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Antiviral activity of glycyrrhizin against varicella-zoster virus in vitro

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Summary

One of the plant extracts, glycyrrhizin (GL) was investigated for its antiviral action on varicella-zoster virus (VZV) in vitro. When human embryonic fibroblast (HEF) cells were treated with GL after inoculation of virus (post-treatment), the average 50%-inhibitory dose (ID_{50}) for five VZV strains was 0.71 mM, and the selectivity index (ratio of ID_{50} for host-cell DNA synthesis to ID_{50} for VZV replication) was 30. GL was also effective against VZV replication when HEF cells were treated 24 h before the inoculation (pretreatment). Furthermore, at a concentration of 2.4 mM GL inactivated more than 99% of virus particles within 30 min at 37°C. In combination with other anti-herpes drugs (acyclovir, adenine arabinoside, bromovinyldeoxyuridine, and phosphonoformate) or human native beta-interferon, GL had an additive or slightly synergistic effect on VZV replication. The mechanism of anti-VZV action is still unclear. We postulate that GL inhibits the penetration, uncoating or release of virus particles.

Glycyrrhizin; Varicella-zoster virus

Introduction

Most of the antiviral agents, in particular anti-herpes drugs, are nucleoside analogues: i.e. 9-(2-hydroxyethoxymethyl)guanine (ACV), 9-β-D-arabinofuranosyladenine (Ara-A), and (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU). They are inhibitors of specific virus-induced enzymes.

Glycyrrhizin (GL) is one of the aqueous extracts of licorice root (*Glycyrrhiza*

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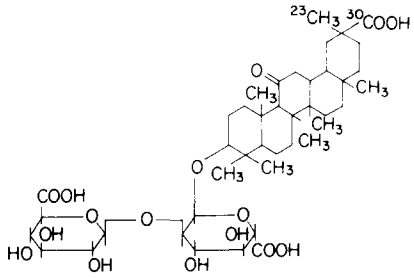

Fig. 1 The structure of glycyrrhizin (GL).

radix), which is known as an anti-inflammatory substance in Chinese medicine. Fig. 1 shows the structure of GL. This compound consists of one molecule of glycyrrhetinic acid (GA) and two molecules of glucuronic acid. In a previous investigation, it was reported that GA had antiviral activities (Pompei et al., 1979). Since the therapeutic and prophylactic effects of GL on chronic viral hepatitis have been claimed (Suzuki et al., 1977; Fujisawa et al., 1980; Ebina and Ishida, 1985), a preparation of GL with glycine and cysteine (designated as 'SMNC') has been used clinically against hepatitis. In addition, some physicians recently reported that administration of 'SMNC' accelerated the healing of herpes zoster (Uchiumi and Imai, 1981). In this study we investigated the inhibitory effects of GL on varicella-zoster virus (VZV) replication in vitro.

Materials and Methods

Viruses

The laboratory strain of VZV, CaQu, was provided by N.J. Schmidt (Viral and Rickettsial Disease Laboratory, Department of Health, Berkeley, Cal., USA). The Kimura, Suzuki, and Hosen strains were isolated in our laboratory from patients with herpes zoster or varicella. The Kanno-Kohmura strain is a deoxythymidine (dThd) kinase-deficient (TK^-) mutant previously described (Shigeta et al., 1983). Cell-free virus was obtained by sonic treatment of infected cells, as described by Schmidt and Lennette (1976), and stored at −80°C until used.

Cells

Human embryonic fibroblast (HEF) cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% heat inactivated newborn calf serum (NCS), 100 units/ml of penicillin G and 50 μg/ml of streptomycin (growth medium).

Drugs

GL was supplied by Minophagen Pharmaceutical Co., Tokyo, Japan. It was dis-

solved in 0.01 M phosphate-buffered saline (PBS) and adjusted to pH 7.2 with 1 N sodium hydroxide. ACV was supplied by Wellcome Japan, Osaka, Japan. Ara-A was purchased commercially from Sigma Chemical Co., St. Louis, MO. BVDU was supplied by E. De Clercq (Rega Institute, Katholieke Universiteit Leuven, Belgium), phosphonoformic acid (PFA) by Astra-Läkemedel AB, Södertälje, Sweden, and human native beta-interferon (HuIFN-β) by Toray Co., Tokyo, Japan.

Antiviral assay

All antiviral assays were carried out in confluent HEF monolayers in Falcon multiwell plates with 24 flat bottom wells of 16 mm diameter (Becton Dickinson Labware, Cal., USA). The anti-VZV activities of GL were determined by using three different treatment procedures. First, the monolayers were exposed to various concentrations of GL (12, 2.4, 0.48, and 0.096 mM) in maintenance medium (MEM, 2% NCS, and antibiotics at the concentrations mentioned above) for 24 h (pretreatment). After treatment, the monolayers were washed thoroughly with maintenance medium and inoculated with 100 plaque forming units (PFU) of VZV which was allowed to adsorb to the cells for 60 min at 25°C. Then the monolayers were incubated with GL-free maintenance medium at 37°C. Second, HEF monolayers were inoculated with VZV, and immediately thereafter exposed to various concentrations of GL in maintenance medium (post-treatment) and further incubated at 37°C. Third, HEF monolayers were treated with various concentrations of GL before and after VZV inoculation (pre- and post-treatment). All tests were run in duplicate. The monolayers were incubated at 37°C for 72 h, and the VZV foci were detected by the immunoperoxidase method. Briefly, after removal of maintenance medium, the monolayers were washed twice with PBS, and fixed with 100% methanol at 4°C for 10 min. Then they were treated with a proper dilution of anti-VZV human convalescent serum for 30 min at 37°C. After being washed twice with PBS, they were treated with peroxidase-conjugated rabbit antibody to human IgG (Dakopatts a/s, Denmark) for 30 min at 37°C, and again washed twice with PBS. To each well was then added 0.5 ml of substrate solution containing 0.5 mg/ml 3,3′-diaminobenzidinetetrahydrochloride, 0.05 M tris(hydroxymethyl)-aminomethane, 0.2 M NaCl and 0.01% H_2O_2. The plates were left for 15 min in a dark room. The VZV foci appeared as light brown spots; they were counted under a magnifying glass.

The antiviral potency is expressed as ID_{50}, that is the concentration of compound required to reduce the number of foci to 50% of that obtained in the control cell cultures (infected with virus but not exposed to the compound).

Assay for combined effect with anti-herpes drugs or interferon

Combined effects with anti-herpes drugs (ACV, BVDU, Ara-A and PFA) or interferon (HuIFN-β) on VZV replication were examined by the checkerboard combinations of various concentrations and analyzed by the isobologram method previously described (Baba et al., 1984). In this experiment, HEF monolayers were treated with GL either before or after inoculation of VZV (CaQu strain). Anti-

herpes drugs were added after virus inoculation, and interferon was added before virus inoculation.

VZV inactivation assay

A suspension of 0.1 ml of cell-free VZV (CaQu strain) containing approximately 5×10^3 PFU was mixed with 0.9 ml of various concentrations of GL (final concentrations: 2.4, 0.48, and 0.096 mM) in test tubes and incubated at 37°C. At 10 and 30 min after incubation, 0.1 ml of each mixture was diluted with 0.9 ml maintenance medium, and then 0.2 ml of these diluted samples were inoculated to HEF monolayers for infectious virus titration. After incubation for 72 h at 37°C, VZV foci appearing in HEF monolayers were counted by the immunoperoxidase method described above.

Cytotoxicity assay

Cytotoxicity of the compounds against HEF was examined by both the trypan blue-exclusion test and the inhibition of host-cell DNA synthesis as previously described (Shigeta et al., 1983). In the former, HEF monolayers were exposed to various concentrations of GL for 2 days, and the host-cell viability was judged microscopically. In the latter, [^{3}H]thymidine was added to HEF monolayers at 0.25 μCi/well together with various concentrations of test compounds. The amounts of [^{3}H]thymidine incorporated into acid-insoluble material of HEF were determined after 24 h incubation. ID_{50} for host DNA synthesis was defined as the concentration of compound required to reduce [^{3}H]thymidine incorporation to 50% of that in the control cell cultures.

Results

Antiviral activity against various VZV strains

The ID_{50} values of GL for five VZV strains are shown in Table 1. There was

TABLE 1

Inhibition of various varicella-zoster virus (VZV) strains by GL

Strain	ID_{50} (mM)		
	Pretreatment	Post-treatment	Pre- and post-treatment
CaQu	3.28	0.87	0.58
Kimura	3.55	0.72	0.50
Suzuki	2.54	0.52	0.33
Hosen	2.99	0.53	0.30
Kanno-Kohmura[a]	3.24	0.89	0.59
Cytotoxicity[b]		21.3	

[a] TK^- mutant strain (Shigeta et al., 1983).

[b] ID_{50} for host DNA synthesis, defined as the concentration of GL required to reduce [^{3}H]thymidine incorporation by 50%.

little variation in the susceptibility of the VZV strains to GL. Under the condition of post-treatment of GL, the average ID_{50} value was 0.71 mM. On the other hand, no reduction in cell viability was observed at this concentration by the trypan blue-exclusion test. The ID_{50} of GL for host cell DNA synthesis was 21.3 mM. Thus, GL was inhibitory to VZV replication at noncytotoxic concentrations. GL was less effective when added to the cells before virus inoculation (pretreatment) conditions (Table 1). The activity of 2′,5′-oligoadenylate (2′,5′-A) synthetase (Shimizu and Sokawa, 1979) was determined in HEF cells treated with various concentrations of GL. There was marked increase of 2′,5′-A synthetase activity after the treatment with HuIFN-β (positive control), while there was no increase in 2′,5′-A synthetase activity following treatment with GL (data not shown). Furthermore, the pretreatment of the cells with GL slightly enhanced the inhibitory effect obtained with post-treatment (Table 1).

The TK^- mutant strain (Kanno-Kohmura), which is resistant to ACV and BVDU, proved equally sensitive to GL as the TK^+ strains (Table 1).

Antiviral activity under several conditions

To assess the mechanism of action of GL, we examined the influence of various experimental conditions on the inhibitory effect of GL on VZV replication. Fig. 2 shows that GL had antiviral activity when the infected cells were treated with GL for only 1 h (pulse-treatment) immediately after adsorption of VZV at 25°C. However, this activity was weakened, when the pulse-treatment was done following a 1- or 2-h incubation with GL-free maintenance medium, or when virus adsorption took place at 37°C (Fig. 2). When GL was added to the infected cells at 16 h after virus adsorption, thus at a late stage of VZV replication (Yamanishi et al., 1980), VZV foci were too small to be detected with a magnifying glass. Therefore, the

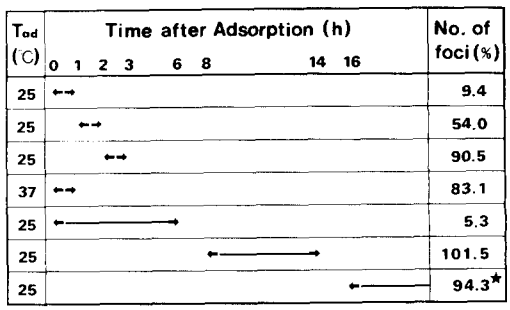

Fig. 2 Antiviral activity of GL under several conditions. Cell-free VZV (CaQu) adsorption to HEF monolayers was carried out at 25 or 37°C (Tad: adsorption temperature) for 1 h. After the virus-adsorption period, the infected monolayers were treated with GL (2.4 mM) for various periods (↔). During the other periods the monolayers were incubated with GL-free maintenance medium at 37°C. Number of foci is expressed as percent of the control not treated with GL. When the cells were exposed to GL at 16 h after virus adsorption, and then further incubated, the foci were too small to be detected under a magnifying glass. However, when the foci were stained by immunofluorescence and counted microscopically (*), no much difference was found between the control number of foci in the group which received GL from 16 h after virus adsorption.

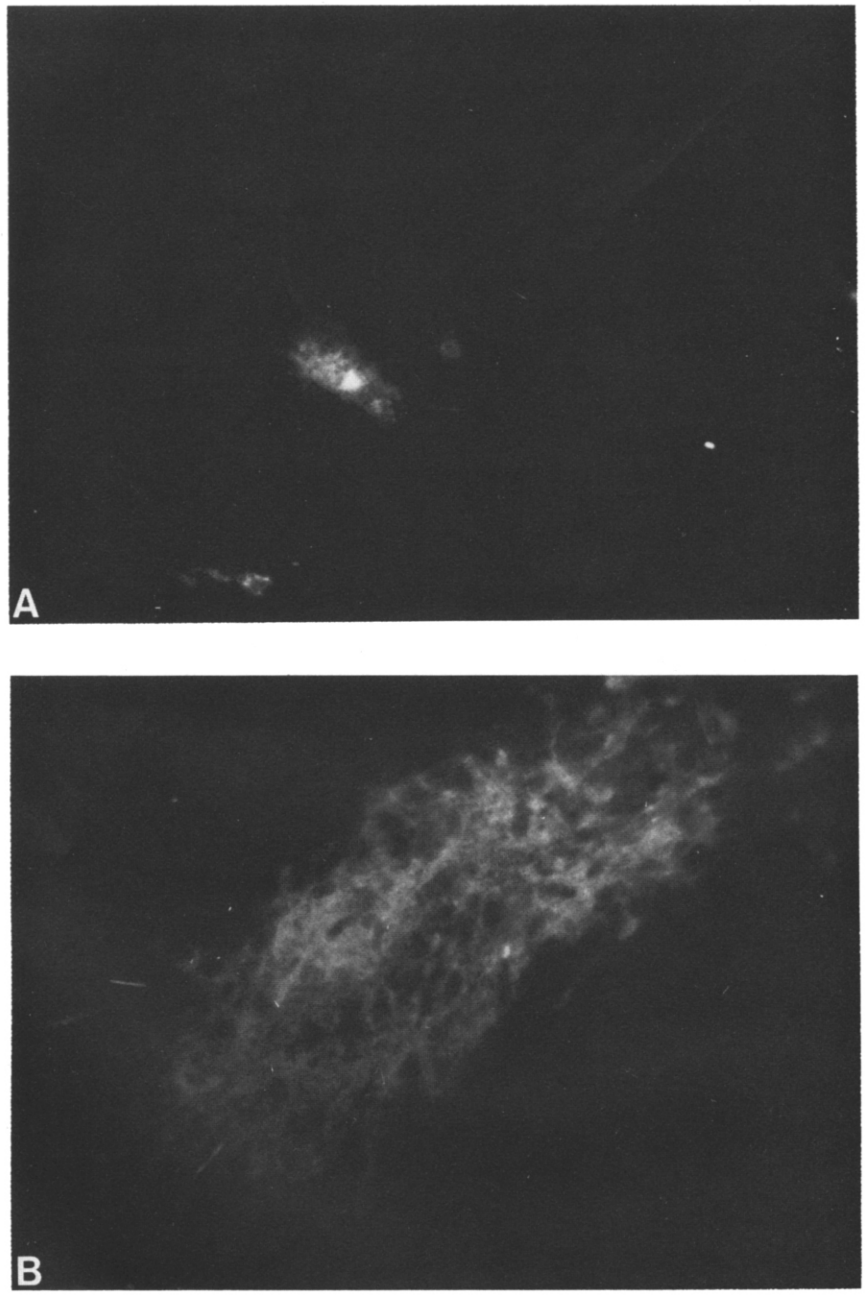

Fig. 3 Indirect immunofluorescence of VZV-infected HEF cells 72 h post-infection. (A) GL (2.4 mM) added to the monolayers at 16 h after virus adsorption. Specific viral antigen was detected in only one or a few cells. (B) Cells incubated with GL-free maintenance medium. Specific antiviral antigens were detected in many neighbouring cells.

TABLE 2

Minimum fractional inhibitory concentration (FIC) index when GL was combined with other anti-herpes drugs

Drug[a]	Minimum FIC index	
	Pretreatment of GL	Post-treatment of GL
ACV	0.80	1.02
BVDU	0.74	1.08
Ara-A	0.75	0.76
PFA	0.90	0.82
β-IFN	N.D.[b]	0.80

[a] Anti-herpes drugs were administered after VZV inoculation (post-treatment) and interferon was added 24 h before VZV inoculation (pre-treatment).

[b] Not determined.

indirect immunofluorescence method with anti-VZV human convalescent serum and FITC conjugated anti-human IgG goat serum was used. Using this method, it was evident that with GL added at 16 h post-infection, the size of the foci was considerably reduced (Fig. 3), although the number of foci did not differ significantly from that of the control (untreated, infected) cells (Fig. 2). These results suggest that GL prevented the virus from spreading from infected to uninfected neighbouring cells.

Combined effect with anti-herpes drugs or interferon

The combined effects of GL with other drugs were evaluated based on the minimum fractional inhibitory concentration (FIC) index which was obtained by the

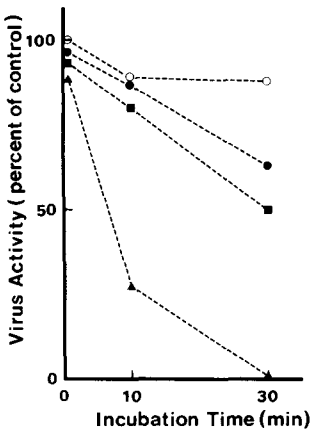

Fig. 4. Inactivation of VZV by GL. A suspension of 0.1 ml of cell-free VZV (CaQu) containing approximately 5×10^3 PFU was mixed with 0.9 ml of various concentrations of GL (final concentrations: ▲, 2.4 mM; ■, 0.48 mM; ●, 0.096 mM; ○, control) in test tubes and incubated at 37°C. At 10 and 30 min after incubation, 0.1 ml of each mixture was diluted with 0.9 ml maintenance medium and then titrated.

isobologram method previously described (Baba et al., 1984). As a rule, the combination is additive, when the minimum FIC index is equal to 1.0. When the combination results in synergy, the minimum FIC index is less than 1.0; when the combination results in antagonistic activity, it is more than 1.0. Table 2 shows that all combinations resulted in an additive to slight synergistic action against VZV replication.

Inactivation of VZV

In addition, GL also had a direct inactivating effect on VZV. Fig. 4 indicated that more than 99% of the virus had lost its activity after incubation with 2.4 mM GL for 30 min at 37°C. This effect was dose-dependent, and even a 0.096 mM of GL was still effective against VZV.

Discussion

It has been reported that some natural compounds have anti-herpesvirus activity (Yamazaki and Tagaya, 1980). One such compound, GL, inhibited VZV replication at an ID_{50} of 0.71 mM. This ID_{50} is higher than that of other anti-herpes drugs such as ACV (20.6 μM) (Shigeta et al., 1983). However, the ID_{50} of GL for host cell DNA synthesis was 21.3 mM, so that its selectivity index (ratio of ID_{50} for host cell DNA synthesis to average ID_{50} for VZV strains) could be estimated at 30.0, which is comparable to the selectivity index of ACV (38.8) and 10 times higher than the selectivity index of Ara-A (3.1) (Shigeta et al., 1983). Unlike ACV or Ara-A, GL was also effective when added to the cell cultures before virus infection, and, in addition, GL also had an inactivating effect on VZV. The mechanism of action of GL upon pretreatment of the cells is unclear, but does not appear to be related to the induction of interferon in HEF cells. Furthermore, our preliminary experiments have indicated that the adsorption of VZV particles is not inhibited by the pretreatment of the cells with GL (data not shown). Following post-treatment, GL was effective even when the cells were treated with GL for only 1 h immediately after virus adsorption at 25°C. This effect was weakened when this 1-h treatment of GL was done after 1- or 2-h incubation with GL-free maintenance medium or when the virus adsorption took place at 37°C (Fig. 2). These results suggest that GL may inhibit virus replication during a very early stage of the replicative cycle, i.e. penetration or uncoating of virion.

GL was also effective when added 16 h post-infection (Figs. 2,3). Yamanishi et al.(1980) have reported that a single cycle of virus growth would require 8 to 14 h and that after 18 h progeny virus may start to spread to neighbouring cells. Thus, when added 16 h post-infection GL may inhibit virus spread from infected cells to uninfected cells.

GL is known as an interferon-inducing substance in vivo (Abe et al., 1982), and its in vivo effects may be at least partly mediated by interferon production. The present findings suggest, however, that its in vitro antiviral activity may be based on a direct interaction of the compound with a very early stage of the virus rep-

licative cycle, the exact nature of which remains to be elucidated. Being a polycarboxylic substance, GL may share some common features in its mechanism of action with other polycarboxylates such as polyacrylic acid and polymethacrylic acid (De Clercq, 1973; De Somer et al., 1968a,b).

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