti-gC and anti-gD IgG2 subclasses may increase neutralizing activity. Notably, the anti-gC IgG2 subclasses have a high probability of effectively preventing the gC-2 mediated immune inhibition function. Both benefits of anti-gC IgG2 subclasses should enhance the efficacy of a gD vaccine. Although there are clear differences between mouse and human IgG subclasses, the principles that have emerged from this study should be applicable to the design of

strategies for the induction of human antibodies. Data on the comparative efficiency of the antibody subclasses in humans will be useful in future design for both passive protection and vaccination regimens.

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VITA

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