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Ontogeny of Glycoprotein gB-Specific Antibody and Neutralizing Activity During Natural Cytomegalovirus Infection

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The envelope glycoprotein gB (gpUL55) is a candidate for inclusion in subunit cytomegalovirus (CMV) vaccines, although data on gB antibody responses after natural infection are limited. [^{35}S]-labeled gB was partially purified from cells infected with an adenovirus recombinant expressing gB and used in radioimmunoprecipitation assays to characterize responses in solid organ transplant recipients with primary (n = 11) or secondary (n = 8) CMV infection. Seropositive transplant patients without evidence of infection (n = 5) and healthy seroconverters (n = 7) were also studied. gB antibody developed concurrently with CMV-specific IgG, IgM, and neutralizing activity in transplant patients with primary infection. Sustained boosts in gB antibody were seen in patients with secondary infection, and healthy seroconverters developed early gB responses. These data imply that gB antibody is an integral part of the humoral response to CMV infection, and, in view of experimental data regarding immunogenicity, support a role for gB in subunit vaccines.

KEY WORDS: CMV, major envelope glycoprotein, gpUL55(gB), neutralizing antibodies, radioimmunoprecipitation

INTRODUCTION

At the present time, the most promising candidate for inclusion in a subunit cytomegalovirus (CMV) vaccine is the abundant envelope glycoprotein complex gB (gpUL55), a membrane-anchored glycoprotein which is conserved among herpesviruses and by inference plays an essential role in virus infectivity [Marshall and Plotkin, 1993]. Support for gB as a subviral immunogen is compelling but necessarily indirect, since only a handful of human inoculations have taken place [Gönczöl et al., 1990]. The protein carries several epitopes recognized by neutralizing monoclonal antibodies, and these antigenic regions are recognized by convalescent human sera as well [Meyer et al., 1992; Qadri et al., 1992; Wagner et al., 1992]. Further, gB induces neutralizing antibodies and cellular responses in animals after introduction as inert protein [Rasmussen et al., 1985; Gönczöl et al., 1986] or in the context of recombinant viruses [Cranage et al., 1986; Britt et al., 1988; Marshall et al., 1990]. Unfortunately, the protective efficacy of vaccination cannot be directly tested in these models, since human CMV is severely host range restricted.

Accordingly, attention in our laboratory and others has turned to studying the response to gB in individuals with natural CMV infection. With the recent availability of cloned reagents for use in immunological assays, greater definition has been given to the role played by gB in the genesis of potentially protective immune responses. For example, absorption studies using cells infected with vaccinia-gB recombinants found that 26% to 100% of the neutralizing activity in convalescent sera was directed against gB [Britt et al., 1990; Gönczöl et al., 1991]. Our study employing gB antigen derived from an adenovirus recombinant showed detectable gB-specific IgG in each of 48 individuals with past CMV infection [Marshall et al., 1992]. Further, there was a strong correlation between gB antibody levels and neutralizing activity, and specific absorption of gB antibody resulted in a median loss of 48% of neutralizing activity.

That study [Marshall et al., 1992] looked at the "steady state" of gB antibody after natural CMV infection. The current study was undertaken to examine the ontogeny of gB antibody during the acute phase of natural infection in both immunosuppressed and healthy individuals. The relationship between gB antibody,

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neutralizing activity, and other serological markers was also investigated.

MATERIALS AND METHODS

Study Population

Serial serum specimens were obtained from 24 patients (median age 37 yr, range 23 to 60; 16 males) undergoing solid organ transplantation (22 kidney, 2 heart) from 1986 through 1989. These sera were collected during prospective surveillance for CMV infection in transplant patients at the University of Ottawa; data from this program have been published before [Landini et al., 1988; Miller et al., 1991]. All patients were monitored by viral culture and serology at approximately monthly intervals. Immunosuppression was routinely induced with antilymphocyte immunoglobulin and maintained with prednisone, azathioprine, and cyclosporine. Rejection episodes were treated with pulse methylprednisolone or, in severe cases, OKT3 monoclonal antibodies. Intravenous immunoglobulin was not used and antiviral prophylaxis was not given.

Eleven patients, including both heart transplant recipients, were CMV-seronegative prior to transplant and developed urinary CMV excretion shortly thereafter; this constituted the "primary infection" group. Since all patients in this group had received organs from seropositive donors, the allograft was probably the source of infection and the date of transplantation was presumed to be the date of infection. Eight patients were seropositive and culture negative at the time of transplantation and subsequently developed urinary CMV excretion. This constituted the "secondary infection" group, and while no attempt was made to rigorously differentiate reinfections from reactivations, it is noteworthy that four of these patients received kidneys from seronegative donors. Five additional patients were seropositive at the time of transplantation but never had virologic or serologic evidence of active CMV infection; this constituted the "no infection" control group.

Cytomegalovirus syndrome, as defined by van den Berg et al. [1989], occurred in all patients in the primary infection group. Eight cases were classified as mild and three severe (one of these received ganciclovir). In the secondary infection group, four patients developed CMV syndrome, one of which was severe.

Sera from seven healthy individuals with primary CMV infection ("healthy seroconverters") were collected as part of a prospective study at the University of Washington during 1983 and 1984 [Gold et al., 1988]. Five were gay men, one of whom was HIV antibody positive but asymptomatic, and 2 were heterosexual attendees at a sexually transmitted diseases clinic. All patients were CMV seronegative at enrollment and had documented seroconversion with at least one positive CMV culture from the genitourinary tract.

In the clinical management of patients, serologies were determined by standard methods including ELISA, anticomplement immunofluorescence, latex particle agglutination, and indirect fluorescent antibody [Waner and Stewart, 1992]. Similarly, CMV isolation was performed in clinical viral diagnostic laboratories according to standard procedures [Starr and Friedman, 1985].

Radioimmunoprecipitation Assay

IgG antibodies to gB were measured by radioimmunoprecipitation (RIP) using antigen derived from cells infected with an adenovirus recombinant expressing gB (Ad-gB; [Marshall et al., 1990]). The technique was modified from that previously reported [Marshall et al., 1992]. Briefly, A549 cells were infected with Ad-gB and labeled between 24 and 48 hr post-infection with 10 μCi/ml [^{35}S]methionine. After labeling, cells were harvested, washed, and lysed in buffer containing 1% Nonidet-P40, 1% deoxycholate, and 0.1 mM phenylmethylsulfonyl fluoride. The lysate was clarified by ultracentrifugation and passed over lentil-lectin Sepharose 4B (Sigma, St. Louis, MO) columns equilibrated with lysis buffer. Columns were washed, then bound glycoproteins eluted with buffer containing 0.2 M methyl α-D-mannopyranoside. Eluted glycoproteins were concentrated by ultrafiltration and stored at −70°C. The specificity of this antigen preparation was previously demonstrated [Marshall et al., 1992].

For RIP, 5×10^4 counts per minute (cpm) of antigen was preabsorbed with pooled CMV seronegative serum then combined with antibody in buffer consisting of 10 mM sodium phosphate, 150 mM NaCl, 1% Triton X-100, and 10 mM EDTA (pH 7.0); final volume of the reaction mixture was 100 μl and final serum dilution was 1:50. After incubation at 4°C overnight, protein-G beads (ImmuBind®; Genex Corporation, Gaithersburg, MD) were added and the mixture was agitated for 1 hr at 4°C. Beads were collected by centrifugation and washed five times in cold buffer. Bound antigen-antibody complexes were eluted from the beads by heating at 90°C for 10 min in 30 μl of 100 mM Tris (pH 6.8), 2% SDS, 40% glycerol, 0.1% Triton X-100, and 10% 2-mercaptoethanol. Five μl of this supernatant was counted in a scintillation counter in order to quantitate gB-specific antibody levels, and the remainder was electrophoresed through reducing 7.5% polyacrylamide gels and autoradiographed.

Since precipitated gB antigen was counted without electrophoretic separation, some counts due to contaminating adenovirus-associated glycoproteins recognized by human serum [Marshall et al., 1992] were included. To account for this, glycoproteins derived from cells infected with the parental adenovirus type-5 (Ad-5) strain were prepared by lectin affinity as above. Forty-nine separate serum specimens from 10 patients were reacted with Ad-5 antigen in the quantitative RIP. The mean number of precipitated counts was 94 cpm, with a range of 60 to 134. Since all CMV seropositive specimens in this study gave greater than 134 cpm in the RIP assay, this value was taken as a conservative determination of background counts and was subtracted from the results of all gB antibody assays.

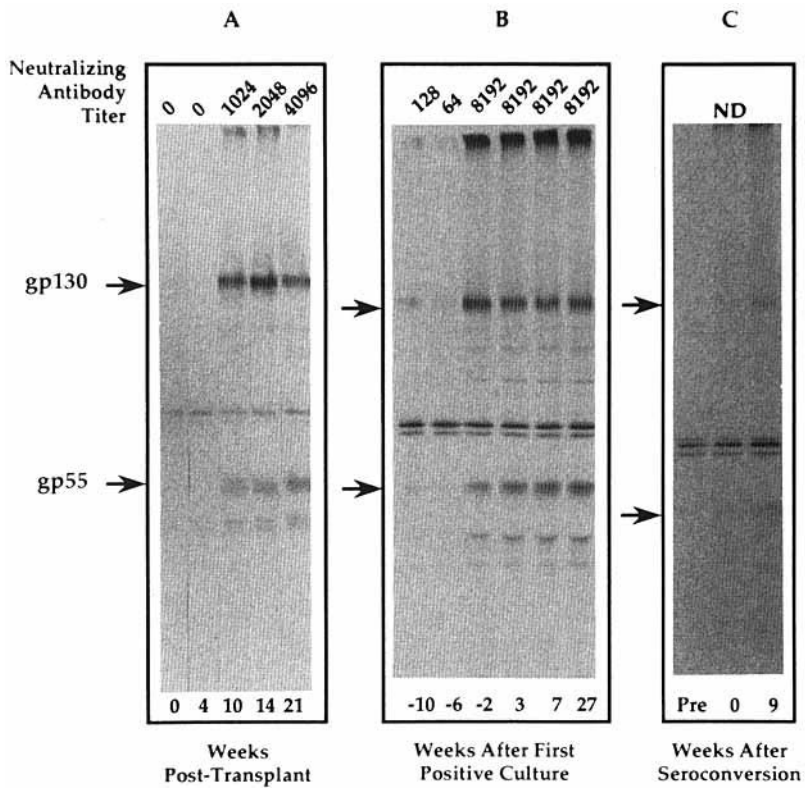

Fig. 1. Autoradiograph of radioimmunoprecipitation assays from 2 representative renal allograft recipients (**panel A** = primary infection, **panel B** = secondary infection) and a representative healthy seroconverter (**panel C**). gB-specific bands corresponding to gp130 and gp55 are indicated by arrows. Sera were collected at times shown beneath each lane. Neutralizing antibody titer of serum specimens is shown at top of lane. ND = not done.

Other Immunological Assays

Total CMV-specific IgG and IgM were determined by commercially available ELISA kits (Cytomegelisa® and CMV STAT M®, respectively; Whittaker, Walkersville, MD) which employ strain AD169 on the solid phase. Neutralizing antibody was measured by an ELISA-based microneutralization assay employing strain Towne [Rabalais et al., 1993].

RESULTS

Transplant Patients With Primary Infection

All 11 patients with primary CMV infection developed antibody to gB within 3 months of transplantation. Figure 1 shows the RIP assay from a representative patient (panel A). As expected, neither gB antibody nor neutralizing activity was present at the time of transplantation (week 0). However, antibody to gp130 and gp55 appeared at 10 weeks and closely paralleled the development of neutralizing activity, shown at the top (this patient began excreting CMV at 4 weeks). Bands seen between gp130 and gp55 represent adenovirus-associated glycoproteins ([Marshall et al., 1992]; note their presence at week 0). Dark bands at the origin of each lane were seen only in lanes demonstrating gp130 and gp55 reactivity; these are likely to represent gB oligomers or aggregates which do not migrate well under the electrophoresis conditions used [Britt and Vugler, 1992]. Note also that gp55 appears as a doublet. The lower band could represent gp55 that is alternatively glycosylated, further processed, or partially degraded.

Figure 2 summarizes quantitative serological data from transplant patients with primary infection (left panel). Two patients (both kidney transplants) who were sampled only once during the first 6 months are not included in this figure. All patients seroconverted to CMV-specific IgG and developed CMV-specific IgM in the first 2 months (data not shown), consistent with a median time to first positive CMV culture of 7 weeks (range 4 to 27). Note the appearance of neutralizing antibody and gB-specific antibody in the same time frame, as well as their persistence over the 6-month period. Follow-up sera showed persistence of gB antibody in all cases at a median of 20 months post-transplant (range 7 to 32 months). It should be noted that five patients were still culture positive at the time of this last sample, and 4 of the remaining 6 patients had a positive culture within 2 months.

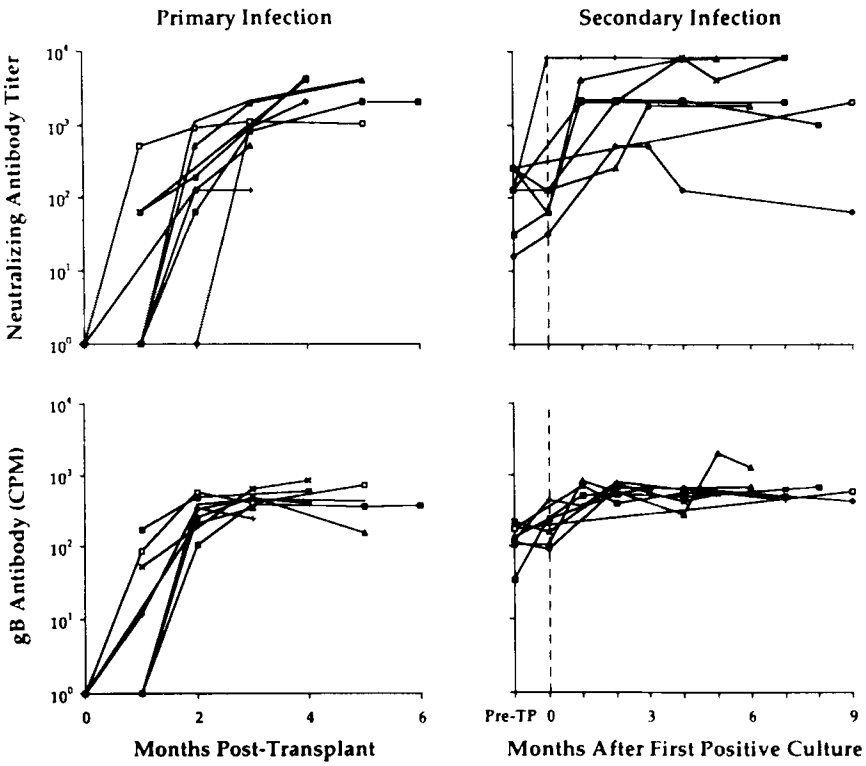

Fig. 2. Quantitative serological data on individuals with primary (n = 9) or secondary (n = 8) cytomegalovirus (CMV) infection. Neutralizing antibody was measured by a microneutralization assay and is expressed as reciprocal titer. gB-specific antibody was measured by quantitative radioimmunoprecipitation assay (see Materials and Methods) and is expressed as counts per minute (CPM).

Transplant Patients With Secondary Infection

All eight patients with secondary CMV infection also had readily detectable antibody to gB. Since reactivations were not distinguished from reinfections in this group, the date of transplantation could not be assumed to be the date of infection. Therefore, the date of first positive culture was used as an index point in the data presentation. Figure 1 shows the RIP assay from a representative patient (panel B). Low levels of gB antibody and neutralizing activity were present 10 weeks before the onset of CMV excretion. However, within weeks of the onset of CMV excretion there were boosts in gp130 and gp55 signal intensity as well as dramatic increases in neutralizing activity. Figure 2 summarizes quantitative serological data on all patients with secondary infection (right panel). Boosts in CMV-specific IgG and CMV-specific IgM were seen around the time of first positive culture (data not shown). Similarly, boosts in neutralizing antibody and gB-specific antibody occurred at times proximate to the first positive culture (month 0); the mean (±SD) gB-specific CPM were 135 ± 57 pre-transplant and 610 ± 145 post-infection ($P < .0001$, paired t-test). These antibodies persisted during the 9 month period. Five of these patients were still culture positive at the time of the last sample, and each of the remaining three patients had a positive culture within 2 months.

Control Group Without CMV Infection

Five renal transplant patients who were CMV seropositive at the time of transplantation were followed for a median of 12 months for evidence of CMV infection. A median of 11 negative urine cultures were obtained per patient, and levels of total CMV-specific IgG did not change significantly. Although each patient had readily detectable gB-specific antibody, there was little change in signal intensity with time (data not shown).

Healthy Seroconverters

All seven healthy individuals with documented primary CMV infection developed antibodies to gB. Figure 1 shows the RIP assay from a representative patient (panel C). As with the transplant patients, gB antibodies were seen within weeks of seroconversion, although the bands were less intense (quantitative studies were not done in this group).

Correlation of Neutralizing Activity With gB-Specific Antibody

In all, 96 seropositive specimens from 19 patients were tested for gB antibody and neutralizing activity. As in our previous study, a positive correlation between these two serologic measures was seen (r = .44, Spearman rank correlation coefficient; $P < .0001$).

DISCUSSION

Previous studies have examined the ontogeny of polypeptide-specific antibody responses in CMV-infected individuals by employing infected-cell lysates [Zaia et al., 1986; Gold et al., 1988; Allen et al., 1991] or purified virions [Hayes et al., 1987; Alford et al., 1988] in immunoblot or RIP assays. Whereas many patients develop antibodies to polypeptides in the molecular weight range of gB components (130–150 kDa, 93–116 kDa, and 55–58 kDa), these studies are difficult to interpret because of the large number of immunoreactive CMV-associated proteins in the antigen preparations. Utilizing partially purified gB derived from an adenovirus recombinant as the antigen in RIP assays, the current study better defines gB-specific responses in a variety of natural infection settings. All patients studied had detectable gB-specific IgG antibodies. Primary responses in CMV-naive transplant patients occurred within 3 months of infection and paralleled the development of total CMV-specific IgG, IgM, and neutralizing activity. Given existing data on the protein-specificity of neutralizing antibody responses [Britt et al., 1990; Gönczöl et al., 1991; Marshall et al., 1992], it is likely that a significant proportion of this neutralizing activity was due to the development of gB antibody. (Although the isotype specificity of CMV-neutralizing antibodies has not been studied, neutralizing activity is readily demonstrable in convalescent sera that lack CMV-specific IgM [unpublished observations]). Primary responses in CMV-naive healthy individuals were also seen in the same time frame but were generally weaker. The explanation for this is unclear but may relate to greater virus burden (and thus antigenic presence) in the transplant patients.

It should be mentioned that there are other CMV envelope glycoproteins that carry neutralizing epitopes [Rasmussen, 1990], and that heterogeneity may exist in responses to these glycoproteins among different individuals. Note, for example, that in some patients (Figure 1, panel A) neutralizing activity increases (week 10 to 21) without apparent change in gB reactivity; such increases could be due to the formation of neutralizing antibodies to other glycoproteins. In other patients, gB antibody was occasionally seen prior to the appearance of neutralizing activity; such antibodies could be directed against non-neutralizing epitopes on gp130. Nevertheless, as in our previous study [Marshall et al., 1992], a significant (albeit weaker) correlation between gB antibody and neutralizing activity was noted.

Early booster responses in gB antibody were seen in transplant patients with reinfection or reactivation, and these paralleled boosts in total CMV-specific IgG, IgM, and neutralizing activity. Boosts in the latter serological markers during secondary infection have been reported by other investigators [Pass et al., 1983]. In all transplant patients examined in our study, gB antibody was long-lived. However, most patients were also persistently culture positive throughout the follow-up period. The persistence of vigorous gB responses may have been related to the continued presence of viral activity in the host, although it should be noted that gB antibody did not wane in the uninfected control group. Continued viral replication despite high titers of neutralizing antibody is not surprising, since after the establishment of infection cell-mediated immunity probably plays a larger role in containment than does humoral immunity [Rook, 1988].

A recent study by Rasmussen and colleagues [Rasmussen et al., 1991] also utilized recombinant-derived gB antigen to investigate the ontogeny of gB responses during natural infection, although the results differ somewhat from ours. That study showed significant responses to gB in three immunocompetent individuals in the first few months after symptomatic CMV infection, but responses were minimal in another three individuals with asymptomatic infection. A dramatic rise in gB antibody was seen within 4 months in only 1 of 5 transplant patients experiencing primary infection, although some patients developed significant responses later in convalescence. In contrast, all 11 of our transplant patients with primary infection developed dramatic increases in gB antibody within 3 months. Further, only modest and inconsistent rises in gB antibody levels were seen with secondary infection in that study [Rasmussen et al., 1991], in contrast to the uniform rise in titer seen in our patients. Finally, gB antibody was detected in only 60% of sera from immunocompetent individuals [Rasmussen et al., 1991]. We previously detected gB antibody in each of 48 immunocompetent seropositives tested [Marshall et al., 1992]. In the current study, gB antibody was detected in each of 11 transplant patients with primary infection, eight transplant patients with secondary infection, five seropositive transplant patients without reactivation or reinfection, and seven healthy seroconverters. Thus, our data suggest that IgG antibody to gB is universally generated after natural infection in a variety of clinical settings.

However, differences between these two studies should be mentioned. For one, the study by Rasmussen and colleagues employed radiolabeled gB antigen derived from conditioned medium of CHO cells stably transfected with a truncated gB gene [Spaete et al., 1988]. That construct is truncated proximal to the transmembrane domain (at Leu-680) and thus retains the immunodominant region of the molecule, recently mapped between Val-552 and Arg-635 [Wagner et al., 1992]. This secreted antigen includes both uncleaved (gp110) and cleaved (gp93, gp31) forms of the carboxy-truncated gB protein. Antibody-binding domains have been mapped by different investigators [Kneiss et al., 1991; Silvestri et al., 1991; Basgoz et al., 1992] to the cytoplasmic tail of the molecule, a region that is not represented in this truncated construct. Since we used a full-length, cell-associated gB protein in our study, it is possible that some differences in recognition by human sera are due to this difference in antigen composition. Second, we used an antigen preparation that was rela-

tively enriched for glycoproteins by lectin-affinity chromatography and ultrafiltration. Therefore, the relative content of gB in our antigen preparation may have been higher. Finally, most of the patients in our study were renal transplant recipients, whereas the patients studied by Rasmussen and colleagues were exclusively heart and heart-lung transplant recipients and may have been more immunosuppressed. However, gB responses in our two heart transplant patients with primary infection were similar to those in the renal allograft recipients.

The gB gene of Towne strain CMV consists of an open-reading-frame of 2,721 nucleotides, with a predicted translation product of 907 amino acids [Spaete et al., 1988]. In CMV-infected cells, this primary polypeptide product is cotranslationally modified by cleavage of the signal peptide and the addition of high mannose oligosaccharides to form a precursor in the range of 130–150 kDa (termed gp130 by some authors [Spaete et al., 1988]). This precursor undergoes oligomerization and folding in the endoplasmic reticulum, after which it is transported to the Golgi where proteolytic cleavage takes place [Britt and Vugler, 1992]. The carboxy-terminal cleavage product is approximately 55 kDa in mass (gp55) and carries an immunodominant epitope [Britt and Auger, 1986; Britt and Vugler, 1989; Silvestri et al., 1991; Kneiss et al., 1991; Wagner et al., 1992]. The amino-terminal cleavage product has a mass of 93-116 kDa (referred to as gp93 by some authors [Spaete et al., 1988] and gp116 by others [Britt and Vugler, 1989]) and also carries antigenic determinants [Meyer et al., 1992]. gp55 and gp93 probably remain associated by disulfide bonds in a heterodimer, although higher order structures probably exist [Spaete et al., 1990; Britt and Vugler, 1992].

In our study, responses to gp130 and gp55 always occurred together, consistent with the precursor-product relationship and the presence of the same immunodominant region on both proteins. However, in the current study and our previous one [Marshall et al., 1992], gB bands in the range of 93–116 kDa were not detected. Since sera were reacted with antigen under nondenaturing conditions, complex structures should have been precipitated whose components should have resolved after polyacrylamide gel electrophoresis under reducing conditions. Indeed, recombinant-derived gB used in the study by Rasmussen and colleagues [Rasmussen et al., 1991] showed the precursor as well as both cleavage products in RIP assays. Given the fact that gp93 should copurify and coprecipitate with gp55, which was readily recognized, our failure to detect gp93 is not likely to be due to the absence of antibodies to this protein. Likewise, this failure should not be due to differential labeling of recombinant gp55 and gp93 with [^{35}S], since they contain nearly equivalent numbers of methionine residues in their primary structure. Using [^{35}S]methionine labeling of CMV-infected fibroblasts and immunoprecipitation, other investigators have seen much weaker signals corresponding to gp93 (gp116) than to gp55 [Britt and Vugler, 1992].

Recent experiments in our laboratory using [^{3}H]mannose to label AdgB-infected A549 cells readily demonstrated gp93 along with gp55 in immunoprecipitated cell extracts, presumably because of the large number of potential asparaginyl glycosylation sites on gp93 (unpublished observations). Potential explanations for the absence of gp93 in our RIP experiments include preferential intracellular degradation or extracellular secretion of gp93, less solubilization of gp93, incomplete or unstable heterodimer formation, and retention of gp93-containing complexes on the lectin columns used to prepare the antigen. Although we are currently investigating these possibilities, the absence of demonstrable precipitation of gp93 in our study does not alter the general conclusions with respect to gB-specific antibody.

Our previous study demonstrated the presence of gB antibody in individuals convalescent from natural CMV infection. The present data extend those observations to the dynamic situation of acute infection in both immunocompetent and immunocompromised patients. They provide further support for the fact that responses to gB are an integral part of the humoral immune response to this virus.

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