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Antibody responses to rhesus cytomegalovirus glycoprotein B in naturally infected rhesus macaques

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Rhesus cytomegalovirus (RhCMV) exhibits strong parallels with human CMV (HCMV) in terms of nucleic and amino acid identities, natural history, and mechanisms of persistence and pathogenesis in its natural host, rhesus macaques (*Macaca mulatta*). To determine whether this non-human primate model would be useful to assess vaccine strategies for HCMV, host immune responses to RhCMV glycoprotein B (gB) were evaluated in RhCMV-infected monkeys. Total protein extracts were prepared from cells transiently transfected with an expression plasmid for either the full-length gB or a derivative (gB Δ , 1–680 aa) lacking both the transmembrane domain and cytoplasmic tail. Western blot analysis showed identical reactivity of macaque sera with full-length gB and its derivative gB Δ , indicating that the immunodominant epitopes of gB are contained in the extracellular portion of the protein. Using gB Δ extract as a solid phase, a sensitive and specific ELISA was established to characterize gB antibody responses in monkeys acutely and chronically infected with RhCMV. During primary infection (seroconversion), gB-specific antibodies developed concurrently and in parallel with total RhCMV-specific antibodies. However, during chronic infection gB-specific antibody responses were variable. A strong correlation was observed between neutralizing and gB-specific antibody levels in RhCMV-seropositive monkeys. Taken together, the results of this study indicate that, similar to host humoral responses to HCMV gB, anti-gB antibodies are an integral part of humoral immunity to RhCMV infection and probably play an important protective role in limiting the extent of RhCMV infection. Thus, the rhesus macaque model of HCMV infection is relevant for testing gB-based immune therapies.

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INTRODUCTION

Human cytomegalovirus (HCMV) is a ubiquitous pathogen that usually causes an asymptomatic infection in immunocompetent individuals. However, it is often associated with serious or fatal diseases in immunocompromised hosts, such as immunosuppressed transplant recipients and AIDS patients (Alford & Britt, 1993). HCMV is also the most common congenital infection in humans. Foetal outcomes can range from an asymptomatic infection to severe development defects and, sometimes, death.

Although the immune responses that control HCMV are not precisely known, the majority of studies in the murine CMV model and observations in humans indicates that cellular immunity may play a role in limiting virus replication and enabling recovery from HCMV infection, whereas humoral immunity likely has an important role in protection against primary infection and in limiting the severity of HCMV-related diseases (Boppana & Britt, 1995; Fowler *et al.*, 1992; Jonjic *et al.*, 1994; Li *et al.*, 1994; Polic *et al.*, 1998; Reusser *et al.*, 1991, 1997, 1999; Walter *et al.*, 1995). For example, in transplant patients the presence of

HCMV-specific cytotoxic T cells correlates with favourable outcome of HCMV infection (Reusser *et al.*, 1991, 1997, 1999), and adoptive transfer of HCMV-specific CD8⁺ T cells prevents CMV viraemia and disease (Walter *et al.*, 1995). On the other hand, preexisting maternal seroimmunity to HCMV limits the frequency of symptomatic infection in the newborn (Fowler *et al.*, 1992), and neutralizing antibodies to HCMV are relevant in controlling the severity of HCMV disease in immunocompromised patients (Alberola *et al.*, 1998, 2001).

HCMV is a highly complex virus potentially encoding 164–167 proteins, including 30 glycoproteins (Chee *et al.*, 1990; Davison *et al.*, 2003; Rigoutsos *et al.*, 2003). Although virus-neutralizing antibodies may target all of the glycoproteins, glycoprotein B (gB) has been shown to be a major target (Britt & Mach, 1996). Antibodies to gB comprise 40–70% of total neutralizing activity to HCMV (Britt *et al.*, 1990; Marshall *et al.*, 1992; Utz *et al.*, 1989). Moreover, gB elicits specific lymphoproliferative responses, as well as CD8⁺ and CD4⁺ cytolytic responses in some infected individuals (Boppana & Britt, 1996; Gonczol *et al.*, 1990;

Hopkins *et al.*, 1996; Liu *et al.*, 1991). Consequently, gB has been studied as a vaccine target for HCMV.

As a non-human primate model for HCMV, rhesus CMV (RhCMV) infection parallels that of HCMV in many aspects, including genome collinearity, immunological and virological parameters of infection, as well as persistence and pathogenesis (Alcendor *et al.*, 1993; Baroncelli *et al.*, 1997; Baskin, 1987; Gibson, 1983; Hansen *et al.*, 2003; Huff *et al.*, 2003; Kaur *et al.*, 1996; Kuhn *et al.*, 1999; Lockridge *et al.*, 1999; Sequer *et al.*, 2002; Swack & Hsiung, 1982). Homologous open reading frames of RhCMV exhibit various degrees of nucleic and amino acid identities to HCMV (Hansen *et al.*, 2003). The gB protein of RhCMV is 60% identical to HCMV gB at the amino acid level with a higher identity within the region of AD-1 (Kravitz *et al.*, 1997; Kropff & Mach, 1997). Cross-reactivity and cross-neutralization of HCMV gB-specific monoclonal antibodies with RhCMV gB also indicate that the two proteins share immunogenic epitopes (Kropff & Mach, 1997). Therefore, the rhesus macaque model may be especially useful for evaluating anti-gB vaccine strategies. To develop the potential of the model, a better understanding of humoral responses of RhCMV gB within infected macaques is required.

A previous report has demonstrated that RhCMV gB is highly immunogenic in RhCMV-infected macaques (Kropff & Mach, 1997). However, some important immunological events about gB following primary and secondary infection or endogenous reactivation remain to be characterized. In this study, we developed a relative simple protocol to detect IgG antibodies to RhCMV gB using transiently transfected cell extracts as a source of antigen. The humoral response to gB and its relationship to the presence of neutralizing activities after RhCMV naturally infection were characterized in both acutely and chronically infected rhesus macaques.

METHODS

Plasma and serum samples. Rhesus macaque plasma and serum samples from three groups of animals at the California National Primate Research Center (CNPRC) at the University of California, Davis were tested by ELISA and Western blot assays. Group I was composed of plasma samples collected longitudinally from a cohort of animals ($n=10$) that were less than 1 year of age. These juveniles had been housed indoors, and their RhCMV serostatus changed from negative to positive following an unknown exposure to RhCMV. Seven of these animals were involved in a project evaluating genetic immunization against herpes B virus (Loomis-Huff *et al.*, 2001). These samples were used to quantify development of anti-gB antibody responses during primary infection. Group II consisted of 44 archived serum samples from eight macaques, selected at random, that had been collected over a period of time spanning 4 to 9 years. All of the Group II monkeys were healthy, immunocompetent animals housed in outdoor corrals at the CNPRC, and all were persistently seropositive for RhCMV. Group III comprised a single serum sample from each of 20 gravid monkeys that were RhCMV seropositive. Sera were obtained either on gestation day 50 (late first trimester) or 65 (early second trimester); gestation is 165 days in rhesus macaques.

Construction of full-length and truncated gB expression vectors. The full-length gB open reading frame (Kravitz *et al.*, 1997) was cloned into the pND expression vector (Loomis-Huff *et al.*, 2001) utilizing restriction sites upstream of the translation start codon (*Nru* at position -107 , relative to the start site at $+1$ (Kravitz *et al.*, 1997), and downstream of the polyadenylation site within the polylinker. The construct, pND/gB, utilized the HCMV immediate-early promoter/enhancer and the homologous gB polyadenylation signal. A truncated version of gB was constructed, pND/gB Δ , by deleting the transmembrane (TM) region and the carboxyl portion of the protein downstream of TM. This was accomplished by deleting the region between an *EcoRV* site at amino acid 680 (Kravitz *et al.*, 1997) and a restriction site (*XbaI*) within the pND polylinker downstream of the gB polyadenylation signal. The TM for RhCMV, most likely, corresponds to amino acids 688–745 (Kropff & Mach, 1997). The deletion shifted the reading frame, and translation of the truncated gB terminated at a novel stop codon positioned six amino acids downstream of the *EcoRV* site. The deletion of the gB polyadenylation signal resulted in the utilization of the bovine growth hormone polyadenylation signal within pND. Plasmids were purified with the Endofree plasmid kits (Qiagen).

Transient transfection of 293T cells and preparation of gB antigen extracts. 293T [human embryonic kidney cells expressing adenovirus E1 and SV40 T antigen (DuBridge *et al.*, 1987)] were used for transfection according to published protocols (Loomis-Huff *et al.*, 2001). Briefly, cells were subdivided the day prior to transfection such that the cells would be 70–80% confluent the following day. For each T-75 flask of cells, 30 μ g of DNA was mixed with 180 μ l of DOTAP/DOPE transfection reagent (Avanti Polar Lipids) in 3 ml of DMEM media (without serum) and incubated at 37°C in 5% CO₂ for 1 h. The cells were washed twice with PBS (Invitrogen), and the DNA/transfection reagent mixture was then added to the flask. Cells were incubated at 37°C in 5% CO₂ for 4 h, the DNA mixture was removed, and the cells were re-fed fresh complete media for 48 h. Cells were harvested by washing twice with PBS and then removing the cells from the surface of the flask by gentle scraping with a rubber-tipped spatula. The cells were pelleted (500 g for 10 min), the supernatant was removed and discarded, and extraction buffer (0.5 ml 2% Triton X-100 in PBS) was added to the cells. The cells were incubated on ice for 30 min with vigorous vortexing every 10 min. Cells could be stored at -80°C after resuspension in extraction buffer until processing, if necessary. The concentration of Triton X-100 was adjusted to 0.5% by addition of PBS. Cell debris was pelleted by a high-speed spin in a microcentrifuge. The supernatant was collected for protein determination (Bio-Rad protein assay), and aliquots were stored at -80°C . Extracts of untransfected cells were similarly prepared for use as control antigen.

RhCMV infection and preparation of RhCMV antigen. Primary rhesus dermal fibroblasts were infected with RhCMV strain 68-1 (Lockridge *et al.*, 1999) and harvested when the cells exhibited 100% cytopathic effect. Whole RhCMV antigen was prepared identically to the protocol described for gB antigen.

RhCMV and gB-specific ELISA. Anti-RhCMV and anti-gB antibodies were quantified by ELISA as follows. Each well of a 96-well plate was coated overnight at 4°C in a humidified chamber with 25 μ g of viral or cell control antigen diluted in 0.1 ml of coating buffer (Hanks' buffered salt solution – HBSS/0.375% bicarbonate buffer). The following day, each plate was washed three times with PBS/0.05% Tween 20 (PBS-T), and non-specific binding was blocked by incubating each well in 0.3 ml of blocking buffer (1% BSA in PBS) for 2 h at 25°C. Plates were washed three times in PBS-T, and then incubated in 0.1 ml of plasma/serum (diluted 1:100 in 1% BSA/PBS-T) for 2 h at 25°C. Wells were washed three times in PBS-T, and then 0.1 ml of horseradish peroxidase-conjugated goat anti-rhesus IgG (KPL Inc.) diluted in 1% BSA in

PBS-T was added to each well for 1 h. Wells were washed again in PBS-T and then incubated in 0.1 ml of tetramethylbenzidine substrate (Sigma) for 30 min. The reaction was terminated by the addition of 50 μ l of 0.5 M H₂SO₄. Absorbance was recorded at 450 nm. Values were considered positive when the absorbance for specific antigen was 0.1 units higher than that of control antigen. The reproducibility of the ELISA was assessed by using pools of known RhCMV seronegative and seropositive samples as controls on each plate.

Western blot analysis. Plasma and serum samples were analysed for reactivity (1:100 or 1:50 dilution) to the full-length and truncated forms of gB by Western blot, according to previously published protocols (Vogel *et al.*, 1994).

Neutralization assay. Neutralization activity was assayed using a previously published method (Lockridge *et al.*, 1999). The titre of samples in this study was calculated as the inverse dilution giving a 90% reduction of IE1-positive cells per well.

Statistical analysis. Statistical analysis was carried out using Prism (GraphPad Software). The relationship between two variables was analysed by linear regression and Spearman rank correlation test.

RESULTS

The high sequence conservation between the gB proteins of HCMV and RhCMV (Kravitz *et al.*, 1997; Kropff & Mach, 1997) implies that the rhesus macaque model of CMV would be especially useful for evaluating gB-based vaccine strategies. A previous study has demonstrated that RhCMV gB is highly immunogenic in RhCMV-infected macaques, although the temporal kinetics of anti-gB antibody development were not described. Utilization of the nonhuman primate model would be enhanced by a better understanding of the relationship between RhCMV infection and development of anti-gB antibodies.

Two expression plasmids containing either full-length or truncated gB under the transcriptional control of the HCMV IE promoter were constructed (pND-gB and pND-gB Δ , respectively), transfected into 293T cells, and analysed by Western blot. Expression of gB was observed following transfection of cells with pND-gB and pND-gB Δ (Fig. 1). The sizes of the proteins corresponded to the predicted uncleaved forms of gB (130 and 110 kDa protein for pND-gB and pND-gB Δ , respectively). The reactivity of the truncated form of gB (gB Δ) was considerably more intense than the full-length gB (Fig. 1), consistent with the interpretation that deletion of the TM and carboxyl domains of gB enhanced expression (Marshall *et al.*, 2000). The proteolytic products (80–110 and 50–55 kDa) of gB (Kravitz *et al.*, 1997; Kropff & Mach, 1997) were not observed in the Western blot. A preponderance of the full-length, uncleaved precursor form of gB has been noted in RhCMV-infected cells (Kravitz *et al.*, 1997). The apparent absence of the cleaved products has also been observed following expression of recombinant HCMV gB in mammalian cells (Marshall *et al.*, 1996; Qadri *et al.*, 1992). It has been postulated that this is due to insignificant amounts of properly processed gB resulting from an insufficient concentration of de novo gB (Marshall *et al.*, 1996). A minor

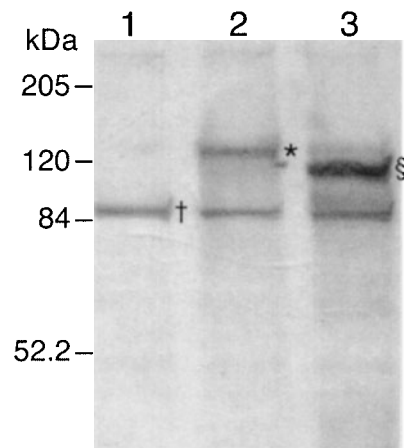


Fig. 1. Analysis of RhCMV gB expressed in 293T cells. Cell lysates were prepared from untransfected 293T cells (lane 1), and cells transiently transfected with either pND/RhCMV gB (lane 2) or pND/RhCMV gB Δ (lane 3). Cell lysates were separated on polyacrylamide gels and analysed by Western blot using sera from RhCMV-seropositive macaques. * Full-length gB band; § gB Δ band; † non-specific band.

band at 41 kDa was detected in extracts of cells transfected with the truncated version of gB, but not in cells transfected with full-length gB or in control cells (data not shown). This band may have represented the carboxy-terminal cleavage product derived from gB Δ . The apparent size, which was slightly higher than the predicted 36 kDa (Kravitz *et al.*, 1997; Kropff & Mach, 1997), may have resulted from extensive glycosylation, similar to what has been found for truncated versions of HCMV gB (Qadri *et al.*, 1992; Spaete *et al.*, 1988).

To determine whether the gB Δ would have utility as a screen for gB seroreactivity, it was necessary to confirm that antibodies to the portion of gB upstream of the TM would sufficiently represent the antibodies to the full-length form. Serum samples from 23 confirmed RhCMV-seropositive macaques (10 from primary infected animals and 13 from seropositive individuals) were analysed by Western blot for reactivity to full-length and truncated gB. All samples (22/22) that had reactivity to the full-length protein also had reactivity with gB Δ (Table 1). This parallel reactivity demonstrated that the vast majority, at least, of gB epitopes reside between the amino terminus and TM, in agreement with a previous report using recombinant subdomains of RhCMV gB (Kropff & Mach, 1997). The localization of immunodominant epitopes on RhCMV gB was similar to observations of HCMV gB (Navarro *et al.*, 1997). One sample (MMU 24165, discussed below) did not react to either version of gB.

Since pND-gB Δ was more efficiently expressed in tissue culture than pND-gB and contained the important humoral determinants, it was used as a source of antigen for an

Table 1. Reactivity of RhCMV-seropositive monkey plasma with full-length gB and its derivative gBΔ

		ELISA*		Western blot†	
		Anti-gBΔ IgG		Anti-full-length gB IgG	
		Ab ⁺	Ab ⁻	Ab ⁺	Ab ⁻
Western blot†	Ab ⁺	19	3‡	22	0
Anti-gBΔ IgG	Ab ⁻	0	1	0	1

*Reactivity of monkey plasma with gBΔ tested by ELISA when diluted 1:100.

†Reactivity of monkey plasma with full-length gB or gBΔ by Western blot when diluted 1:100 or 1:50.

‡These three samples were anti-gB antibody seropositive by ELISA when diluted 1:50 or 1:25.

ELISA. Large-scale extracts of cells transfected with this plasmid construct were prepared and used as the solid phase for indirect ELISA. Untransfected 293T cells were also prepared as control antigen and used in parallel with gBΔ extracts. The optimal concentration of cell extract (10, 20, 30 µg per well) was determined using four RhCMV antibody-positive and three negative sera. Each of the four seropositive serum samples exhibited a substantial absorbance at each of the gBΔ antigen concentrations and correspondingly minimal reactivity to control extracts (data not shown). The three negative sera gave equally low absorbance values with gBΔ and control antigens at all three concentrations (data not shown). These results demonstrated that gB-specific immune responses could be quantified using transiently expressed gBΔ as coating antigen for ELISA (Fig. 2), even though Western blot analysis showed the existence of non-specific reactivity with untransfected cell extracts. One possible explanation for this may have been the fact that a much lower concentration of antigen was used in the ELISA than in the Western blot, thus minimizing non-specificity. Using the same test conditions, no distinct reactivity was detected at any concentration of the full-length gB extract (data not shown) with the seropositive samples, consistent with the low expression level of full-length gB (Fig. 1). Based on the reactivity of sera with both gBΔ and control antigens, subsequent assays used 25 µg per well as antigen concentration.

A threshold for a seropositive response was determined by screening 50 confirmed RhCMV-seronegative samples. Minimal reactivity was detected in these samples, and the net absorbance ($A_{450} \text{ gB}\Delta - A_{450} \text{ control}$) was always less than 0.02 (data not shown). The cut-off value for determining a positive gB antibody response was arbitrarily chosen as any net A_{450} value exceeding 0.1. Moreover, the reliability of the assay was further demonstrated by the consistent results of 23 samples detected by ELISA and Western blot (Table 1). This included three samples from two monkeys (MMU 27108 and 28731) that were negative

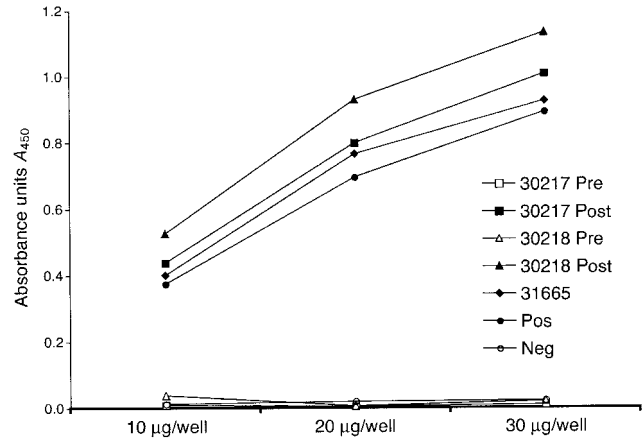


Fig. 2. Optimizing the concentration of gBΔ antigen for ELISA. Three concentrations of gBΔ extract were analysed using plasma samples from two monkeys (30217, 30218) experimentally inoculated with RhCMV, a naturally infected monkey (31665), and pools of sera derived from RhCMV antibody-positive and -negative animals. Twenty-five µg of antigen per well was chosen for ELISA and the net A_{450} absorbance is shown in the figure. Pre, pre-RhCMV inoculation; Post, post-inoculation of RhCMV; Pos, RhCMV seropositive pool; Neg, RhCMV seronegative pool.

by ELISA to gB at 1:100 dilution but had weak reactivity by Western blot at 1:50 dilution. All of them were gB antibody positive at a dilution of 1:25 or 1:50 in ELISA.

To characterize the ontogeny of the antibody response to gB during primary infection, longitudinal plasma samples were analysed from seven macaques involved in another study (Loomis-Huff *et al.*, 2001). Seven macaques that had been genetically immunized against the gB protein of herpes B virus (BV) seroconverted to RhCMV during the course of observation. The source of infectious RhCMV was unknown, but RhCMV is endemic in colonies of rhesus macaques (Vogel *et al.*, 1994) and can be readily transmitted to naive cohorts (unpublished observation). The change in immunostatus from RhCMV seronegative to seropositive was unrelated to the vaccination responses to BV gB. Animals had been immunized in February 1999 and developed detectable antibody responses during April and May of that year, prior to seroconversion to RhCMV (Loomis-Huff *et al.*, 2001). For each monkey, antibodies specific to RhCMV gB developed concurrently and in parallel with antibodies to total RhCMV antigens (Fig. 3). The same pattern of immune responses has been observed in three other naturally infected monkeys (data not shown). These results indicated that antibodies to RhCMV gB developed soon after infection and, accordingly, antibody responses to gB may be used as a marker of seroconversion.

Macaques infected with RhCMV maintain relatively stable antibody responses to total viral antigens for the life of the infected host (Baroncelli *et al.*, 1997, and unpublished

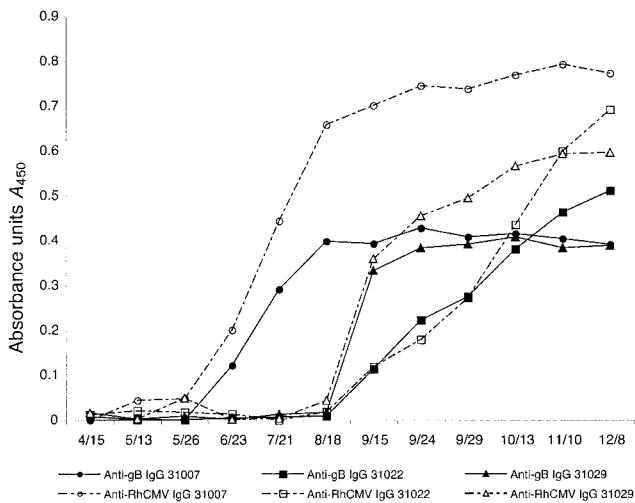


Fig. 3. Representative results for the ontogeny of gB-specific and RhCMV-specific antibody during primary infection. Plasma samples from three monkeys were sequentially collected in 1999 and analysed for the development of antibodies to gB (solid lines) and total RhCMV antigens (dashed lines).

observations). Longitudinal responses to gB were evaluated in eight macaques using banked serum samples that spanned a period of 4 to 9 years. Antibodies against gB were found in all the animals. Generally, antibody responses to both total RhCMV antigens and gB in these animals exhibited small fluctuations over time, and remained within a relative narrow range over a timeframe of up to 8 years (Fig. 4). Of eight animals, four were noted for robust gB and RhCMV antibody levels. The other four were characterized by initial weak gB antibody responses and vigorous RhCMV responses (Fig. 4a). However, two monkeys (MMU 25525 and 27740) were noted for 30–50% increases in RhCMV antibody responses and a relative stable pattern up to 5 years. gB antibody responses in MMU 25525 matched the pattern of RhCMV antibody responses during this timeframe (Fig. 4b), whereas the gB responses in MMU 27740 did not (data not shown). In addition, MMU 28731 had barely detectable gB antibodies at the first two time-points, which increased slightly over the course of 5 years (Fig. 4c). MMU 27108 had a similar change from barely detectable to, in this case, a strong gB antibody response (Fig. 4d).

The gB envelope glycoprotein of HCMV is a predominant

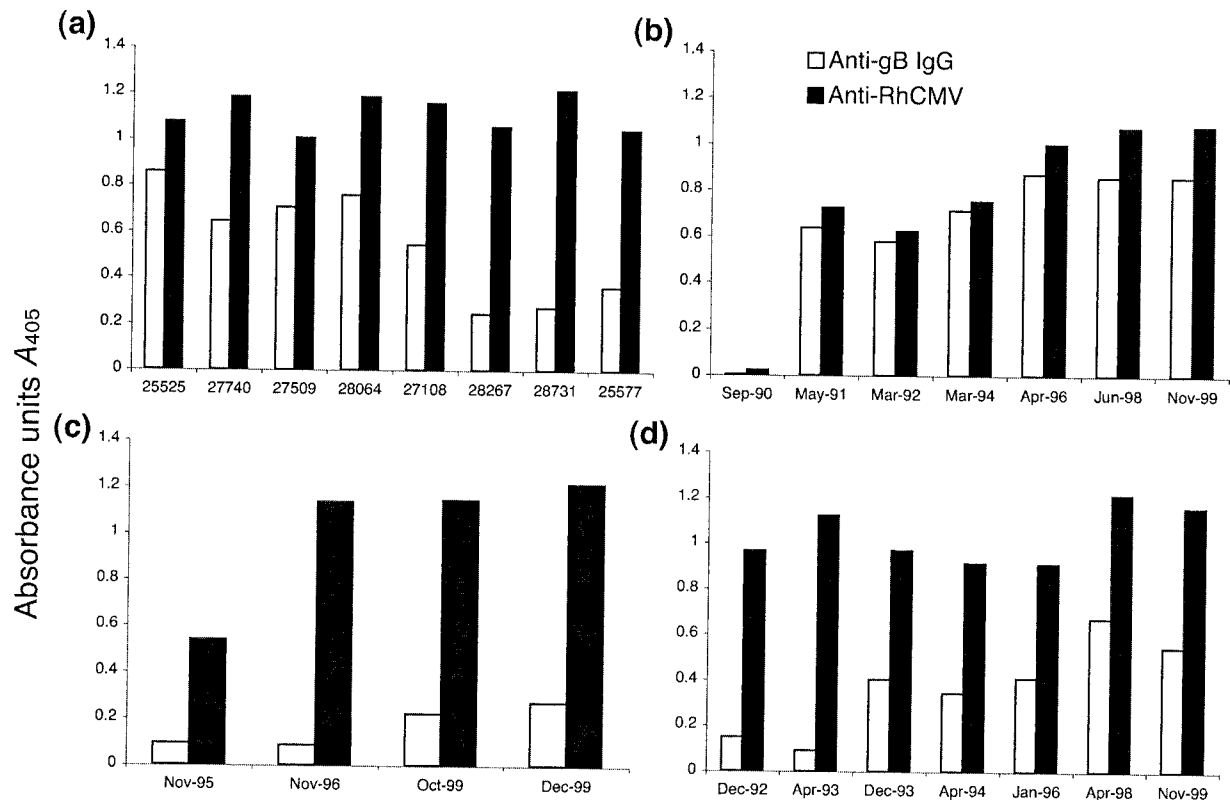


Fig. 4. Analysis of antibody responses to gB and total RhCMV antigens during chronic infection. Eight macaques were analysed for antibody responses to gB (open bars) and RhCMV (solid bars) for periods of up to 9 years following primary infection. (a) Individual sera from eight monkeys; (b) sequential sera from MMU 25525; (c) sequential sera from MMU 28731; (d) sequential sera from MMU 27108.

target of neutralizing antibodies following human infection (Britt *et al.*, 1990; Marshall *et al.*, 1992; Utz *et al.*, 1989), and neutralizing monoclonal antibodies to the AD-1 domain of HCMV gB can cross-neutralize RhCMV (Kropff & Mach, 1997). Sera from RhCMV-infected macaques also have neutralizing activity, although it has not been determined whether RhCMV gB encodes neutralizing epitopes (Kropff & Mach, 1997; Lockridge *et al.*, 1999). To determine if there is a relationship between neutralizing activity and antibodies to gB, the 90% neutralizing antibody titres and the end-point titres to the truncated version of gB were analysed in 20 seropositive macaques. There was a strong correlation between gB and neutralizing titres (Fig. 5) (linear regression, $r=0.775$, $P<0.001$; Spearman rank, $r=0.641$, $P<0.005$). The correlation was similar to that reported for HCMV gB (Marshall *et al.*, 1992). All 90% neutralizing titres exceeded 128; 18 of 20 monkeys had gB titres between 800 and 12 800. One animal had a gB titre of 50, and the other (MMU24165) had no detectable gB antibody at 1:50 dilution. Neutralizing titres for these two monkeys were 231 and 128, respectively. The data indicated that gB represents a major, but not complete, determinant of neutralizing activity to RhCMV infection.

DISCUSSION

This study demonstrates the feasibility of developing a protein-specific ELISA using transiently expressed protein products. In this case, extracts of cells transfected with a plasmid expression vector for RhCMV gB Δ enabled specific and sensitive detection of gB antibodies in rhesus macaques. The primary limitation appears to be the level of gene expression in the transfected cells. Expression of the full-length form of gB was insufficient to discriminate

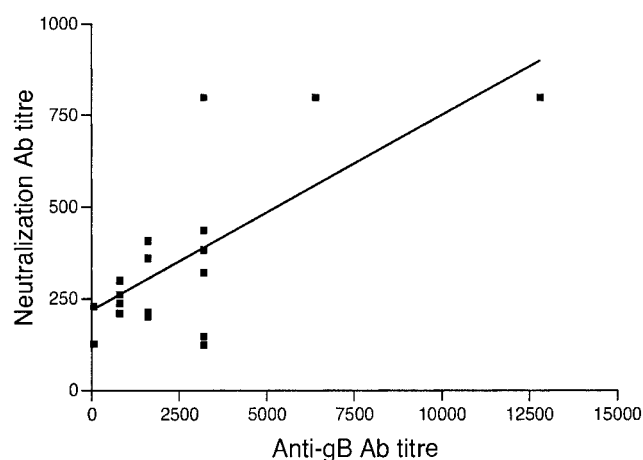


Fig. 5. Relationship between gB-specific antibody and neutralization antibody. Both were expressed as reciprocal titre. Correlation coefficient ($r=0.775$) and P values (<0.001) were determined by linear regression test.

between seropositive and seronegative macaques. Truncation of the transmembrane domain and carboxyl portion of the protein resulted in a higher level of protein expression enabling a clear distinction between naive and infected monkeys. Truncation of the coding region to achieve higher expression levels must be judicious to avoid deletion of epitopes. The parallel reactivity of RhCMV seropositive samples with full-length and the truncated version of gB suggested that the immunodominant epitopes of gB are predominantly contained in the ectodomain of gB. Together with a previous study (Kropff & Mach, 1997), gB Δ is a suitable antigen for detection of RhCMV gB antibodies.

The humoral immune responses to gB in RhCMV-infected macaques were similar to those observed in HCMV-infected humans (Marshall *et al.*, 1994). The ontogeny of the antibody response to gB paralleled the response to total viral antigens during primary infection (Fig. 3). Thus, antibodies to gB arise early after infection, indicating that gB can be used as a marker of seroconversion. Analysis of neutralizing and anti-gB antibody titres (Fig. 4) strongly suggests that RhCMV gB encodes neutralizing epitopes. The absence of a complete correlation between total neutralizing antibody titres and gB titres in naturally infected monkeys emphasizes that there are neutralizing epitopes within other RhCMV virion proteins. While HCMV gB represents the predominant target of neutralizing antibodies, significant neutralization activity is directed against gH (Urban *et al.*, 1996) and gN (Britt & Auger, 1985). Both proteins are encoded within the RhCMV genome (Hansen *et al.*, 2003) and may also represent important immunological targets.

The pattern of gB seroreactivity in chronically infected animals highlights the complex interplay between the virus and the infected host. As with all herpesviruses, RhCMV establishes a persistent and asymptomatic infection in immunocompetent hosts. Persistence is characterized by a relatively stable immune response to viral antigens (Fig. 4) (Baroncelli *et al.*, 1997; Kaur *et al.*, 1996, 1998) and frequent detection of viral DNA (Huff *et al.*, 2003) or infectious virus (Asher *et al.*, 1974) in mucosal fluids during the life of the infected host. Four of the macaques infected long-term (4–9 years) developed robust antibodies to gB that were maintained at high levels for many years. The other four had relatively low levels of gB antibodies over time, discordant to the high antibody levels to the total viral antigens. The reasons for this are not known. The results support the view that infected monkeys are under chronic antigenic assault, either from reinfection and/or reactivation, despite the existence of humoral and cellular antiviral immunity. The variability between infected monkeys in the antibody responses to total viral antigens, as well as to gB, is consistent with the notion that the course of viral infection is variable in different animals.

Several studies of HCMV have noted an inverse correlation between systemic viral loads, as measured by antigenemia and DNAemia, and anti-gB and neutralizing antibody titres (Alberola *et al.*, 1998, 2001; Rasmussen *et al.*, 1995; Schoppel

et al., 1997, 1998). It has been proposed (Alberola, 1998) that when viral loads are high, gB antibody may be complexed with soluble antigen or virus, potentially leading to a reduction in the level of protective antibody responses and an increase in the potential for HCMV sequelae. It remains to be determined whether the differences in RhCMV gB antibody levels inversely reflect the level of gB antigen (Schoppel *et al.*, 1997). If they do, then it would indicate persistent production of gB for very long periods of time in some monkeys (e.g. 3 to 4 years in animals 28267 and 28731). Alternatively, minimal humoral immune responses to gB may have developed during primary infection in these same animals for some unknown reason. The latter possibility has potential implications for vaccine design.

RhCMV has extremely low pathogenic potential in immunocompetent macaques. Antiviral immunity can be considered protective in that it protects the host from disease, even if it is insufficient to eliminate persistent viral reservoirs. Precedence from studies of HCMV establishes both humoral and cellular correlates of protection (Boppana & Britt, 1996; Riddell *et al.*, 1992). Studies in animal models of HCMV have confirmed the importance of both humoral and cell-mediated immunity (Jonjic *et al.*, 1994; Polic *et al.*, 1998; Reddehase *et al.*, 1987). Since most monkeys develop strong antibody responses to gB, including neutralizing antibodies, it is likely that gB represents a critical target for protection, similar to HCMV. However, the absence of vigorous responses to gB in some infected macaques implies that protective humoral immunity can be generated against viral proteins other than gB. Of course, this statement must be qualified by the uncertainty of what level of immune response is required to achieve a minimal threshold of protection. Notwithstanding, optimization of protective antibody development will require multiple target proteins, with gB constituting a core element of any multiple antigen approach.

The RhCMV model of HCMV persistence and pathogenesis offers a relevant system to address vaccine strategies that can limit infection and prevent disease. The availability of reagents and techniques to study infection and manipulate the genome opens new avenues of investigation in a primate host that recapitulates HCMV infection in humans (Chang & Barry, 2003; Chang *et al.*, 2002; Hansen *et al.*, 2003; Kaur *et al.*, 2002; Pitcher *et al.*, 2002). This report describes a simple method for analysing host immune responses to viral antigens using transiently transfected cell extracts as a source of antigen. The data about the humoral responses of gB, which are similar to those of HCMV gB after infection, further highlight the utility of the rhesus macaque model of CMV.

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