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Suppression of In Vitro Growth of Virulent and Avirulent Herpes Simplex Viruses by Cell-Mediated Immune Mechanisms, Antibody, and Interferon

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A rounding cell-forming −GC strain, which is a variant of a syncytial giant cell-forming herpes simplex virus (+GC Miyama strain), was highly attenuated for Swiss, BALB/c nu/nu, and nu/+ mice, whereas +GC was highly virulent to all the mice tested. +GC and −GC were antigenically indistinguishable from each other by cross-neutralization and cross-immunization. Immunosuppression induced by cyclophosphamide converted the nonlethal −GC infection of mice into a fatal infection. −GC replication in tissue culture was more effectively suppressed by spleen cells immunized with either +GC or −GC than was the +GC replication. −GC replication was also inhibited more effectively by antibody or the antibody-dependent cell-mediated system than was the +GC replication. −GC is highly sensitive to mouse interferon, but +GC was relatively resistant. These findings indicate that attenuation of this avirulent −GC strain may be due to a high susceptibility of its replication to humoral and cell-mediated defense factors. The probable roles of each defense factor in recovery from the infection with virulent and attenuated herpes simplex virus are also discussed.

The biological behavior of herpes simplex viruses (HSV) has been studied in terms of their relationship to virulence. The findings have shown that virulence is not parallel with susceptibility of the virus to antibody or with their thermostability (25). No correlation of virulence with plaque size or cytopathic effect (CPE) of viruses in tissue culture cells has been found (13, 25, 27). Studies of the effect of a proposed defense on the viruses with different virulence often permit further evaluations of the importance of the proposed defense factor and causes of attenuation of virus. Little information, however, has been reported on the effect of humoral factors such as antibody or interferon (IF) on the replication of virulent and avirulent strains of virus (15, 22). Cumulative evidence indicates that the cell-mediated immunity plays a major part in recovery from herpesvirus infection (10, 16, 21). In vitro systems using purified effector cells, humoral factors, and defined targets have now clearly established the reality of these immune mechanisms (10, 16, 17, 21). Whether these cell-mediated immune factors affect differently the infection by virulent and avirulent herpesviruses also has not been investigated. Sensitivity tests of the virulent and avirulent viruses to these humoral and cell-mediated factors may provide us with information whether any given factors exhibit relative importance to the process of recovery from HSV infection. The aim of this study was to determine the effect of humoral factors, such as antibody or IF, and cell-mediated immune systems, such as immune lymphocytes or antibody-dependent cell-mediated cytotoxicity, on a virulent strain of HSV and its attenuated variant. The results show that the in vitro growth of avirulent strain is more effectively suppressed by these cell-mediated immune systems as well as IF and antibody than is the virulent strain. The probable roles of these immune and nonimmune mechanisms in recovery from herpesvirus infection also are discussed.

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MATERIALS AND METHODS

Cells and viruses. 3T3 Swiss mice, green monkey kidney, human embryonic lung, and FL cells were used. All were grown in Eagle minimal essential medium (MEM) supplemented with 10% heat-inactivated calf serum (CS), penicillin (100 U/ml), and streptomycin (100 μg/ml). MEM containing 2% CS was used as maintenance medium for the cultures.

+GC and −GC clones of the Miyama strain of HSV type 1 were used throughout the study. Both clones were obtained from S. Nii, Osaka University, Japan. The −GC clone, which causes rounding of infected cells, was isolated from the +GC strain which produces large syncytial giant cells (7). Each of these clones was propagated in monolayers of green monkey kidney

cells as previously reported (18). Virus infectivity was assayed by counting the plaque-forming units (PFU) in 3T3 cells and FL cells or 50% tissue culture infectious doses per milliliter in human embryonic lung cells. Stock viruses of +GC and −GC clones contained 3×10^7 and 8×10^6 PFU/ml, respectively. Vesicular stomatitis virus (Indiana strain) propagated in human embryonic lung cells was used as an indicator virus for interferon assays.

Antiserum. Rabbit antisera against +GC or −GC were prepared as previously described (18), and used after heat inactivation.

Mice and virus infection. Ten-week-old Swiss mice and eleven-week-old nu/nu and nu/+ BALB/c mice were used. The virulence of +GC and −GC was investigated by an intraperitoneal (i.p.) injection of each virus at different doses in 0.2 ml of tris(hydroxymethyl)aminomethane buffer. Mortality rate was determined 4 weeks after infection.

Cyclophosphamide-treated mice. Cyclophosphamide (CP)-induced immunosuppression was performed by the methods of G. D. Stockman et al. (23). CP (Shionogi and Co., Ltd., Osaka, Japan) was dissolved in distilled water at a final concentration of 30 mg/ml, and 0.2 ml of the solution was injected i.p. into the mice (300 mg per kg of body weight, a dose which is nonlethal to mice).

Effector cells used for cell-mediated inhibition assays. The peripheral blood lymphocytes (PBL) and spleen cells were purified by Ficoll-Isopaque centrifugation as described previously (1, 17). The PBL preparation used regularly was composed of more than 99% lymphocytes, less than 1% monocytes, as judged by the peroxidase-staining test, and a very few neutrophils. The viability of cells was more than 98% as judged by the trypan blue dye exclusion test. The nonimmune spleen cell preparations were composed of 90 to 95% peroxidase-negative small lymphocytes, 2 to 5% macrophage-like cells, and a few neutrophils (17).

From the spleens of mice 9 days after intravenous inoculation with −GC (10^6 PFU per mouse) or +GC (10^3 PFU), the lymphocytes were prepared by the same procedures as that for the normal spleens, incubated for 30 min at 37°C to remove the passively adsorbed immunoglobulins (5, 10, 11), and then used as immune lymphocytes.

Assay for inhibition of virus growth by antibody-dependent cell-mediated systems. The assay methods have been described previously (17). To be brief, confluent monolayers of 3T3 cells in trays (Falcon Chemical Co., Inc.) containing 24 wells (16 mm in diameter) were infected with approximately 30 PFU of HSV in 0.1 ml of MEM containing 2% CS and then incubated for 60 min at 37°C in a humidified atmosphere, plus 5% CO_2. After this incubation period, monolayers were washed twice with 1 ml of phosphate-buffered saline (PBS) (pH 7.2), and then either PBL or antiserum against +GC, or both, in a total volume of 0.6 ml of MEM containing 5% CS was added to the culture. For controls, only the medium (MEM containing 5% CS) was added to the culture. For other controls of plaque development, infected cultures overlaid with 1% methylcellulose in MEM containing 5% CS were prepared. The monolayers were reincubated for about 72 h at 37°C, and 0.2 ml of medium was then harvested for assay of the virus yield.

Assay for inhibition of virus yield by immune spleen cells. The assay was performed by methods similar to that used for antibody-dependent cell-mediated systems. 3T3 cells inoculated with 30 PFU of +GC or −GC were incubated for 60 min at 37°C, and either immunized or unimmunized spleen cells in MEM containing 5% CS was then added to the culture. For the virus controls, only MEM containing 5% CS was added. The monolayers were reincubated for 72 h at 37°C and assayed for virus titers in the culture fluids.

Assay of IF sensitivity. IF (8,000 U/ml), derived from mouse L-MS cells stimulated by Newcastle disease virus, was obtained from N. Ishida, Tohoku University School of Medicine, Sendai, Japan. The IF was serially diluted in a maintenance medium. A 5-ml portion of each was added in triplicate to 3T3 monolayers in plaque bottles. After incubation for 20 h at 37°C, the cultures were washed once with PBS, and 0.2 ml of maintenance medium containing 100 to 120 PFU of +GC or −GC was added to each culture. After 1 h of adsorption, each culture was washed once with PBS, overlaid with 5 ml of maintenance medium containing 1% methylcellulose, incubated for 70 h at 37°C to allow for plaque development, and then stained with crystal violet. For control assays, vesicular stomatitis virus was used as previously described (26).

RESULTS

Virulence for mice. Table 1 shows the mortality rates of Swiss, BALB/c nu/nu and nu/+ mice after receiving an i.p. inoculation of +GC and −GC viruses. The results clearly show that the 50% lethal dose of +GC to Swiss mice was about 3.9×10^3 PFU per mouse. In contrast, −GC virus even at an inoculation dose as high as 3×10^6 PFU per mouse was not lethal to Swiss mice. BALB/c nu/nu mice were killed by less infectious +GC virus than were nu/+ BALB/c mice and Swiss mice. A large inoculum of −GC (3×10^6 PFU), however, was still nonlethal to BALB/c nu/nu as well as nu/+ mice. Next, the virulence of −GC virus in immunosuppressed mice induced by CP treatment was ex-

TABLE 1. *Mortality rates of nu/nu and nu/+ BALB/c and Swiss mice after i.p. inoculation with +GC or −GC*

Inoculated virus	Dose of virus (PFU/mouse)	Ratio of dead/treated (%)		
		nu/nu	nu/+	Swiss
+GC	3×10^5	ND[a]	ND	8/8 (100)
+GC	3×10^4	ND	6/8 (75)	6/8 (75)
+GC	3×10^3	4/4 (100)	3/8 (38)	4/8 (50)
+GC	3×10^2	3/4 (75)	0/8 (0)	1/8 (12.5)
+GC	3×10^1	0/4 (0)	0/8 (0)	ND
−GC	3×10^6	0/4 (0)	0/8 (0)	0/8 (0)

[a] ND, Not done.

amined. Swiss mice were inoculated i.p. with different doses of −GC, followed by an i.p. injection of 6 mg of CP 72 h after virus inoculation. The results (Table 2) indicate that the nonlethal infection with −GC in control mice was converted into a lethal infection in such immunosuppressed mice, 50% of which were killed by inoculation of 1.0×10^5 PFU per mouse.

These results suggest that the −GC virus was infective in the mice per se, as it was in tissue cultures, but the establishment of infection in healthy mice was hindered by host defense mechanisms mediated by certain cellular elements which could be destroyed by CP.

Protective effect of −GC virus against infection with +GC virus. −GC doses of 1.4×10^4 and 1.4×10^2 PFU per mouse were inoculated into the Swiss mice i.p. The mice then were challenged with 3×10^5 PFU of +GC per mouse, the amount which killed 100% of nonimmune control animals (Table 3). The results show that the preinoculation of 1.4×10^4 PFU of −GC per mouse conferred complete protection against +GC challenge. This clearly indicates that −GC had enough potency to confer immunogenicity and that there were no antigenic differences between two strains, which is consistent with the results of the cross-immunization test (data are not shown).

Suppression of in vitro growth of +GC and −GC viruses by immune specific spleen cells. 3T3 cells infected with either of the viruses were overlaid with immune spleen cells from mice infected with +GC or −GC viruses. For control cultures, spleen cells from nonimmune mice were added. After cultivation of cells for 72 h at 37°C, virus yields in the culture fluids were assayed (Table 4). Replication of −GC was remarkably inhibited by addition of either −GC- or +GC-immune spleen cells. In contrast, +GC virus growth was slightly inhibited by spleen cells immunized with its own virus, although it was significantly inhibited by −GC-immune cells. These results indicate that the in vitro replication of −GC was much more susceptible to immune lymphocytes than was +GC. The results also suggest the possibility that +GC infection of mice produced immune cells with a low activity or less amounts, as compared with those of −GC-induced immune lymphocytes.

TABLE 2. *Fatal infection of CP-treated mice by −GC inoculation*[a]

Dose of inoculated virus (PFU/mouse)	No. of mice	CP treatment	% of mortality (mean surviving days)
1.4×10^6	4	−	0
1.4×10^6	6	+	100 (13.8 ± 2.2)
1.4×10^5	8	+	50 (13.5 ± 3.5)
1.4×10^4	8	+	25 (15.3 ± 5.5)
—	10	+	0

[a] Swiss mice were inoculated i.p. with different doses of −GC and followed by i.p. injection of 6 mg of CP (300 mg/kg of body weight) after 72 h of virus inoculation.

TABLE 3. *Protective effect of −GC preinoculation against the fatal infection by +GC*

Pretreated −GC (PFU/mouse)	Challenged[a] +GC (PFU/mouse)	No. of mice	% of mortality (mean surviving days)
0	3×10^5	6	100 (6.3 ± 1.4)
1.4×10^4	3×10^5	8	0
1.4×10^2	3×10^5	8	75 (6.8 ± 1.2)

[a] Challenge of mice with +GC was performed i.p. after 4 weeks of i.p. inoculation of −GC.

TABLE 4. *Inhibition of +GC and −GC replication in 3T3 cells by immune spleen cells*

Challenge virus	Overlaid[a] with spleen cells from mice			MEM
	+GC immunized	−GC immunized	Nonimmunized	
+GC	7.0[b]	6.0[b]	7.5	7.8
−GC	5.0[c]	3.0[c]	6.0	7.0

[a] 3T3 cells monolayers containing 1×10^5 cells in a well were inoculated with approximately 30 PFU of +GC or −GC. After adsorption periods, spleen cells (2×10^6) from mice immunized with +GC (sublethal dose) or −GC were added. For virus yield control, MEM alone was added. Values are expressed as the $\log_{10}$ 50% tissue culture infective dose per milliliter.

[b] $P < 0.01$ by Student's t test.

[c] $P < 0.01$ by Student's t test.

Effect of antibody and ADCC systems on the in vitro replication of +GC and −GC viruses. To the 3T3 cells infected with +GC or −GC virus, either nonimmune mouse peripheral blood lymphocytes (PBL) or antibody, or both, were added and then incubated for 72 h at 37°C. After the incubation period, the cultures were examined for the virus yield in culture fluids. Figure 1 shows that −GC virus replication was more susceptible to suppression by ADCC than was +GC replication. −GC virus replication could be suppressed by a 1:6,400 dilution of antibody in the presence of PBL, whereas +GC virus required 1:1,600 dilution of antibody for ADCC. Furthermore, −GC virus replication was inhibited at a lower concentration of antibody alone (1:1,600) than in the case of +GC virus (1:400). These results clearly show that the in vitro replication of −GC virus was extremely susceptible to the restriction by either antibody or the ADCC system, whereas that of +GC was suscep-

tible to the ADCC system, but relatively resistant to the inhibition by antibody alone.

Sensitivity of +GC and −GC growth to mouse IF. The 3T3 monolayers were incubated with mouse IF at serial dilutions for 24 h at 37°C, repeatedly washed, and then infected with approximately 100 PFU of +GC or −GC virus. After incubation for 72 h at 37°C, infectivity of viruses in the culture fluids was estimated. Susceptibility of each virus to IF was calculated as an interferon unit producing 50% reduction of PFU (Fig. 2). The results show that the susceptibility of −GC to IF (6 to 7 U) was much higher than that of +GC (160 U).

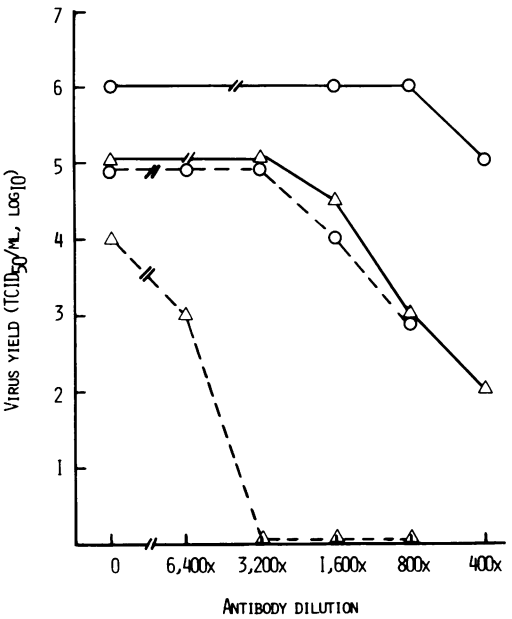

FIG. 1. *Inhibition of +GC and −GC replication in 3T3 cells by antibody-dependent cell-mediated systems. Confluent 3T3 cell monolayers containing 1 × 10^5 cells in a well were inoculated with approximately 30 PFU of +GC or −GC. After adsorption periods of 60 min at 37°C, either PBL (2 × 10^6 lymphocytes) or serially diluted antiserum, or both, were added to the culture. Symbols: ○——○, +GC (antibody alone); △——△, +GC (antibody plus PBL); ○- - -○, −GC (antibody alone); △- - -△, −GC (antibody plus PBL).*

Virulence for mice and in vitro susceptibility to cellular and humoral mechanisms of +GC and −GC viruses obtained in this study are summarized as follows. Parent strain +GC was highly virulent for healthy mice as well as immunosuppressed mice (data not shown) and their in vitro growth was less susceptible to suppression by antibody, ADCC, immune spleen cells, and interferon. Avirulent strain −GC was not lethal to healthy mice and nude mice, but was lethal to drug-induced immunosuppressed mice. Its in vitro growth was effectively suppressed by all the immune systems tested and interferon.

DISCUSSION

The present studies demonstrate that the growth of an avirulent variant, −GC of herpes simplex virus Miyama strain was much more susceptible to suppression by immune spleen cells and ADCC systems as well as antibody and interferon than was the virulent strain +GC.

−GC virus was avirulent for nu/nu as well as nu/+ BALB/c mice and Swiss mice. Immunosuppression of mice by CP treatment, however, converted a nonlethal infection into a lethal

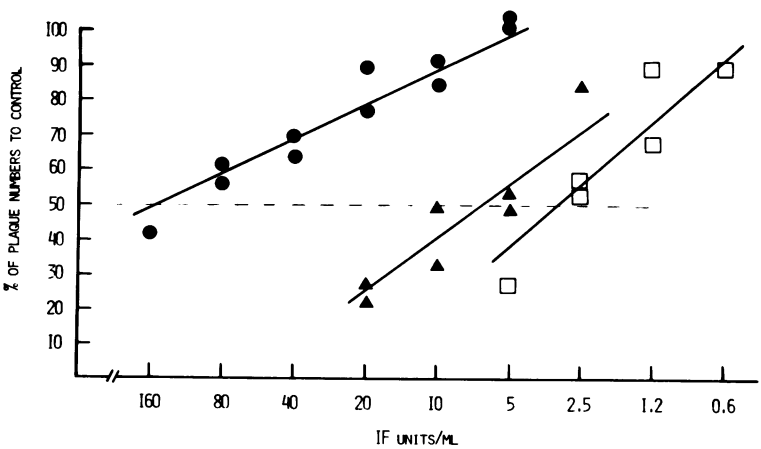

FIG. 2. *Sensitivity of +GC and −GC to mouse IF. 3T3 monolayers were pretreated with mouse IF for 20 h and then challenged with each virus at 100 to 200 PFU per culture. Symbols: ●, +GC; ▲, −GC; □, vesicular stomatitis virus. Two of the same symbols, at the same dose of IF, represent each of the results obtained from two separate experiments.*

infection. The results suggest that the cellular components such as macrophages (14), K (8), or NK (4) cells other than T cells or humoral factors such as IF may play a major role in recovery from the −GC infection in nude mice. −GC infection in mice produced immune spleen cells with a high activity by which the growth of −GC was effectively suppressed. During the process of −GC infection in healthy mice, therefore, immune specific, probably T, cell-mediated suppression of virus growth also may play a part as described in previous reports (6, 10).

The results show that −GC replication, compared with +GC, was highly susceptible to suppression by immune spleen cells. However, the virus was slightly inhibited by nonimmune cells as well. Whether the cells inducing such viral suppression are macrophages or NK cells or other cells remains undetermined.

The present results also show that the +GC infection elicited the immune spleen cells with much less activity in suppressing the growth of its own virus as well as −GC, whereas −GC infection elicited the immune spleen cells capable of effectively suppressing both the −GC and +GC replications. The reason for this is not known. Some investigators have reported that the immune-specific killer T cells could be produced by HSV infection (2, 21), but others have reported that such cells could not be easily produced (9, 12). Our results, therefore, suggest the possibility that, between the viruses causing lethal and nonlethal infections, there is a difference in activity eliciting the immune killer T cells in mice, although further investigation will be required to resolve this problem. It seems unlikely that the antigenic difference between +GC and −GC resulted in a production of immune spleen cells at different levels of activity since no antigenic differences were found between the two viruses as revealed by cross neutralization (data are not shown) and cross immunization.

The role of ADCC in herpesvirus infection has been discussed in many reports by the assay methods of ^{51}Cr release from infected cells (11, 19, 20). The findings suggest that this system may also play an important role in recovery or protection from herpesvirus infection. We have recently shown that this system indeed inhibits virus dissemination in HSV-infected cell cultures (17). The present report further shows that an avirulent strain was inhibited more effectively than a virulent strain by this system.

IF may play an important role in host defense mechanisms since it occurs at a very early stage of infection when virus concentrations are still at relatively low levels (24). The present studies show that −GC is very sensitive to mouse IF as is vesicular stomatitis virus, whereas +GC is highly resistant. Using human leukocyte IF instead of mouse IF, comparable results were obtained (unpublished data). It has been reported that HSV may be relatively resistant to IF (3). However, our results show that there may be a relationship between lack of virulence and sensitivity to IF among the different strains of HSV. These results coincide with previous reports that the attenuated strains of foot-and-mouth disease virus and some other viruses, compared with their virulent strains, were much more sensitive to IF (15, 22). Our preliminary experiments showed that +GC and −GC infections produced approximately the same levels of IF and antibody. Therefore, it seems unlikely that a differing +GC and −GC virulence is due to their different activities inducing IF or antibody.

Overall, our results indicate that antibody, alone or in cooperation with macrophages or K cells, and immune T cells, effectively play a part in recovery of mice with avirulent, but not virulent strains, of HSV from infection and that nonimmune defense mechanisms mediated by macrophages and other cells or humoral factors such as interferon also may be effective. Immune T cell-mediated suppression systems may be required for recovery from infection by virulent strains such as +GC. However, growth of such virulent viruses generally may be less susceptible to T cell-mediated immune systems and essentially not susceptible to interferon. It is also possible that the immune T cells, which are enough to suppress virus growth, may not be produced in mice.

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