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Little Role of Anti-gB Antibodies in Neutralizing Activity of Patient's Sera with Human Cytomegalovirus (HCMV) Infection

Human cytomegalovirus (HCMV) gB is known to play important roles in cell surface attachment, virion penetration, spread of infection from cell to cell, and provocation of neutralizing antibody. This study was performed to determine the role of anti-HCMV gB antibody in overall neutralizing response in patients with HCMV infection and healthy control with past infection. HCMV gB was stably expressed in 293 cells. With the stable cell line expressing gB as a specific immunosorbent, anti-gB antibody was removed from the current and past HCMV-infected sera and the remaining neutralizing activity was measured by plaque assay. It was shown that 19-50% of the total virus-neutralizing activity of sera with past HCMV infections was derived from anti-gB antibody, but anti-gB antibody had little effect on the total serum virus-neutralizing activity in patients currently infected with HCMV. This result suggests that neutralizing antibody to HCMV gB may reflect disease status.

Key Words: *Cytomegalovirus; Human; Antibodies, Viral; Neutralization Tests; Immunosorbents*

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INTRODUCTION

Human cytomegalovirus (HCMV) is a ubiquitous infectious agent that is responsible for a subclinical course in normal hosts. HCMV infection is frequent and of considerable clinical relevance after placental transmission and in immunocompromised patients such as transplant recipients or AIDS patients (1-4).

HCMV glycoproteins, which contain major antigenic determinants of the virus, are inserted into the membranes of infected cells and virion envelope. Immunoprecipitation studies of human sera against HCMV antigens have shown that late HCMV-infected cell polypeptides, in particular viral glycoproteins, are highly immunogenic in the human host. And the glycoproteins are located on the surface membranes of HCMV-infected cells and contained neutralizing sites (5-7).

Passively transferred anti-HCMV antibodies can attenuate the severity of HCMV diseases (8-11). Antibodies against several gene products of HCMV were developed after the infection. A major fraction of neutralizing antibody in the sera from HCMV-infected patients was directed to HCMV gB (12-16). HCMV gB plays roles in cell surface attachment, virion penetration, and spread of infection from cell to cell (17).

To date, many studies have documented the neutralizing activity of anti-gB antibodies. However, there have been some problems of reports in verifying the neutralizing activity of anti-gB antibodies. Most of the methods verifying the neutralizing activity of anti-gB antibodies were positive selection, for example, characterization of monoclonal antibodies against HCMV gB (5, 18-20) or postimmune sera (13). Positive selection is adequate as a screening method for qualitative analysis, but it is somewhat inadequate for quantitative analysis in total serum pool. Investigations by using the human sera were documented according to the quantitative correlation between anti-gB titer and neutralizing antibody titer (16). However, it gives only some indirect evidences on the neutralizing activity of anti-gB antibodies. There have been no data verifying the differences in the immune reaction pattern between patients with current HCMV infection and healthy persons with past HCMV infection.

There has been few studies concerning the proportion of anti-gB antibodies in the neutralizing antibody pool in human serum from healthy persons or patients with current HCMV infection.

To verify the importance of anti-gB antibodies, we performed this study by using the immunoabsorption method to remove anti-gB antibodies specifically in human sera and tried to determine the contribution of

anti-gB antibodies to the overall neutralizing response in human sera.

MATERIALS AND METHODS

Materials

Human fetal lung fibroblasts (FLFs) were used for HCMV infection and propagated in Dulbecco's modified Eagle medium (DMEM, GIBCO Lab., Grand Island, NY, U.S.A.) supplemented with 10% fetal bovine serum. Cell line 293 (ATCC CRL-1573) was obtained from ATCC and propagated in DMEM supplemented with 10% fetal bovine serum. HCMV isolated from Korean patient was used. It has almost the same sequence as that of AD 169 strain in major antigenic domains of gB (21). Human sera used in this study came from two groups. One group was composed of nine patients currently infected with HCMV infection, which was proven by clinical findings and isolation of HCMV in urine or blood, and the other group was made of nine healthy persons with past HCMV infection (Table 1). Monoclonal antibodies used in this study were MCMVA 57, MCMVA 66, MCMVA 98 and MCMVA 135, which are reactive to the conformational epitope on D3, D2b, D3 and D2b domain of HCMV gB, respectively (22). MCMVA 135 is the neutralizing antibody (23). T4 DNA ligase, alkaline phosphatase, and all restriction endonucleases were obtained from Promega Co. (Madison, WI, U.S.A.). All cell culture reagents were purchased from Gibco Lab (Grand Island, NY, U.S.A.), and all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Construction of recombinant vector

Primers for polymerase chain reaction (PCR), designated as gB1 and gB2, were designed to amplify the gene encoding HCMV gB. The sequences of primers were gB1: TGGATTTGGCAAGCTTCGAACATGGA and gB2: GACACGGCCAAAGCTTGTCATGACTC. These oligonucleotides were commercially synthesized (Bioneer Co., Daeduck, Korea). Temperature cycling for PCR, performed in a programmable thermal cycler (Perkin Elmer 9600, U.S.A.), was set at 94℃ for 11 sec, 55℃ for 30 sec, and 72℃ for 60 sec for 30 cycles. The resulting PCR product of 3.1 kbp and pcDNA3 (Invitrogen Co., Netherlands) were digested with *Hin*dIII and ligated to generate the recombinant vector named as pcDNA3-gB.

Establishment of stable cell line expressing gB

pcDNA3-gB was transfected into 293 cells by calcium phosphate (24). Expression of gB in 293 cells was screened by immunofluorescent staining with monoclonal antibodies against gB as described above. The transfected cells were cloned by limiting dilution method to establish a stable cell line expressing gB. Expression of gB in the

Table 1. Results of antibody titers against HCMV in human sera

Individual No.	Sex	Age (year)	Underlying condition	IF titer		Nt titer†
				FLF-CMV*	gB†	
1	M	21	BMT	1:40,960	1:40	1:50
2	M	43	KT	1:20,480	1:160	1:80
3	M	53	KT	1:5,120	1:160	1:500
4	F	22	SLE	1:10,240	1:160	1:20
5	M	34	KT	1:5,120	1:1,280	1:320
6	F	32	KT	1:10,240	1:1,280	1:1,000
7	M	32	BMT	1:10,240	1:40	1:20
8	F	35	BMT	1:5,120	1:320	1:200
9	M	18	AML	1:2,560	1:160	1:20
10	M	24	Healthy	1:10,240	1:160	1:20
11	M	26	Healthy	1:20,480	1:40	1:20
12	M	30	Healthy	1:10,240	1:80	1:10
13	M	24	Healthy	1:10,240	1:80	1:60
14	M	40	Healthy	1:10,240	1:80	1:10
15	M	24	Healthy	1:20,480	1:160	1:20
16	F	36	Healthy	1:20,480	1:160	1:20
17	F	28	Healthy	1:10,240	1:80	1:20
18	M	31	Healthy	1:10,240	1:160	1:10

*FLF-CMV: Antigen was fetal lung fibroblasts (FLFs) infected with HCMV AD169, †gB: Antigen was 293 cells expressing HCMV gB, ‡Nt titer: Neutralizing antibody titer which reduced the plaque number to 50%

M, male; F, female; BMT, bone marrow transplantation; KT, kidney transplantation; SLE, systemic lupus erythematosus; AML, acute myelogenous leukemia

cells was confirmed by immunofluorescent staining with monoclonal antibodies against gB and human sera. For cell surface expression, immunofluorescent staining of the live cell was carried out as described elsewhere (25).

Neutralizing antibody assay

Neutralizing activity of human sera was assayed by plaque reduction assay. Briefly, 0.2 mL of human serum diluted serially in phosphate buffered saline (PBS) was added to 0.2 mL of 10^3 pfu/mL titrated virus. Following a 60-min incubation at 37℃, 0.2 mL of the mixture was added in duplicate to tissue culture plate containing fibroblasts. After 1 hr adsorption, the inoculum was removed, the monolayer was washed once with PBS, and methyl cellulose overlay media supplemented with 2% fetal bovine serum was added. The plate was incubated for 15 days and new overlay media was added every fifth day. After the 15-day incubation period, the number of plaques was quantitated under microscope. Results are expressed as the reciprocal of the highest dilution of serum, showing 50 percent reduction in plaque number.

Removal of anti-gB antibodies

To achieve a preabsorption neutralizing activity of approximately 50% and complete reduction in input infectivity, titer of human serum samples was determined prior to using the samples in the absorption experiments. A 0.5-mL amount of appropriately diluted sera was then mixed with a pellet of 3×10^7 293 cells expressing HCMV gB. The mixture was briefly vortexed, and the absorption was allowed to proceed on rotator at 4℃ for 60 min. This absorption procedure was repeated seven times. Following centrifugation, the serum was carefully removed, heated at 56℃ for 30 min, and assayed for neutralizing activity. Percent residual infectivity was calculated as follows: {infectivity (experimental)/infectivity (control)} × 100, where infectivity (experimental) represented the plaque number after the treatment of human serum, and infectivity (control) represented the plaque number without human serum. The standard error of the mean was at most 10% in all experiments.

RESULTS

Establishment of stable cell line expressing HCMV gB

HCMV gB gene was cloned into the *Hin*dIII site of pcDNA3 and the resulting pcDNA3-gB plasmid was transfected into 293 cells. The HCMV gB expression in 293 cells was initially screened by immunofluorescent staining with specific monoclonal antibodies against gB,

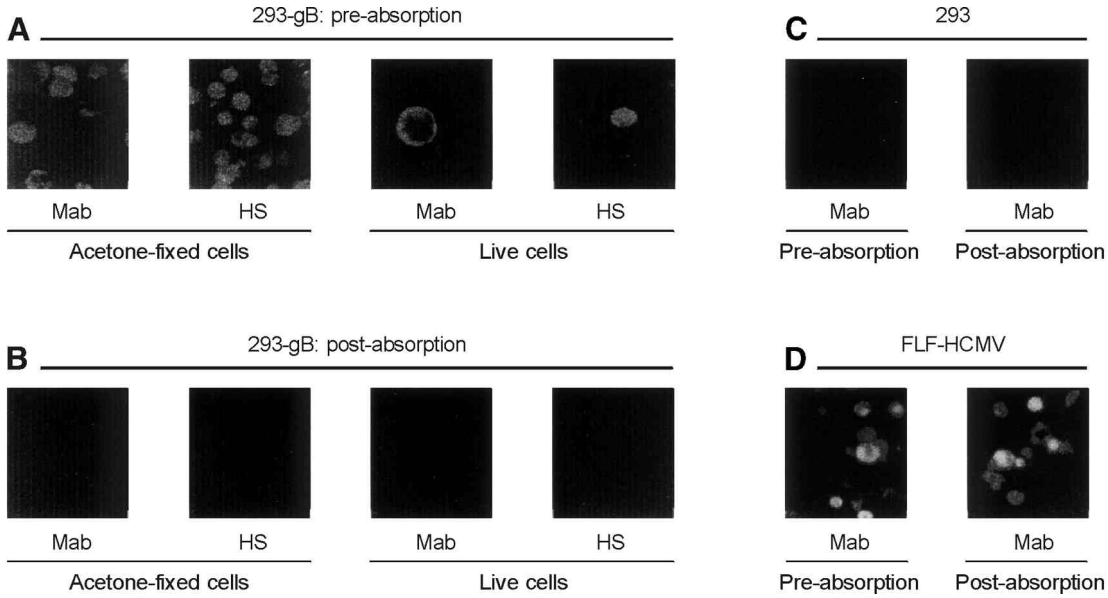

Fig. 1. Immunofluorescent staining patterns. **A:** Acetone-fixed 293-gB cells and live 293-gB cell with specific monoclonal antibody and human sera before immunoabsorption. Acetone-fixed cells are stained in the cytoplasm and live cells on the surface with monoclonal antibody and human serum. **B:** Acetone-fixed 293-gB cells and live 293-gB cell with specific monoclonal antibody and human sera after immunoabsorption. None of the cells are stained with monoclonal antibody and human serum. **C:** Acetone-fixed 293 with specific monoclonal antibody before and after immunoabsorption. None of the cells are stained. **D:** Acetone-fixed HCMV-infected fibroblasts with human sera before and after immunoabsorption. Cells are stained strongly before and after immunoabsorption. FLF-HCMV: HCMV infected fetal lung fibroblasts, MAb: representative monoclonal antibody to HCMV gB (MCMVA 135). HS: representative positive serum from HCMV-infected patients.

MCMVA135. Monoclonal antibodies used in this staining were MCMVA 57, MCMVA 66, MCMVA 98 and MCMVA 135, which were reactive to the conformational epitope on D2b or D3 domain of gB (22). Immunofluorescent assay revealed that the acetone-fixed stable cell line expressing gB was stained in the cytoplasm whereas control 293 cell was not (Fig. 1A). On the other hand, the live stable cell line expressing gB was stained on the cell surface with all antibodies described above and the live 293 cell was not (Fig. 1A). Immunofluorescent assay with positive human serum showed the same staining pattern in the acetone-fixed and live cell line expressing gB as monoclonal antibodies (Fig. 1A). The final 293 cells expressing HCMV gB were named 293-gB.

Characterization of human neutralizing antibody

As shown in Table 1, immunofluorescent antibody titers to HCMV-infected fibroblasts and 293-gB were 1:2,560 to 1:40,960 and 1:40 to 1:1,280 in the patient group, respectively, and 1:10,240 to 1:20,480 and 1:40 to 1:160 in the healthy control group, respectively. Neutralizing antibody titers in patient and healthy control groups were 1:20 to 1:1000 and 1:10 to 1:60, respectively.

The availability of human cells expressing gB allowed us to investigate the relative contribution of antibodies against gB to the overall HCMV neutralizing antibody response in humans. We utilized 3×10^7 cells as immunosorbents to remove anti-gB antibody in human sera. The immunoabsorption was repeated seven times. Anti-gB antibodies were removed by repeated immunoabsorption with 293-gB (Fig. 1B). When human serum was absorbed by the 293 cells, the intensity of fluorescence was almost the same in spite of repeated immunoabsorption (data not shown). This result indicated that it was possible to exclude the possibility of non-specific removal of antibodies by immunoabsorption to 293 cells. Immunofluorescent staining of mock- or HCMV-infected fibroblasts with the human sera immunoabsorbed to 293-gB revealed that the immunoabsorption to gB removed anti-gB antibodies specifically and did not have any influence on the other antibodies (Fig. 1D). After the final immunoabsorption to 293-gB, anti-gB antibody was not detected (Fig. 1A and 1B). Absorption of the human sera to 293 cells failed to reduce anti-gB antibody as compared with that of unabsorbed sera (Fig. 1C).

Neutralizing assay was carried out in duplicate. Absorption of the healthy human sera with 293 cells failed to reduce neutralizing activity of the sera significantly as compared with that of unabsorbed sera, whereas absorption with 293-gB cells reduced the neutralizing activity of the sera, ranging from 50 to 81% of their original activity. But postabsorbed patient sera absorbed by 293-gB cells retained the same activity as their original activity. These results are shown schematically in Fig. 2. In this diagram, the percent reduction of input infectivity of patients did not change significantly, but that of healthy controls was reduced by 32% on average, ranging from 19 to 50%.

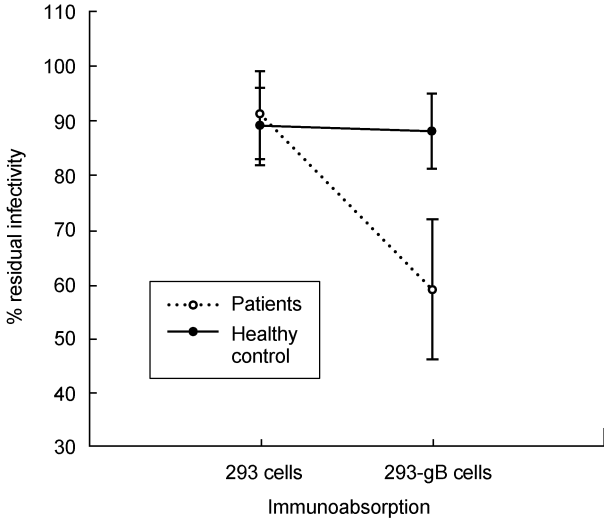

Fig. 2. Percent reduction of input infectivity of human sera before and after immunoabsorption with 293 cells or 293-gB cells (293 cells: parental cells, 293-gB cells: 293 cells expressing HCMV gB). Input infectivity is reduced to 19-50% in healthy control, but not in patient group. Dotted line with open circle: healthy control, Line with closed circle: HCMV-infected patients.

DISCUSSION

Glycoproteins on the viral envelope are of interest because of their potential importance as antigens in both humoral and cellular response. HCMV glycoproteins show complex electrophoretic profiles. It has been estimated that there are 3-8 glycoproteins on the HCMV envelope (7, 18, 26, 27). HCMV gB plays roles in cell surface attachment, virion penetration, and spread of infection from cell to cell (17).

Monoclonal antibodies used in the staining were reactive to the conformational epitope on D2b or D3 domain of gB (22). Immunofluorescent staining with monoclonal antibody and HCMV-positive human serum revealed that HCMV gB was stably expressed on the surface of 293 cells as an immunoreactive form (Fig. 1A). This result suggested that conformational epitopes were expressed effectively on mammalian cells.

All patients had a broad range of antibody titer to HCMV, that is, IF titer 1:2,560 to 1:40,960 to HCMV-

infected fibroblasts, IF titer 1:40 to 1:1,280 to 293-gB, and 1:20 to 1:1000 in neutralizing antibody (Table 1). There were no correlation between antibody titer to whole HCMV and gB, between antibody titer to whole HCMV and neutralizing antibody titer, and between antibody titer to gB and neutralizing antibody titer.

All anti-gB antibody present in the sera were removed by immunoabsorption with 293-gB to measure the proportion of neutralizing antibody in anti-gB antibody.

Thirty-two percent in average of the total serum virus-neutralizing activity of healthy control group with past HCMV infections was directed against HCMV gB. This result is similar to the report by Gönczol et al. (28) using recombinant vaccinia virus expressing HCMV gB. But anti-gB antibodies have little effect on the total serum virus-neutralizing activity in patients with current HCMV infection even though patients had high titer of antibody to gB generally (Table 1 and Fig. 2). Several possibilities can be considered for this result. First, relative ratio of neutralizing anti-gB antibodies in the total neutralizing antibody pool could be decreased whereas the total amount of anti-gB antibodies was the same as that of healthy persons, because the production of neutralizing antibodies against other antigenic components was increased and concurrently the increase of neutralizing antibody production against gB was inhibited. Total virus-neutralizing antibody titer was higher than that of healthy persons in the other patients and this supported the First possibility (Table 1 and Fig. 2). Second, in current HCMV-infected patients, the production of neutralizing anti-gB antibodies could be reduced whereas the total amount of serum virus-neutralizing antibodies was almost the same as that of healthy persons with past HCMV infection. Some of the patients currently infected with HCMV (patients Nos 1, 4, 7, 9 in Table 1), total virus-neutralizing antibody titer was almost the same as that of healthy persons whereas the contributory extent of anti-gB antibodies was reduced. This phenomenon supported the second possibility. Although humoral immunity, especially neutralizing antibody alone, could not prevent HCMV disease progression (Table 1), there must have been existing preventive factors in the sera of healthy persons because the passively transferred anti-HCMV antibodies could attenuate the severity of HCMV diseases (8-11). The antibody to neutralizing epitopes, not the antibody to epitopes on the whole HCMV gB, could be the main factor in disease prevention.

The most interesting result of our study was the analysis of the neutralizing response to HCMV in human sera and its result indicated that the contributory extent of anti-gB antibodies of patients with present HCMV infection was different from that of healthy persons with past HCMV infection. Although at present this hypothesis is speculative, we hope that future prospective studies will clarify these issues and provide directions towards development of effective immune prophylaxis against HCMV infection.

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