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Studies on mechanism of action of glycyrrhizin against hepatitis A virus replication in vitro

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Summary

Glycyrrhizin (GL) achieved a concentration-dependent inhibition of the replication of hepatitis A virus (HAV) in PLC/PRF/5 cells. GL has been shown to inhibit an early stage of the HAV replication. GL was not virucidal and had no measurable effect on the adsorption of [^{3}H]uridine-labelled virions to cells. GL inhibited HAV penetration of the plasma membrane as measured by the amount of infective virus no longer neutralizable by specific antibody over time.

Hepatitis A virus; Glycyrrhizin; Virus penetration

Introduction

Hepatitis A virus (HAV) is an hepatotropic picornavirus classified within the genus Hepatovirus (Francki et al., 1991). Infection with HAV remains a worldwide health problem. In the United States at least 25 000 cases of hepatitis A are reported annually (Gust, 1988). In many developing nations, improved hygiene has increased the population of susceptible adults. Thus, local outbreaks can cause extensive disease, such as the 1988 epidemic in Shangai that resulted in 300 000 cases (Halliday et al., 1991). Killed vaccines are undergoing clinical trials and one of them is now commercially distributed (André et al., 1992). However HAV growth in tissue culture is considerably lower than this obtained with most other picornaviruses and production of

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vaccine quantities of antigen is difficult and expensive. Recent research efforts have focused on the development of effective antiviral compounds (Widell et al., 1986; Biziagos et al., 1987; Superti et al., 1987; Passagot et al., 1988; Superti et al., 1989; Crance et al., 1990; Biziagos et al., 1990; Girond et al., 1991; and Divizia et al., 1992).

Among the antiviral substances screened in our laboratory (Biziagos et al., 1987; Passagot et al., 1988; Crance et al., 1990; Girond et al., 1991), glycyrrhizin (GL) has proved to be an attractive candidate as anti-HAV drug. This compound exhibited potent antiviral activity against HAV replication at concentrations which had no measurable effect on the viability or the protein synthesis of PLC/PRF/5 cells (Crance et al., 1990). GL is the aqueous extract of licorice root, which consists of one molecule of glycyrrhetinic acid and two molecules of glucuronic acid. This drug is known for its various biological activities, such as anti-inflammatory activity (Finney et al., 1958), antitumorigenic activity (Agarwal et al., 1991), activity against human immunodeficiency virus type 1 (HIV-1) (Ito et al., 1987) and several other viruses (Pompei et al., 1979). Moreover, GL has already been used for its therapeutic and prophylactic effects on chronic viral hepatitis (Fujisawa et al., 1980).

The aim of the present work was to study inhibitory effects of GL on HAV replication in vitro under different experimental conditions in order to elucidate its mechanism of action.

Materials and Methods

Antiviral compound. GL was purchased from Sigma (St. Louis, MO). It was dissolved in RPMI 1640 medium and adjusted to pH 7.0 with ammonia/water.

Cell culture. Human hepatoma cell line PLC/PRF/5 (obtained from the American Type Culture Collection, Rockville, MD) was grown at 37°C in 5% CO_2 in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and containing 100 IU/ml of penicillin and 100 μg/ml of streptomycin. HAV-infected cells were maintained, at 32°C, in RPMI 1640 medium without FCS.

Virus. The CF53 strain of HAV was isolated in our laboratory and adapted to PLC/PRF/5 cells by serial passage (Crance et al., 1985). The virus was passaged 26 times in these cells before use. After 12 days of incubation at 32°C, virus-infected cells were frozen and thawed four times and the virus suspension was clarified by centrifugation at $10\,000 \times g$ for 20 min at 4°C. This virus pool contained $10^{7.0}$ 50% tissue culture infective doses ($TCID_{50}$s) per ml.

Antiviral assays and cytotoxicity evaluation. For determination of antiviral activity, confluent monolayers in 24-well tissue culture plates were infected at

the appropriate multiplicity of infection (MOI) in the presence of various concentrations of GL (4 wells/concentration). For each panel quadruplicate wells were used as virus controls and cell controls. The infected cells were incubated at 32°C for 12 days (multiple rounds of replication). Then the virus was extracted by four cycles of freezing and thawing and antiviral activity was determined by the inhibition of viral antigen expression and by the reduction of virus yield. Viral antigen expression was evaluated by solid-phase radioimmunoassay (RIA) endpoint titration (Crance et al., 1987). The drug concentration required to reduce viral antigen expression by 50% in comparison to the control was designated as 50% effective dose (ED_{50}). The infectious virus titer was determined by titration in 48-well tissue culture plates (Crance et al., 1990).

Cytotoxicity measurements were achieved by determining the inhibition of trypan-blue exclusion, as described previously (Crance et al., 1990). The 50% cytotoxic dose (CD_{50}) was defined as the concentration required to reduce cell viability by 50%.

Virus growth inhibition. GL was examined for its effect on the growth of HAV in PLC/PRF/5 cells (single-cycle conditions). Confluent cells in 48-well tissue culture plate were washed and inoculated at a MOI of 1.0. After 60 min of adsorption at 4°C, all wells were washed twice with maintenance medium. Twenty wells received 1 ml of maintenance medium and 20 other wells received 1 ml of maintenance medium with 2000 μg/ml of GL. After incubation at 32°C for 0, 2, 5, 10, 24, 36, 48, 72, 96 or 120 h, two wells per group were harvested and stored frozen at −80°C. Finally, all samples were frozen and thawed four times and the homogenates were used for titration of virus infectivity in cell culture.

Virus inactivation. The putative virucidal effect of GL was tested by incubating the virus ($10^{6.0}$ $TCID_{50}$s/ml) in culture medium (controls) or in culture medium containing GL at 4000 μg/ml for 4 h at 32°C. Then the virus suspension was diluted 100 times and its infectious titer was determined in cell culture.

Effect of time of addition or removal of GL on virus yield from single-round replication. The effect of time of addition or removal of GL (2000 μg/ml) on the yield of HAV was studied at a MOI of 1.0 for a single cycle of virus replication (96 h) by performing four types of experiments: (1) cell pretreatment with drug which was removed just before infection, (2) cell pretreatment followed by incubation in the presence of GL, (3) drug addition at the time of infection, with drug removal at different times post-infection, then incubation in drug-free maintenance medium, (4) drug addition at different times post-infection and cell incubation in the presence of the drug. All tests were run in triplicate. At 96 h post-infection, cells were washed and HAV was extracted by freezing and thawing and the HAV infectious titer was determined in cell

culture.

Virus adsorption. Virus adsorption was measured with [^{3}H]uridine-labelled HAV. For the preparation of [^{3}H]uridine-labelled virus, the following procedure was used. Confluent PLC/PRF/5 cell monolayers (in 225 cm^2 bottles; Costar) were infected with HAV (MOI = 1.0). After 60 min of adsorption at 4°C, the viral inoculum was removed and 20 ml of maintenance medium containing 50 μCi/ml of [5,6-^{3}H]uridine (46 Ci/mmol) were added. At 96 h post-infection, cells were extensively washed with maintenance medium and the virus was extracted by four cycles of freezing and thawing. The extract was centrifuged at 4°C at 20 000 × *g* for 15 min to remove cell debris. The supernatant was centrifuged at 100 000 × *g* for 3 h in an SW27 rotor (Beckman). The pellet was suspended in PBS and the virus suspension was centrifuged for 20 h at 100 000 × *g* at 4°C through a 20% sucrose cushion in an SW27 rotor. The pellet was suspended in PBS and layered onto a preformed linear cesium chloride gradient (1.17 to 1.45 g/cm^3) and centrifuged for 20 h at 200 000 × *g* at 4°C in a type 70.1 Ti rotor. Fractions were collected and assayed for their radioactivity and for HAV antigen by RIA. Fractions corresponding to the 1.33 g/cm^3 HAV peak were pooled and desalted by ultrafiltration (Centricon 30, Amicon). To carry out adsorption experiments, confluent PLC/PRF/5 cells in 24 well tissue culture plates were incubated at 4°C with 0.1 ml of purified [^{3}H]uridine-labelled virus (10 000 cpm per well, MOI = 1.0) in the presence or absence of GL (2000 μg/ml). At different times post-infection, cells were washed four times with ice-cold PBS and lysed in 0.5 ml of 2% sodium dodecyl sulfate to solubilize the cells for radioactivity determinations.

All experiments were performed in triplicate.

Virus penetration studies. Virus penetration was studied using previously published procedures (Eggers, 1977; Kenny et al., 1988) with some modifications. Confluent PLC/PRF/5 cell monolayers in 24 well tissue culture plates were incubated with HAV (MOI = 1.0) for 60 min at 4°C. Then the infected cells were washed two times and cell cultures, except the 0 time set (see Table 2), were incubated at 32°C in the presence or absence of GL (2000 μg/ml). At various times post-infection (0, 1, 3, 5 or 7 h) neutralizing immunoglobulin (human immunoglobulin from an high anti-HAV neutralizing titer serum, CTSA, Paris, France) was added. The treatment with neutralizing antiserum (used at a 10-fold dilution in maintenance medium) included an initial 30-min incubation at 4°C followed by an additional incubation for 30 min at room temperature. The same procedure was followed for the 0 time cultures. Monolayers not treated with antiserum were used as controls. The cells were then washed four times with maintenance medium and incubated at 32°C until 96 h post-infection. The virus was extracted by four cycles of freezing and thawing and the infectious titer was determined in cell culture (Crance et al., 1990). Two independent experiments were achieved. Each experiment was performed in duplicate.

Statistical analysis. Statistical analysis of the data was carried out using Student's *t*-test.

Results

Inhibitory effect of GL on HAV antigen expression

Inhibition of HAV antigen expression, measured 12 days after infection, at three MOIs (1.0, 0.1 and 0.01 $TCID_{50}$/cell) in the presence of various non-toxic concentrations of GL, is shown in Fig. 1. For each MOI tested, a concentration-dependent inhibition of expression of HAV antigen in PLC/PRF/5 cells was observed. The inhibitory effect of GL was also dependent on the MOI. As the MOI was decreased, the antiviral effect of the drug increased. The concentrations required to inhibit HAV antigen expression by 50% (ED_{50}) were 267, 100 and 26 μg/ml at a MOI of 1.0, 0.1 and 0.01, respectively. The CD_{50} value of GL for cellular viability was 4283 μg/ml. The selectivity indices, calculated as CD_{50}/ED_{50} ratios were 16, 43 and 165 at a MOI of 1.0, 0.1 and 0.01, respectively. On the other hand, the effect of the compound on RNA synthesis of non-infected cells was examined after 12 days of treatment at 32°C using previously published procedure (Girond et al., 1991). Incorporation of [^{3}H]uridine in trichloracetic acid insoluble fraction of cells was not inhibited up

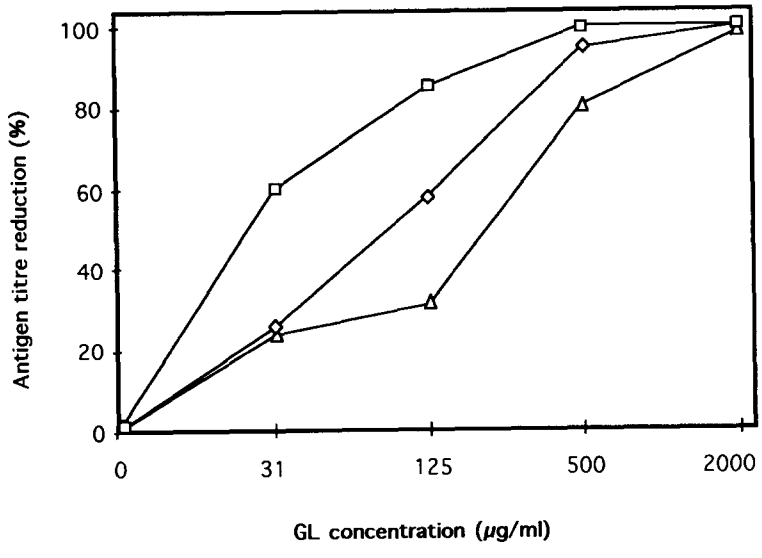

Fig. 1. Inhibitory effect of GL on the expression of HAV antigen in PLC/PRF/5 cells at three multiplicities of infection. □, 0.01 $TCID_{50}$/cell; ◇, 0.1 $TCID_{50}$/cell; △, 1.0 $TCID_{50}$/cell. PLC/PRF/5 cells were infected with HAV at the appropriate MOI in the presence of GL and further incubated at 32°C for 12 days. On day 12, antiviral activity was evaluated by the determination of the HAV antigen titer by RIA endpoint titration. Toxicity was evaluated by trypan-blue staining. All of the concentrations used were non-toxic (*t*-test; $P > 0.05$). Means of quadruplicate experiments.

to 4000 μg/ml (data not shown). These results indicate that GL proved to be highly selective in its antiviral action.

Inhibitory effect of GL on HAV infectivity

GL was also examined for its inhibitory effect on HAV infectivity at three different MOIs (1.0, 0.1, 0.01 $TCID_{50}$/cell). The infected cells were incubated in the presence of various concentrations of GL for 12 days (multiple rounds of replication). The results are shown in Fig. 2. For the three MOIs, GL caused a concentration-dependent reduction in HAV infectivity. The drug significantly reduced the infectious titer at a concentration as low as 31 μg/ml at the MOI of 0.01. At 2000 μg/ml of GL (the highest non-toxic concentration of GL used in this study) the viral titer reduction was 1.9, 2.3 and 3.0 $\log_{10}$ at the MOI of 1.0, 0.1 and 0.01, respectively.

Inhibitory effect of GL on virus growth

GL was examined for its inhibitory effect on the growth of HAV in PLC/PRF/5 cells for a single round of replication. At the concentration of 2000 μg/ml drug inhibited the growth of HAV in cells, as measured at 48, 72, 96 and 120 h after infection (Fig. 3). After 96 h, i.e., one step growth cycle, GL reduced the viral replication by about 98%.

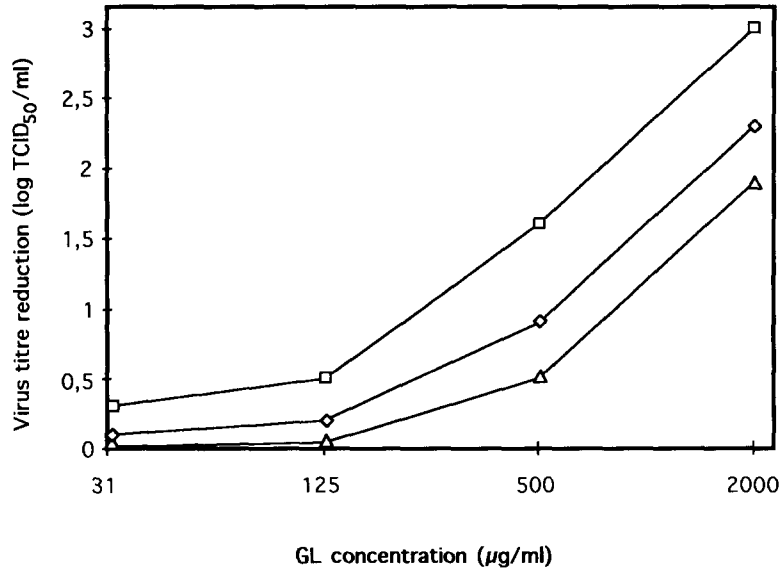

Fig. 2. Inhibitory effect of GL on HAV infectivity in PLC/PRF/5 cells at three multiplicities of infection. □, 0.01 $TCID_{50}$/cell; ◇, 0.1 $TCID_{50}$/cell; △, 1.0 $TCID_{50}$/cell. PLC/PRF/5 cells were infected with HAV at the appropriate MOI in the presence of GL and further incubated at 32°C for 12 days. On day 12, antiviral activity was evaluated by the determination of the infectious titer in cell culture. Means of quadruplate experiments.

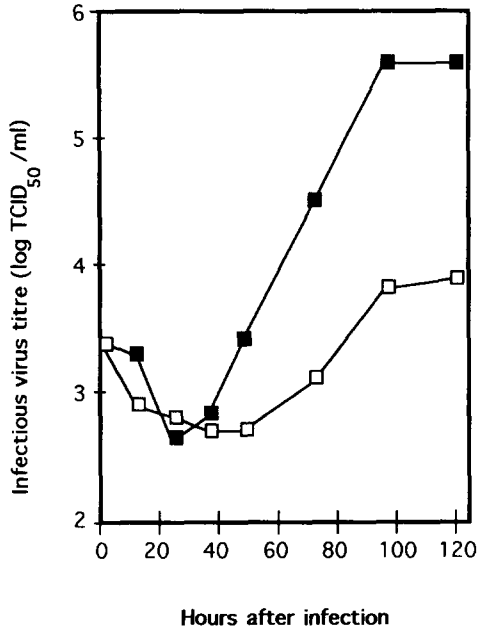

Fig. 3. Inhibitory effect of GL on growth of HAV in PLC/PRF/5 cells. ■, control (no drug); □, 2000 μg/ml GL. PLC/PRF/5 cells were infected with HAV at a MOI of 1.0. After 60 min of adsorption at 4°C, cells were washed and incubated at 32°C in the presence or absence of GL (2000 μg/ml). At indicated times cells were harvested and the infectious titer was evaluated in cell culture. Means of duplicate experiments.

Virucidal test

GL did not significantly reduce the virus infectivity titer of HAV after 4 h incubation at 32°C. The titer of the virus suspension incubated with or without drug was $10^{6.0}$ $TCID_{50}$/ml. The dose used (4000 μg/ml) was about 15 times the inhibitory concentration in cell culture at the MOI of 1.0. These results indicate that the antiviral effect of GL cannot be attributed to a direct inactivation of the virus.

Effect of time addition or removal of GL on virus yield

The effect of time of addition of 2000 μg/ml of GL on HAV replication is shown in Table 1.

Pretreatment of cells before virus inoculation induced a weak but significant decrease (t-test; $P=0.05$) in infectious virus titer (0.4 $\log_{10}$).

When the drug was added at the time of HAV inoculation and removed at 5 h after adsorption, its antiviral activity was completely reversible; no significant reduction of virus titer (t-test; $P>0.05$) was observed. A 8 h treatment caused only a weak effect (0.4 $\log_{10}$) on the infectivity of HAV compared with a treatment for 96 h (1.6 $\log_{10}$).

The greatest inhibition of HAV replication (2.1 $\log_{10}$) was observed when GL was added 24 h before virus inoculation and incubated with cells for a

TABLE 1

Effect of time of addition and removal of GL on HAV infectivity[a]

Duration of the treatment (h)[b]	Infectious HAV titer (10^2 $TCID_{50}$s/ml)[c]	Virus titer reduction ($log_{10}TCID_{50}$/ml)
Pretreatment[d]		
(−24 → 0)	460 ± 257	0.4
Treatment[e]		
(0 → + 5)	1688 ± 676	−0.2
(0 → + 8)	488 ± 215	0.4
(0 → +24)	141 ± 60	0.9
(0 → +96)	28 ± 11	1.6
Pretreatment + Treatment[f]		
(−24 → +96)	10 ± 3	2.1
Post-treatment[g]		
(+ 0 → +96)	29 ± 7	1.6
(+ 2 → +96)	143 ± 47	0.9
(+ 4 → +96)	294 ± 57	0.6
(+ 5 → +96)	707 ± 284	0.2
(+24 → +96)	1345 ± 97	−0.1
Control[h]	1145 ± 197	–

[a]PLC/PRF/5 cells were infected at a MOI of 1.0. The titers of infectious virus in cell samples were determined at 96 h after viral inoculation.
[b]GL, at the concentration of 2000 μg/ml, was added before (−), after (+), at the same time of the inoculation of the virus (0) or immediately after the adsorption of the virus (+0).
[c]Values are means ± S.D. of triplicate experiments.
[d]Cells were pretreated with drug for 24 h at 32°C. Then the cells were washed, infected and incubated in drug-free maintenance medium for 96 h.
[e]Cells were infected and simultaneously treated with GL for the indicated time. Then the cells were washed and incubated in drug-free maintenance medium until 96 h post-infection.
[f]Cells were pretreated ([d]) and treated ([e]) with drug.
[g]GL was added at various times after the adsorption, and the infected cells were incubated with the drug until 96 h post-infection.
[h]Virus-infected, non-drug-treated cells.

single cycle of replication (pretreatment + treatment).

When GL was added immediately after HAV adsorption (post-treatment), the virus titer was reduced by 1.6 log_{10}. The antiviral activity was weakened when GL was added at 2 h or 4 h after adsorption; the reduction of the virus titer was only 0.9 and 0.6 log_{10}, respectively. The inhibitory effect of GL on HAV replication was completely lost when the drug was added at 5 h post-infection or there after. No significant difference (t-test; $P > 0.05$) was observed between the values obtained in the control and GL assays.

Effect of GL on adsorption of HAV

The kinetics of adsorption of [^{3}H]uridine-labelled HAV to PLC/PRF/5 cells

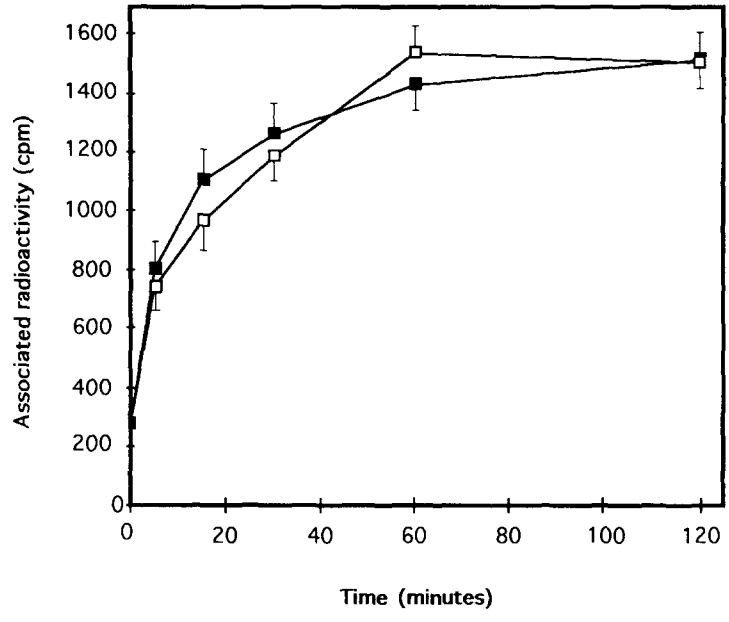

Fig. 4. Adsorption of HAV to PLC/PRF/5 cells in the presence or absence of GL. ■, without GL; □, with GL (2000 μg/ml). Cells were incubated with [^{3}H]uridine-labelled virus at a MOI of 1.0 $TCID_{50}$ per cell in the presence or absence of GL (2000 μg/ml) for up to 120 min at 4°C. At indicated times, cells were washed four times and solubilized before counting radioactivity. Experiments were performed in triplicate. Standard deviations are shown in the figure.

in the presence or absence of GL are shown in Fig. 4. The amount of radioactive HAV associated with cells as a function of time was not reduced by GL (t-test; $P > 0.05$), even at a high concentration (2000 μg/ml) of the compound. Therefore, GL has no detectable inhibitory effect on virus adsorption.

Effect of GL on penetration of HAV

Results of the effect of 2000 μg/ml of GL on penetration of HAV into PLC/PRF/5 cells are shown in Table 2 and Fig. 5. Penetration was measured by the amount of infective virus that was no longer neutralizable by specific antibody after adsorption to the cell surface.

Immediately after adsorption at 4°C (time 0) antibody reduced the infectious titer by about 1 $\log_{10}$ in both the untreated and drug-treated cells. This indicates that only about 10% of the virus was not accessible to neutralization by specific antibody and had penetrated the cell.

After the temperature shift to 32°C, increasing fractions of virus penetrated the untreated infected cells. After 7 h incubation at 32°C, about 70% of virus was no longer neutralizable by specific antibody.

In contrast, in the cultures treated with GL the amount of virus which had

TABLE 2

Effect of GL on HAV penetration[a]

Expt no.	Time after adsorption[c] (h)	Amount of virus penetrated ($log_{10}TCID_{50}/ml$)[b]			
		untreated cells		GL treated cells (2000 μg/ml)	
		−NA	+NA	−NA	+NA
1	0	5.2	4.2	5.2	4.5
	1	5.6	4.5	5.3	4.6
	3	5.6	5.3	5.2	4.4
	5	5.7	5.5	5.7	4.8
	7	5.4	5.3	5.2	4.4
2	0	5.3	4.1	5.0	4.0
	1	5.1	4.4	4.6	3.8
	3	5.3	5.0	5.1	4.2
	5	5.1	4.8	4.8	4.0
	7	5.2	5.0	5.0	3.8

[a]Penetration was determined by measuring the virus titer after neutralization by a specific neutralizing antiserum (NA) (1:10) at various times after adsorption, as described in Materials and Methods.

[b]Data represent the means of duplicate determinations.

[c]After 1 h of adsorption at 4°C, all cultures, except the 0 time set, were placed at 32°C. At the indicated times infected cells were exposed or not to NA for 1 h.

penetrated the cells, did not increase in the course of time of incubation at 32°C. After 7 h incubation at 32°C, about 90% of virus was still neutralized by

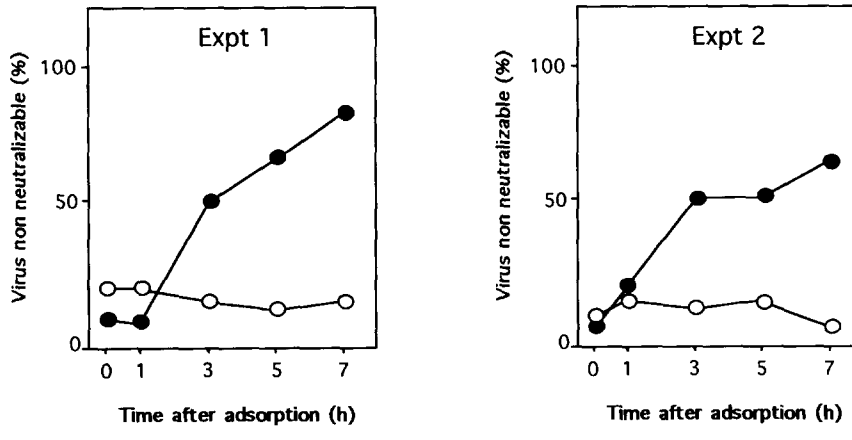

Fig. 5. Effect of GL on HAV penetration. ●, without GL; ○, with GL (2000 μg/ml). After adsorption at 4°C, cells were incubated at 32°C in the presence or not of GL (2000 μg/ml). At indicated times, infected cells were exposed to specific neutralizing antiserum (NA) as described in Materials and Methods. Penetration was determined as measuring the infectious titer after neutralization by NA. Data from two experiments are presented. Each is the average of duplicate determinations.

specific antibody. This indicates that penetration was inhibited by GL. This inhibition was approx. 90% when the titer for cultures treated with antiserum was compared with the control not treated with antiserum.

Discussion

As observed previously (Crance et al., 1990) we found that GL inhibits HAV replication in PLC/PRF/5 cells at concentrations below the cytotoxic concentration. GL exerts a concentration-dependent inhibitory effect on both virus-antigen expression and virus yield. The MOI was found to have a substantial influence on the antiviral activity: a lower virus input MOI increased antiviral activity. GL also proved to be highly selective in its antiviral action: at the MOI of 0.01 the concentration required to inhibit virus replication was only 26 μg/ml, while the cytotoxic concentration (CD_{50}) for the host cells was above 4000 μg/ml.

In order to elucidate the mechanism of inhibition, the effect of GL was studied under different conditions. We showed that the antiviral effect could not be attributed to a direct inactivation of the virus. The partial prophylactic effect of the drug indicates that it rather interacts with the host cell. Analysis of virus growth when GL was added to infected cells at various times, showed that GL acts on the early stage of the HAV replicative cycle: the inhibition process occurs during the first 4 h after infection. GL had no effect on the adsorption of HAV to PLC/PRF/5 cells, as measured by the kinetics of [^{3}H]uridine-labelled virus bound to cells in the presence or absence of drug. GL appears to block selectively the penetration of HAV into PLC/PRF/5 cells: after infection, the amount of infective virus that was no longer neutralizable by specific antibody did not increase in GL-treated cells, whereas increasing fractions of virus penetrated the untreated infected cells.

HAV, a member of the family Picornaviridae, enters susceptible cells by receptor-mediated endocytosis followed by endosomal and/or lysosomal uncoating (Superti et al., 1987). Receptor-mediated endocytosis was studied by electron microscopy in poliovirus-infected HEp-2 cells (Zeichhardt et al., 1985). Invagination of the cell membrane with attached virus commenced at coated pits often situated at the base of microvilli and led to the formation of virus-containing coated vesicles in the cytoplasm. Such mechanisms might be blocked by the biologically potent saponins like GL. Thus, saikosaponins were found to cause a significant decrease in the negative charge on the cell surface and the destruction of microvilli, suggesting an alteration of the negatively charged carbohydrate portions of the membrane (Abe et al., 1981). Saikosaponins must also interact with the lipid layer of membranes since they have been found to cause a remarkable decrease of membrane fluidity (Abe et al., 1978), which could prevent penetration of the virus into the cell.

The mechanism of inhibition of HIV-1 replication by GL in CD4+ cells has been studied (Ito et al., 1988). GL partially prevents the adsorption of HIV-1

particles to CD4+ cells. The drug inhibits also protein kinase C (PKC) activity but the precise role of PKC in the replication process of HIV-1 has not been clarified. Conflicting reports have been published on stimulation of PKC activity by the binding of HIV-1 to CD4 molecule at the surface of CD4 T cells and the role of the phosphorylation of CD4 on viral penetration (Fields et al., 1988; Orloff et al., 1991).

GL has been reported to have therapeutic and prophylactic effects on chronic active viral hepatitis (Fujisawa et al., 1980), and is widely used in Japan in the treatment of chronic viral hepatitis (Ito et al., 1987). It is generally accepted that hepatocellular injury in chronic hepatitis B virus infection is caused by immunopathologic mechanisms (Mondelli et al., 1982). Such cytotoxic mechanisms due to an immunopathological reaction of sensitized cytotoxic T lymphocytes against infected hepatocytes has also been proposed in hepatitis A (Vallbracht et al., 1989). These cytotoxic processes could be inhibited by GL which has been shown, moreover, to prevent the development of hepatic injury induced by some hepatotoxins (Shibayama, 1989) and to inhibit lysis of hepatocyte membranes induced by anti-liver cell membrane antibody (Shiki et al., 1992). These therapeutic activities and the results obtained in the present study on the inhibitory effect of GL on HAV replication in vitro suggest that GL should be further evaluated for its efficacy in the treatment of acute hepatitis A.

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