Role of Anti–Glycoproteins D (Anti–gD) and K (Anti–gK) IgGs in Pathology of Herpes Stromal Keratitis in Humans

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Purpose. To assess the relative impact of antibodies specific for HSV-1 glycoproteins on eye disease in response to HSV-1 infection, the composition of antibodies specific for 10 of the viral glycoproteins, and the effect of anti–glycoprotein (g)D and anti–gK antibodies on antibody-dependent enhancement (ADE).

Methods. In a prospective case-control study, sera from patients with a history of herpes stromal keratitis (HSK) were compared with sera from nonocular HSV-1–seropositive and HSV-seronegative control subjects. HSV-1 neutralizing antibody titer and type-specific IgG and IgM were measured. In addition, the presence of anti–HSV-1 gD and gK antibodies in the sera of all patients also was determined by ELISA using gD and gK antigens. Finally, the role of anti–gD- and gK-specific antibodies to ADE was investigated.

Results. Average neutralizing antibody titers and levels of HSV-1 IgG were similar between HSK- and non–HSK-seropositive patients. However, the contribution of gD to the neutralizing antibody titer in HSK sera was significantly lower than that in non–HSK-seropositive patients, despite higher anti–gD ELISA titers. Overall, sera from patients with HSK had higher anti–gK antibody titers and induced ADE in vitro compared with non–HSK or seronegative sera. The ADE response in HSK sera was attributed to anti–gK antibody.

Conclusions. These results suggest that sera from HSK patients had higher anti–gD and –gK antibody titers than sera from seropositive patients who had no history of HSK despite similar levels of neutralizing antibody titers and HSV-1 IgG, that HSK sera induced ADE whereas sera from non–HSK patients did not induce ADE, and that anti–gD antibody in sera of HSK patients contributed less to the HSV-1 neutralization antibody titer than did sera from non–HSK patients. (*Invest Ophthalmol Vis Sci.* 2007;48:2185–2193) DOI:10.1167/iovs.06-1276

Ocular infection with HSV-1 causes eye disease ranging in severity from blepharitis, conjunctivitis, and dendritic keratitis to disciform stromal edema and necrotizing stromal keratitis.[1–3] The most important ocular herpes virus-related clinical problem is herpes stromal keratitis (HSK), which is related to recurrent rather than primary infection.[1,4,5] HSV-1–induced corneal scarring, also broadly referred to as HSK, can lead to blindness. In developed countries, HSV-1 is the leading cause of corneal blindness by an infectious agent.[1,6,7] HSV-1–induced HSK is caused by an as yet undefined immune response, presumably to one or more as yet undefined HSV-1 proteins.

Of the more than 80 known HSV-1 genes, at least 11 encode glycoproteins.[8,9] Viral glycoproteins are the major inducers of cell-mediated and humoral immune responses against HSV-1 infection.[9–11] Vaccination with glycoprotein gB, gC, gD, gE, or gI induced strong neutralizing antibody titers and provided a high level of protection against lethal ocular and intraperitoneal HSV-1 infection in mice.[12–16] In contrast, vaccination with gG, gJ, gH, gK, gL, or gM induced little or no neutralizing antibody titer or protection against HSV-1 infection.[17–21]

However, in contrast to vaccination with gB, gC, gD, gE, gG, gH, gI, gJ, gL, or gM, vaccination of mice with gK resulted in severely exacerbated HSK after ocular HSV-1 infection.[9,22] The exacerbated HSK was independent of mouse or virus strains and also occurred after passive antibody transfer of anti–gK sera before ocular infection.[23] Vaccination with gK also appeared to block viral clearance from the trigeminal ganglia, resulting in a chronic productive infection.[24] Previously, we showed that gK vaccination induced a very strong T_H1+T_H2 response in the eye.[25] In contrast, vaccination with highly efficacious gD induced mostly a T_H1 response, whereas vaccination with ineffective gG, or mock vaccination, induced mostly a T_H2 response, neither of which was as strong as that induced by gK.[25]

Similar to our animal studies, we hypothesized that one or more of the immune responses induced by gK are the direct cause of corneal disease in patients with HSK. The studies in this report were directed at determining what role, if any, anti–gK and anti–gD IgGs play during the course of ocular HSV-1 recurrences leading to HSK. Thus, we evaluated the contribution of HSV-1 glycoproteins to protection and disease using sera from patients with a history of HSK and compared it with results from patients with no history of HSK (also known as cold sore or herpes labialis) and from seronegative control patients.

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Supported by Public Health Service Grants EY13615 and EY14966 from the National Eye Institute (HG).

Submitted for publication October 23, 2006; revised December 4, 2006; accepted February 13, 2007.

Disclosure: **K.R. Mott**, None; **Y. Osorio**, None; **E. Maguen**, None; **A.B. Nesburn**, None; **A.E. Wittek**, None; **S. Cai**, None; **S. Chattopadhyay**, None; **H. Ghiasi**, None

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Materials and Methods

Virus and Cells

Triple plaque-purified McKrae, a neurovirulent HSV-1 strain, was grown in RS (rabbit skin) cell monolayers in minimal essential media (MEM) containing 5% fetal calf serum (FCS), as described previously.[26] The human monocytic leukemia cell line THP-1 was cultured at 37°C in 5% CO_2 using RPMI 1640 medium with 10% FBS (fetal bovine serum). Before each experiment, 1×10^5 THP-1 monocytes were plated in a 48-well culture plate and treated with 10 ng/mL phorbol-12-myristate-13-acetate (PMA; Sigma-Aldrich, St. Louis, MO) for 48 hours to differentiate the cells into macrophagelike cells, as described.[27,28]

Classifications of Collected Sera

After informed consent was obtained from the participants, the research was performed in accordance with the tenets of the Declaration of Helsinki and with an institutional review board (IRB 3663)–approved protocol. Classification of collected sera was based on the use of neutralizing antibody titers, IgG, and IgM. All patients were 18 years of age or older, of either sex, and not pregnant or breastfeeding. All patients (other than corneal scarring [CS]) were healthy and had no ocular infection (other than HSV-1). Sera from patients who were positive for both HSV-1 and HSV-2 or who were IgM positive were excluded. HSK sera were collected from patients with a well-documented history of recurrent infections. If a patient had a history of recurrent HSV-1 keratitis, the last episode must have occurred within 5 years for inclusion of the patient in this study. Sera from HSK patients who did not have detectable neutralizing antibody titers or HSV-1–specific IgG were excluded.

Collected sera were divided into HSV-1 seropositive and HSV-2 seronegative. For clarification, HSK sera were those collected from patients with HSK, and non–HSK sera were those collected from patients with no HSK (and no history of any recurrent disease). In addition, sera from HSV-seronegative patients with no history of ocular or nonocular HSV-1 or HSV-2 were collected as controls.

Serum Neutralization Assay

All collected sera were analyzed for HSV-1–neutralizing antibody titers. Briefly, collected sera were diluted in MEM, mixed with 100 plaque-forming units (PFUs) of HSV-1 strain McKrae, and incubated for 60 minutes at 37°C. Samples were added to RS cells in six-well culture plates, and residual HSV-1 infectivity was assayed. Plates were incubated at 37°C for 72 hours and stained with 1% crystal violet, and the plaques were counted. Means of the antibody titers (50% plaque reduction) were expressed as the reciprocal of the serum dilution. Neutralizing antibody titers were determined for 132 serum samples.

Enzyme-Linked Immunosorbent Assay for Detection of HSV-1 and HSV-2 IgG

ELISAs (Focus HerpeSelect HSV-1 and HSV-2 ELISA; Focus Diagnostics, Cypress, CA) were performed on each serum sample according to kit instructions. Sera with index values lower than 0.9 were considered negative, those with values greater than 3.5 were considered positive, and those with values ranging from 0.9 to 1.1 (inclusive) were considered equivocal. Index values greater than 1.1 to 3.5 were considered low-positive.

ELISA for Detection of HSV-1 and HSV-2 IgM

HSV-1 and HSV-2 IgM in the sera were determined with an enzyme immunoassay test kit as described by the manufacturer (Diamedix, Miami, FL). Sera with index values lower than 0.9 were considered negative, those with values of 1.1 and higher were considered positive, and those with values ranging from 0.9 to 1.09 were considered equivocal.

Expression of HSV-1 Glycoproteins

Sf9 insect cells were infected with 10 PFUs/cell of each baculovirus recombinant expressing gB, gC, gD, gE, gG, gH, gI, gJ, gK, or gL, as described previously.[9] Infected cells were grown for 72 hours, collected, washed, suspended in PBS, freeze-thawed, and homogenized by sonication. As control, Sf9 cells were similarly infected with wild-type baculovirus.

Blocking Experiments

To determine the ideal concentration of recombinant HSV-1 glycoprotein to block the corresponding antibody titer in each serum sample, we subjected 100 μL of each sample to overnight incubation with 1×10^4, 1×10^5, or 1×10^6 infected cells. The absence of antibody specific for each glycoprotein was confirmed by ELISA, and subsequent blocking experiments were conducted using 1×10^6 infected cells because this was determined to be the most ideal concentration. Briefly, 100 μL sera from HSK or non–HSK patients were incubated overnight with lysates from 1×10^6 Sf9 infected cells expressing each of the following HSV-1 glycoproteins: gB, gC, gD, gE, gI, 2gP (gB, gD), 5gP (gB, gC, gD, gE, gI), 10gP (gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL). In addition, sera were incubated overnight with 1×10^6 HSV-1–infected RS cells in which HSV-1 had been inactivated by a combination of heat and UV treatment. As controls, all sera were incubated similarly with wild-type baculovirus-infected Sf9 cells or with uninfected RS cells. Neutralizing antibody titers in the blocked sera were determined as described. Experiments were performed with sera from 10 patients per group.

Ten samples were randomly chosen from the stromal keratitis HSK serum samples that had at least three episodes of reactivation in the past 5 years and that had neutralizing antibody titers similar to the total mean titer ± 5 and IgG titers in the range of ± 0.5. Similar criteria were used for analysis of the non–HSK serum samples. No information was collected with respect to the number of recurrences in the non–HSK patients. The same serum samples were used for antibody-dependent enhancement (ADE) measurement and ADE-blocking experiments.

ELISA for Detection of Anti-gD and -gK

For gD coating, ELISA plates were coated with purified *Escherichia coli*–expressed gD antigen corresponding to aa266 to aa394 of gD (United States Biological, Swampscott, MA), or affinity-purified baculovirus-expressed gD[12] was used to detect gD antibody. We compared gD expressed in a baculovirus system with that expressed in *E. coli* using serum from HSK and non–HSK patients and obtained similar results. Therefore, the gD expressed in baculovirus was used as antigen in ELISA experiments for 70 of the serum samples, and gD expressed in *E. coli* was used for the remainder.

For gK coating, we affinity purified gK antigen from baculovirus-gK infected Sf9 cells[21] using an affinity-purified polyclonal antibody raised in rabbit (BAbCo, Richmond, CA) that was specific for the amino acid sequence of LIAGRVVPFQVPPDAMNRRI of gK (Peptide Synthesis Core Facility, University of California at Los Angeles) based on published sequence data.[8]

Serum concentrations of antibody to gD or gK were measured by ELISA. Microtiter plates were coated with approximately 5 ng/100 μL of each purified recombinant glycoprotein in carbonate buffer (pH 9.5) and were incubated overnight at 4°C. The plates were washed five times with 1× Tris-buffered saline (TBS; pH 8.0) containing 0.05% tween 20 (TBS-T). Subsequently, 200 μL blocking buffer (3% BSA in TBS-T) was added to each well and incubated for 2 hours at 37°C, followed by five washes with TBS-T. For testing, 100-μL blanks or samples diluted 1:50 to 1:12,800 in TBS-T containing 0.1% BSA were introduced to the wells and incubated for 1 hour at room temperature before five washes with TBS-T. Subsequently, 100 μL peroxidase-conjugated anti–human IgG-Fc–specific antibody (Sigma-Aldrich) at a 1:35,000 dilution in TBS-T/0.1% BSA was added, incubated for 1 hour

at room temperature, and washed five times with TBS-T. Samples were developed by the addition of 100 μL substrate 3,3,5,5-Tetramethylbenzidine (TMB, SIGMA-Aldrich) for 15 minutes. Finally, the reaction was stopped by the addition of 100 μL of 1 N HCl, and the optical density was read at 450 nm in a spectrophotometer (microplate reader; Bio-Rad, Hercules, CA). The antibody titer in each serum sample was defined as the highest dilution giving an optical density reading greater than 0.1. Results were expressed as the reciprocal geometric mean titer for each select group of patient serum samples.

In Vitro Assay for Antibody-Dependent Enhancement of HSV-1

One thousand PFUs of HSV-1 strain McKrae were incubated for 1 hour at 37°C with various serum dilutions of HSK, non–HSK, or seronegative sera in 100 μL volumes. Serum concentrations ranged from 10^{-2} to 10^{-9}. Virus–antibody mixtures were added to each well containing 1×10^5 THP-1 cells. The final multiplicity of infection (MOI) was 0.01. Wells were incubated at 37°C for 1 hour, and the cells were washed with fresh media. After two washes, 300 μL fresh media was added, and the plates were incubated for 24 hours. Twenty-four hours after infection, the cultures were freeze-thawed twice and assayed for plaque formation in five replicates on RS cell monolayers in six-well tissue culture plates. Plaques were stained and counted 3 days after infection. Experiments were repeated two times with 10 sera per group.

Blocking ADE Experiments

Previously, we showed that sera from gK-immunized mice ADE of HSV-1 infection in tissue culture.[29] To determine whether ADE in HSK patients was caused by anti–gK antibody as it was in gK-immunized mice, we blocked anti–gK antibody in the sera of HSK and non–HSK patients by incubating 100 μL of each serum sample with 1×10^6 Sf9 cells infected with baculovirus expressing gK. As a control, we incubated the same sera similarly with baculovirus-expressed gD. Mixtures of antibody–antigen were incubated overnight, and serial dilutions of the sera were incubated with 1000 PFUs of HSV-1 strain McKrae, as described. After 24-hour infection of THP-1 cells with the virus-antibody mixtures, the infected cells were freeze-thawed and assayed for plaque formation on RS cell monolayers.

Statistical Analysis

Protective parameters were analyzed by the Student's *t*-test and Fisher exact test (Instat; GraphPad, San Diego, CA). Results were considered statistically significant when the $P < 0.05$.

Results

Inclusion Criteria

One hundred thirty-two human sera were collected from 1989 through June 2006; 59 samples were from patients with a history of ocular infection, and 73 samples were from patients with no history of ocular infection. Collected sera were analyzed for HSV-1–neutralizing antibody and for HSV-1 and HSV-2 IgG and IgM, as described in Materials and Methods. For patients with a history of HSV infection (ocular or nonocular), only sera from patients with no HSV-2 IgG were considered in the study. In addition, sera from patients positive for IgM were excluded. In general, all the IgM-positive sera came from patients who were both HSV-1 and HSV-2 seropositive. Sera from HSK-positive patients with no history of recurrent infection for at least 5 years were excluded from the study. Finally, sera from three patients in the HSK group who did not have HSV-1 antibody titers based on neutralizing antibody titer and IgG measurements were excluded. The absence of any detectable level of HSV-1–specific antibody in these sera could have been the result of seroreversion.

Sera from the patients with no history of HSK, based on the presence or absence of HSV-1–neutralizing antibody titer and IgG, were further classified into non–HSK-seropositive and -seronegative groups, respectively, as shown in Table 1. Overall, based on IgG, IgM, and neutralizing antibody measurements, we excluded 21 sera that did not meet our criteria.

Detection of HSV-1 Antibody Titers in Collected Sera

Antibody titers were determined by neutralization assay and ELISA for IgG, as described in Materials and Methods. Neutralization titers of all sera were determined by a 50% plaque

Table 1. Classification of Sera Collected from Patients with and without History of HSV-1 Infection

	Neutralizing Antibody Titer†		IgG‡		IgM‡	
Type of Sera*	**Mean**	**Range**	**Mean**	**Range**	**Mean**	**Range**
HSK ($n = 53$)	115 ± 14	57–185	5.14 ± 0.32	3.6–6.3	0.23 ± 0.05	0.10–0.52
Non-HSK ($n = 31$)	111 ± 35	15–345	4.76 ± 0.29	3.0–6.0	0.32 ± 0.08	0.08–0.62
Seronegative ($n = 27$)	0	0	0.24 ± 0.06	0.05–0.8	0.29 ± 0.05	0.13–0.76

* HSK indicates sera from patients with ocular disease sufficient to warrant a physician visit. Non-HSK indicates sera from seropositive patients with no history of ocular infection or CS. Seronegative indicates patients with no neutralizing antibody titer to HSV infection. Serum was extracted from patients with recurrent stromal keratitis. Patients showed no sign of virus reactivation and were not on any antiviral treatment at the time of blood extraction. Patients with primary and recurrent ocular herpes were treated with antiviral medication and topical steroids. Patients received the antiviral acyclovir (Zovirax; GlaxoSmithKline, Brentford, Middlesex, UK; 1–5 g/d) unless resistance was detected, in which case they received valacyclovir (Valtrex; GlaxoSmithKline, Research Park Triangle, NC; 1–3 g/d). When keratouveitis was present, patients were treated with topical steroids such as prednisolone acetate 1% (pred forte), fluorometholone 0.1% (FML), loteprednol etabonate ophthalmic suspension (Lotemax; Bausch & Lomb, Rochester, NY), or Allrex. No systemic steroids were used. None of the patients whose serum was used in this study underwent corneal surgery. Infection was controlled by antiviral medication in most patients with primary HSV-1 infection within a number of days of the first episode. The rate of recurrence in these patients was low. In patients with recurrent disease, healing required several weeks to several months. Sera from patients who experienced no recurrences in the past 5 years were excluded. Non-HSK serum was collected from members of the general population with no history of ocular or genital HSV infection and was classified as HSV-1 seropositive and seronegative based on IgG titers and the presence or absence of HSV-1–neutralizing antibody. Any serum positive for IgM was excluded from analysis. None of the samples collected from HSK or non-HSK patients showed signs of ocular infection or cold sores, respectively, at the time of collection.

† Neutralizing antibody titers were determined by 50% plaque reduction assay.

‡ IgG and IgM titers were measured as described in Materials and Methods.

reduction assay, and the neutralizing antibody titers in the sera that met our criteria are shown in Table 1. The average neutralizing antibody titer for the HSK group was similar to that of the non–HSK group ($P = 0.9$; Student's *t*-test). However, as expected, the HSK and the non–HSK groups had significantly higher neutralizing antibody titers than did the seronegative group ($P < 0.0001$).

Similar to our 50% plaque reduction assay, the HSK and non–HSK groups had similar IgG titers ($P = 0.4$) that were significantly higher than those of the seronegative control group ($P < 0.0001$). Our results suggest that the antibody titers of our collected sera for HSK and non-HSK were similar.

With regard to the IgM ELISA, four of the sera from HSK and non–HSK patients were IgM positive and HSV-2 positive (not shown). However, when we excluded these four sera, the level of IgM between HSV-1 seropositive (HSK or non-HSK) and seronegative sera was similar ($P = 1$; Table 1).

Contribution of Patient HSV-1 Glycoproteins to Neutralizing Antibody Titers in HSK Versus Non-HSK Sera

The studies described here and shown in Table 1 indicated that the mean neutralizing antibody titers between HSK and non–HSK sera were similar. However, our animal studies revealed that different HSV-1 glycoproteins induced different levels of neutralizing antibody titers and protection in mice.[9,12–24] To determine whether corneal versus nonocular clinical presentation of HSV-1 infection can contribute differently to the induction of neutralizing antibody titer associated with each glycoprotein in HSK versus non–HSK sera despite the similarity in total neutralizing antibody to HSV-1, we selected human sera from 20 patients in the HSK and non–HSK groups. All the selected sera had a neutralizing antibody titer greater than 100. For each HSK serum sample, we chose a comparable non–HSK serum with a neutralizing antibody titer within ±5. Sera from each group were incubated with gB, gC, gD, gE, or gI alone or with a mixture of gB and gD (2gP), a mixture of gB, gC, gD, gE, and gI (5gP), or a mixture of gB, gC, gD, gE, gG, gH, gI, gJ, gK, and gL (10gP), as described in Materials and Methods. Control sera were similarly incubated with Sf9 cells infected with wild-type baculovirus, heat- and UV-inactivated RS cells infected with HSV-1 strain McKrae, or uninfected RS cells.

In HSK and non–HSK sera (Fig. 1), gB and gC antigens blocked approximately 40% of neutralizing antibody. These differences were not statistically significant ($P > 0.5$). In contrast to gB and gC, gD antigen blocked 20% of neutralizing antibody in the HSK group (Fig. 1, gD) compared with 40% blocking of neutralizing antibody in the non–HSK group (Fig. 1, gD). The difference between HSK and non–HSK groups for gD was statistically significant ($P = 0.04$). gE and gI blocked the neutralizing antibody titers less efficiently in both groups compared with gB and gC (Fig. 1), and the differences between the two groups of sera were not significant ($P > 0.5$). A mixture of gB and gD (2gP) acted additively compared with gB or gD alone in both groups, and the 2gP mixture was more effective in blocking neutralizing antibody in the non–HSK group (Fig. 1) than in the HSK group (Fig. 1). However, the differences between the two groups were not statistically significant ($P = 0.25$).

A mixture of 5gP was even more effective in blocking HSK- and non–HSK-neutralizing antibody than when 2gP (Fig. 1, 2gP vs. 5gP) was used for blocking. Furthermore, the differences between 5gp and 2gp for both groups and between 5gP from HSK sera and 5gP from non–HSK sera were not statistically significant ($P > 0.09$). Finally, the effect of blocking with a mixture of 10gP was similar to that of 5gP ($P > 0.7$). These

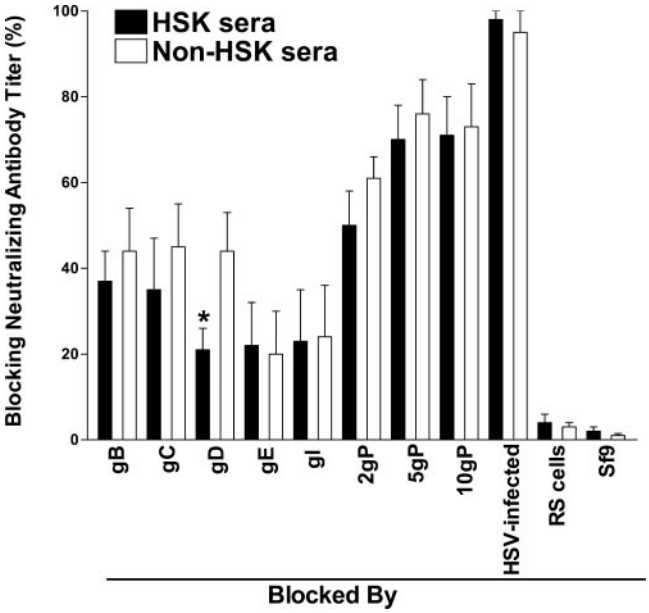

FIGURE 1. Contribution of HSV-1 glycoproteins to neutralizing antibody titers in sera from HSK and non–HSK patients. To determine the contribution of each of the 10 HSV-1 glycoproteins (gB, gC, gD, gE, gG, gI, gJ, gH, gK, gL) to antibody titers in seropositive patients, sera from 10 patients in the HSK group who had at least three episodes of reactivation were compared with sera from 10 patients in the non–HSK group who had similar levels of neutralizing antibody titers. Sera (100 μL) from the HSK or the non–HSK group were incubated overnight with 1×10^6 Sf9-infected cell lysates expressing each of the following HSV-1 glycoproteins: gB, gC, gD, gE, gI, 2gP (gB, gD), 5gP (gB, gC, gD, gE, gI), 10gP (gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL). In addition, sera were incubated with heat- and UV-inactivated 1×10^6 HSV-1 strain McKrae infected RS cells overnight. As controls, sera from 10 patients were incubated similarly with wild-type baculovirus-infected Sf9 cells or uninfected RS cells. Neutralizing antibody titers in the blocked sera were determined. Experiments were repeated twice. Error bars indicate SEM. *Significantly different from non–HSK group ($P = 0.04$; Student's *t*-test).

results suggested that the five additional HSV-1 glycoproteins (gG, gH, gJ, gK, gL) did not have any effect on the induction of neutralizing antibody titers in either group. In contrast to the mixture of the 5gp, which could block approximately 76% for non–HSK and 70% for HSK sera, when we used the cell lysates from the HSV-1–infected RS cells, we completely blocked the neutralizing antibody titers in both groups (Fig. 1, HSV-1). Differences between blocking by the HSV-1 cell lysates and blocking by expressed 5gP proteins were statistically significant for both groups ($P < 0.03$). In summary, results from our blocking experiments suggested that approximately 75% of the neutralizing antibody titers detected in both HSK and non–HSK sera were associated with gB, gC, gD, gE, and gI.

Induction of Antibody-Dependent Enhancement by Sera from HSK Patients

ADE occurs when IgG binds to the virus and the virus–antibody complex attaches to and enters cells containing Fc receptors. This mode of virus entry bypasses the normal mode by which virus enters cells after attachment to specific receptors on the cell. Thus, ADE can result in increased viral entry or infection of cells that the virus normally does not infect, leading to the exacerbation of disease. To determine whether ADE plays any role in HSK, sera from 10 HSK patients were tested for ADE induction. For controls, we used sera from 10 patients in the

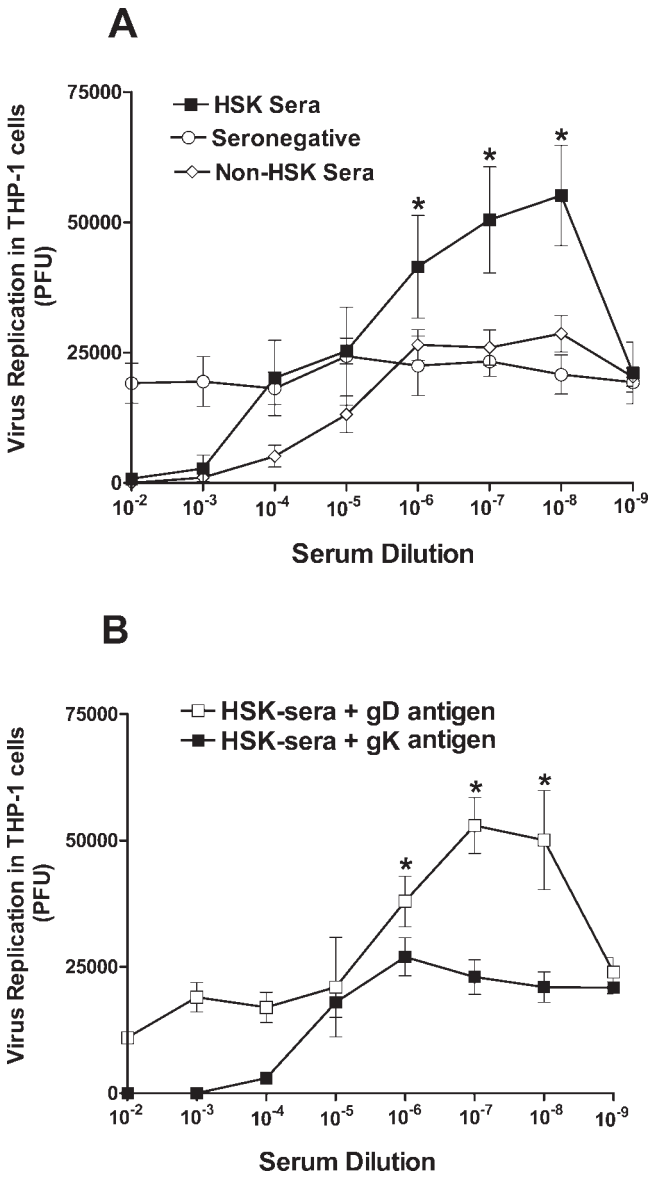

FIGURE 2. Measurement of ADE in the sera. (**A**) Enhancement of HSV-1 infection by HSK sera. THP-1 or RS cells were infected with HSV-1 strain McKrae at an MOI of 0.01 PFU/cell in the presence of sera from HSK and non–HSK patients used in the competition assay described in Figure 1. Twenty-four hours after infection, supernatants were assayed for infectious virus by plaque assays on RS cells. For each point, the virus titer (*y*-axis) represents the average of the titers from sera of 10 patients, with four replicates per dilution. Sera from 10 HSV-1 seronegative patients were used as controls. Experiments were repeated twice. Error bars indicate SEM. *Significantly different from the same dilution in the other groups ($P < 0.05$; Student's *t*-test). (**B**) Enhancement of HSV-1 infection by HSK sera is associated with gK antibody. Sera were incubated with baculovirus-expressed gD, gK, or wild-type–infected Sf9 cells, as described in the legend to Figure 1. THP-1 cells were infected with HSV-1 strain McKrae at an MOI of 0.01 PFU/cell in the presence of blocked sera from HSK or non–HSK patients. Twenty-four hours after infection, supernatants were assayed for infectious virus by plaque assay on RS cells. For each time point, the virus titer (*y*-axis) represents the average of the titers from sera of 10 patients, with five replications per dilution. Sera from 10 HSV-1 seronegative patients were used as controls. Experiments were repeated twice. Error bars indicate SEM. *Significantly different from the same dilution in the other groups ($P < 0.05$; Student's *t*-test).

non–HSK group and from 10 patients seronegative for HSV-1. Sera were heat-inactivated, and ADE was determined using THP-1 cells, as described in Materials and Methods. THP-1 is a human monocyte–derived cell line that can be induced to differentiate into macrophages by treatment with PMA.[27,28] The THP-1 cells are Fc receptor-positive.

Compared with sera from non–HSK or seronegative control samples, sera from the HSK group enhanced the virus yield in THP-1 cells (Fig. 2A). The mean yield of infectious progeny virus in THP-1 cells infected with HSV-1 at an MOI of 0.01 was higher in the presence of HSK sera than in the presence of sera from non–HSK or seronegative control (Fig. 2A). At dilutions of serum between 10^{-6} and 10^{-8}, the differences were statistically significant ($P < 0.05$). The highest level of enhancement by HSK sera was observed at a dilution of 10^{-8} (Fig. 2A). As expected, no significant ADE by HSK sera was detected using RS cells (not shown), which are Fc receptor-negative. Sera from the HSK and non–HSK groups, but not the seronegative controls, at dilutions of 10^{-2} and 10^{-3} resulted in decreased virus titer because of the presence of HSV-1–neutralizing antibody at the lower dilutions but not at the higher dilutions (Fig. 2A). These results suggested that sera from HSK patients could induce ADE in vitro, whereas sera from non–HSK or seronegative patients did not cause ADE.

Role of gK in ADE

Our results suggested that sera from the HSK patients induced ADE in a macrophage cell line but that sera from the non–HSK patients or the seronegative control group did not induce ADE. We previously reported that the inoculation of BALB/c mice with the recombinantly expressed HSV-1 gK or the passive transfer of gK-purified IgG into naive BALB/c mice causes severe exacerbation of HSV-1–induced CS after ocular challenge.[21,23] In addition, we showed that gK-vaccinated mice had significantly more virus in their eyes than mock-vaccinated mice and that anti–gK antibody enhanced HSV-1 infection in the macrophage cell line.[29,30] These results were consistent with ADE of HSV-1 by antibody to gK and suggested that the severely exacerbated corneal disease seen after HSV-1 ocular challenge of gK-vaccinated mice was the result of ADE. To determine the possible involvement of gK in ADE in patients with HSK, we blocked the anti–gK antibody in the sera from 10 patients in the HSK group by incubating 100 μL serum with 1×10^6 cells infected with baculovirus-expressed gK, as described in Materials and Methods. As a positive control, the same sera were blocked with cell lysates infected with baculovirus-expressed gD.

Incubation of HSK sera with baculovirus-expressed gK eliminated ADE (Fig. 2B, HSK-sera + gK antigen). In contrast, incubation of the sera from the same patient with baculovirus-expressed gD did not block ADE (Fig. 2B, HSK-sera + gD antigen). Similar to the results in Figure 2A, sera that were blocked with gD induced ADE at serum dilutions between 10^{-6} and 10^{-8} (Fig. 2B). Significant differences were observed between gK-blocked and gD-blocked sera ($P < 0.05$). Because of the presence of neutralizing antibody, sera from both groups, especially the gK-blocked group, showed decreased virus titer at dilutions between 10^{-2} and 10^{-4} (Fig. 2B). These results confirmed our previous studies with mice immunized with gK showing that anti–gK antibody is involved in ADE in humans and mice.

Anti–gK and –gD Antibodies in Sera of HSK Patients

As shown in Table 1 and as discussed, we analyzed sera from 53 patients with a clinical history of ocular disease (HSK group), sera from 31 patients who were seropositive for HSV-1 but had no clinical history of recurrent disease (and no CS; non–HSK group), and sera from 27 patients with no history of HSV-1 infection (seronegative group). Results from the block-

ing experiments suggested that the neutralizing antibody titers associated with gD were lower in HSK sera than non–HSK sera, whereas ADE experiments suggested that the higher titer of anti–gK antibody in HSK sera led to ADE. These studies suggested that the level of anti–gD antibody in HSK sera was lower than in non–HSK sera; the reverse is true for the anti–gK antibody. Thus, to confirm this hypothesis, the concentrations of gK- and gD-specific antibodies in sera from all patients were determined by ELISA, as described in Materials and Methods.

In contrast to lower levels of gD-induced neutralizing antibody titers in HSK sera, ELISA results showed that HSK sera had significantly higher levels of anti–gD antibody than non–HSK sera (Fig. 3A; $P = 0.003$). As expected, HSK and non–HSK patients had higher anti–gD titers than did seronegative patients (Fig. 3A; $P < 0.0001$). The average anti–gK antibody titer of the HSK group also was significantly higher than that of non–HSK group (Fig. 3B; $P = 0.04$). Sera from the HSK and non–HSK groups had significantly higher antibody titers overall compared with the seronegative group (Fig. 3A; $P = 0.009$).

These results suggested that patients with ocular HSV-1 have higher anti–gD and anti–gK antibody levels than seropositive patients with no history of ocular HSV-1. We observed a negative correlation between neutralizing antibody titer and ELISA titer for gD in the HSK group but not in the non–HSK group.

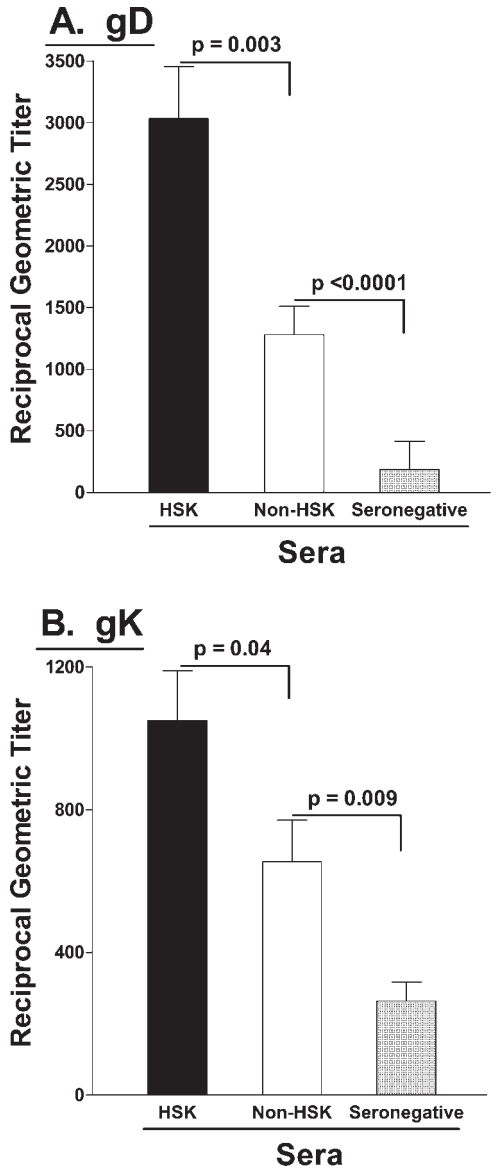

FIGURE 3. Titration of gK and gD antibodies in sera of HSK and non–HSK patients. Concentrations of gD and gK in sera from all HSK and non–HSK patients were determined by ELISA assays using gD- and gK-expressed proteins as coating antigen. Sera from seronegative patients were used as controls. Each bar represents the average ELISA titer expressed as geometric mean ± SEM from sera of 53 patients in the HSK group, 31 patients in the non–HSK group, and 27 patients in the seronegative group. (**A**) gD antigen. (**B**) gK antigen.

DISCUSSION

Recurrent infection with HSV-1 is the leading cause of infectious corneal blindness in the United States.[5,31,32] Periodic ocular reactivation produces permanent visual disability by HSK, uveitic glaucoma, and cataract formation. Although the specific immune response leading to HSK remains an area of controversy, it is well established that HSV-1–induced HSK, and hence subsequent HSV-1–induced corneal blindness, is caused by an immune response to the virus.[33–35] HSK is the most important pathologic response associated with ocular HSV-1 infection in humans.[6,36–38]

It is well established that HSV-1–induced HSK and subsequent HSV-1–induced corneal blindness are caused by immune responses triggered by the virus.[7,33,39] Different HSV-1 genes have been implicated in HSK. For example, autoreactive $CD4^+$ T cells specific for UL21, UL49, UL46, and UL47 have been isolated from the corneas of HSK patients.[40] Similarly, it has been shown that HSK in mice occurs as the result of molecular mimicry involving HSV-1 UL6 and a corneal autoantigen that also forms part of the $IgG2a^b$ isotype of immunoglobulin.[41,42] However, various human studies have shown that HSK occurs independently of HSV UL6.[40,43,44] Thus, the HSV-1 protein(s) against which this harmful immune response is directed is unknown and remains an area of intense controversy. Furthermore, the role of the humoral immune response, especially intracorneal, is also debatable. In this study, we have characterized sera from three groups of patients: those with recurrent clinical ocular HSV-1, those seropositive for HSV-1 (i.e., herpes labialis or cold sores) but with no clinical history of recurrent eye disease (and no CS), and those with no history of HSV-1 infection and no detectable level of neutralizing antibody or IgG to HSV-1.

Our blocking experiments using sera from HSK and non–HSK patients confirmed our previous animal studies suggesting that gB, gC, gD, gE, and gI are the major contributors of neutralizing antibody titers in human sera to HSV-1, whereas gG, gH, gJ, gK, and gL have no effect on neutralizing antibody titers to HSV-1. With regard to the contribution of gB, gC, gE, or gI to neutralizing antibody titers, we did not detect any differences between HSK and non–HSK sera. However, gD contributed significantly less to neutralizing antibody titer in the sera from HSK patients than in the sera from non–HSK patients. Most HSV vaccine studies have focused on HSV gD.[11,32,45–47] Our results also suggest that a vaccine based on gD alone may not be as effective against ocular HSV-1 as against nonocular HSV-1. Recently, it was shown that a gD2 vaccine against genital herpes had approximately 73% efficacy in women but did not show any efficacy in men.[46] Results from the blocking experiments also showed that none of the five protective HSV-1 glycoproteins contributed to more than 40% of neutralizing antibody titers, suggesting that sterile immunity against HSV-1 using any patient glycoprotein may not be attainable. This may be why a phase 3 clinical trial using recombi-

nant HSV-2 gB and gD showed no overall efficacy against HSV-2 infection.[45]

Results from our blocking experiments suggested that approximately 75% of the neutralizing antibody titers detected in HSK and non–HSK sera were associated with gB, gC, gD, gE, and gI, possibly explaining why a mixture of 5gP was more effective than any expressed protein or a mixture of gB and gD.[48] Furthermore, our blocking experiments indicated that a subunit vaccine based on gD may not be as effective in controlling ocular HSV-1 as nonocular HSV-1. Finally, with a mixture of as many as 10 HSV-1 glycoproteins, approximately 25% of the neutralizing antibody was not blocked. In contrast, blocking the sera with HSV-1–infected cell lysates resulted in 100% loss of neutralizing antibody titers in HSK or non–HSK sera. The primary interpretation of this result is that other nonglycoproteins, such as immediate-early proteins,[49] VP16,[50,51] or some unidentified HSV-1 proteins might have contributed to unblocked neutralizing antibody titers. Alternatively, these differences could be attributed to the absence of proper folding or protein–protein interaction or to differences in glycosylation patterns when proteins are expressed in a baculovirus system.

In this study, we did not detect any differences in the level of neutralizing antibody titer or of HSV-1 IgG between the HSK and non–HSK groups, suggesting that route of infection (e.g., ocular vs. nonocular) did not contribute to overall similarities in HSV-1 neutralizing titers. However, despite similarities in neutralizing antibody titers between HSK and non–HSK groups, gD-induced neutralizing antibody titers were significantly lower in the HSK than in the non–HSK group. This finding suggests that the route of infection may not contribute to the ability to detect total neutralizing antibodies against the whole virus; with regard to gD, a difference was observed between the ocular and the nonocular route of infection. In addition, though the gD-induced neutralizing antibody titer was significantly lower in the HSK group than in the non–HSK group, sera from the HSK group had significantly higher anti–gD and anti–gK antibodies, as measured by ELISA, than sera from non–HSK patients. Moreover, the ELISA measured neutralizing and nonneutralizing epitopes of gD, whereas the plaque reduction assay measured gD epitopes involved in neutralizing antibody titer. Thus, the lower gD-induced neutralizing antibody titer in HSK patients might have resulted because the ocular route of infection prevented proper presentation of certain neutralizing epitopes of gD to immune cells, causing a reduction in gD-specific neutralizing antibody titer. Alternatively, the lower gD-induced neutralizing antibody titer in the HSK group, despite the presence of higher gD ELISA titer, might have resulted because of higher gK antibody titers. In mice, we previously demonstrated that nonneutralizing anti–gK antibody correlated with a higher prevalence of ocular disease.[23] Similarly, our animal studies showed that when mice are immunized with gD+gK, levels of neutralizing antibody titers in immunized mice were reduced by approximately 30% compared with mice immunized with gD alone (not shown). Thus, the higher anti–gK antibody titer in the HSK group might have had a reverse correlative effect on the gD-specific neutralizing antibody titer because of a shift from a T_H1 to a T_H1+T_H2 response in the presence of higher gK antibody. Previously, we showed that mice immunized with gD exhibited a T_H1 response, whereas mice immunized with gK exhibited a T_H1+T_H2 response.[25] In gK-immunized mice, this T_H1+T_H2 response was correlated with the increased prevalence of eye disease.

Previously, we showed that HSV-1 titers in the eyes of gK-immunized mice after ocular infection were significantly higher in mice vaccinated with gK than in mock-vaccinated mice.[29] Higher virus replication in the eye of gK-immunized and ocularly infected mice likely was caused by ADE because anti–gK antibody caused ADE in macrophages in vitro. In this study, we demonstrated that HSK sera induced ADE in macrophage-infected cells and that sera from the non-HSK– or the HSV-1–seronegative group did not induce ADE. The ADE effect of HSK sera was blocked by incubation of sera with baculovirus-expressed gK but not gD antigen. This result strongly suggested that the ADE in HSK sera was caused by anti–gK antibody. ADE of virus infection occurs when complexes of virus plus antibody result in more efficient cell infection than by virus alone.[52–55] ADE occurs as follows: the antibody binds to the virus without neutralizing the virus; the target cells contain Fc receptors on their surfaces (i.e., monocytes and macrophages); and the antibody-virus complex binds to the Fc receptors, resulting in increased efficiency of cell infection than by virus alone.[53,55–57] Thus, ADE can enhance the virus load in an infected host by increasing the efficiency of viral infectivity or by allowing the virus to infect additional types of cells, which can lead to increased pathogenesis ranging from enhanced disease[58] to early death.[59–61] Dengue virus and related flavivirus, respiratory syncytial virus, influenza virus type A, rabies virus, and, most recently, HIV have all been shown to promote ADE.[53,56,59,60,62,63]

Recently, we demonstrated that gK vaccination of mice causes an overall increase in T cells and macrophages in the cornea after ocular HSV-1 infection.[64] The immunopathology induced by gK vaccination was related to $CD8^+$ T-cell activity because depletion of these cells, but not of other immune cells, reduced HSK. Thus, the results implicate $CD8^+$ T cells in the exacerbation of HSK observed in these mice, a finding consistent with results indicating that $CD8^+$ T cells play a major role in HSV-1 corneal pathogenesis.[39,65–67] Excessive accumulation of activated $CD8^+$ T cells also increases mortality rates in perforin-deficient mice.[68,69] Our unpublished results (2006) suggest that $CD8^+$ T cells in the corneas of gK-immunized mice after ocular infection with HSV-1 are also $CD25^+$. It has been shown that $CD8^+CD25^+$ T cells can contribute to the disease process in anterior chamber-associated immune deviation,[70] experimental autoimmune encephalitis,[71] Hashimoto disease,[72] and psoriasis.[73] The ability of sera from HSK patients to promote ADE indicates that a similar mechanism could be operational in patients with HSK. Thus, the ability of gK sera to induce ADE can explain how gK sera can increase ocular viral replication and induce $CD8^+CD25^+$ T cells, resulting in HSK.

Finally, our ELISA data from seronegative patients, especially when we used gD as a coating antigen, showed higher than expected titers with some of the sera, despite the fact that these sera showed no detectable level of HSV-1 neutralizing antibody titers or HSV-1– or HSV-2–specific IgG or IgM. These higher-than-expected ELISA titers, in the absence of any detectable levels of HSV-1 neutralizing antibody, IgG, or IgM titers, have resulted from seroreversion. Previously, it had been shown that transient reversion to negative, as detected by the HerpeSelect test, occurred in 10% of HSV-1–infected patients.[74]

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