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Antiviral effect of octyl gallate against DNA and RNA viruses

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Abstract

The effects of gallic acid (3,4,5-trihydroxybenzoic acid) and its alkyl esters on virus growth and virion infectivity were examined. All the compounds tested showed an inhibitory effect on the growth of herpes simplex virus type 1 (HSV-1) in HEp-2 or Vero cells. The antiviral activity of gallic acid alkyl esters was enhanced by increasing the number of carbon in the alkyl moieties of the compounds, reaching maximum at a carbon number of 12 (lauryl gallate), but both cytocidal activity and cytopathic effect of the compounds were also significantly increased simultaneously. Among these compounds, octyl gallate showed a marked antiviral effect with a relatively moderate cytotoxity. In addition, octyl gallate suppressed the multiplication of RNA viruses, such as vesicular stomatitis virus and poliovirus. Quantitative characterization of the HSV-1 infection in the presence of octyl gallate revealed that: (1) this reagent can directly inactivate HSV-1 (virucidal activity), (2) it suppresses both the intracellar multiplication and the release of the virus, (3) it selectively accelerates death of the virus-infected cells and (4) the addition of the reagent even at 6-h post infection completely abolishes the formation of progeny virus in the infected cells.

Keywords: Octyl gallate; Gallic acid; Cell death; Herpes simplex virus; Poliovirus

1. Introduction

It is well-known that many plants produce a variety of compounds carrying characteristic biological activities, which have been used as a traditional medicine and a healthcare beverage throughout the world. These compounds often have shown an antimicrobial effect (Cowan, 1999) and have been a good source for new chemotherapeutic drugs against a variety of pathogenic microbes. One of such biological active compounds is gallic acid (3,4,5-trihydroxybenzoic acid), which is obtained by alkaline or acid hydrolysis of the tannins from nutgalls. Some of gallate derivatives have been well characterized in terms of the physiological activity and cytotoxicity (Van der Heijden et al., 1986) and have been used as antioxidant food additives with the European community codes E-310 (propyl gallate), E-311 (octyl gallate) and E-312 (lauryl gallate). In addition, both propyl and octyl gallates are recognized as quasi drugs in Japan by the Ministry of Health, Labour and Welfare of Japan.

Previously, we found that some gallate derivatives intensify beta-lactam susceptibility in methicillin-resistant and methicillin-sensitive strains of *Staphylococcus aureus* (Shibata et al., 2005). During the clinical trials of a topical application of octyl gallate for the treatment of oral bacterial infection, we accidentally found that the reagent was effective against herpes labialis as well. As to an antiviral activity of gallate derivatives, several studies have revealed that epigallocatechin-3-gallate and prodelphinidin B-2 3′-*O*-gallate have antiviral activities against a variety of viruses (Cheng et al., 2002, 2003; Lin et al., 2000; Mukoyama et al., 1991; Nakayama et al., 1993; Song et al., 2005; Weber et al., 2003), but there are few reports about the antiviral activity of *n*-alkyl esters of gallic acid. Earlier studies on the activity of methyl gallate indicated that it inhibits a plaque formation of herpes simplex virus type 1 (HSV-1) or type 2, but is ineffective against RNA viruses, such as vesicular stomatitis virus (VSV) and influenza virus (Kane et al., 1988). In this report, we characterized the antiviral

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activity of gallic acid and its alkyl-derivatives, in particular the octyl-derivative.

2. Materials and methods

2.1. Cells and viruses

HEp-2 and Vero cells were grown in Eagle's minimum essential medium (MEM) containing 10% newborn calf serum. Herpes simplex virus type 1, strain F (HSV-1), poliovirus type 1, Sabin strain and vesicular stomatitis virus, New Jersey strain (VSV), were used throughout the experiments. These viruses were propagated in Vero cells in MEM supplemented with 0.5% fetal bovine serum and stored at −80 °C until use. The amount of virus was measured by a plaque assay on Vero cells as described previously (Koyama and Uchida, 1989; Koyama, 1995; Koyama et al., 2001).

2.2. Reagents

Gallic acid, methyl gallate, ethyl gallate, propyl gallate and lauryl gallate were obtained from Wako Pure Chemicals; butyl gallate, octyl gallate, cetyl gallate, stearyl gallate, isoamyl gallate and isobutyl gallate were from Tokyo Kasei; hexyl gallate was from APIN Chemicals Ltd. Commercially unavailable alkyl gallates were synthesized as described in the reference (Shibata et al., 2005). Stock solution (2.0 mg/ml) of gallic acid or its derivatives was prepared by dissolving the reagent in dimethylsulfoxide (DMSO), followed by a filtration through Millipore DIMEX membrane (pore size 0.22 μm) and was stored in the dark at room temperature until use.

2.3. Direct virucidal activity of octyl gallate

A series of virus preparations (10^5 plaque-forming units (PFU) of the virus in 900 μl) in Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS) containing 1% calf serum received 100 μl octyl gallate solution at various concentrations and were incubated at the indicated temperature for 10 min. The viruses were diluted with ice-cold PBS containing 1% calf serum and the number of infectious virus in each preparation was measured by a plaque assay.

2.4. Virus yields in the presence of gallic acid or its alkyl esters

To examine the effect of the respective reagent on the virus growth, monolayered cells in 35-mm dishes were infected with the virus at an indicated multiplicity of infection (MOI). The infected cells were further incubated at 37 °C for the indicated period in the serum-free MEM containing 0.1% bovine serum albumin (BSA) and the indicated concentrations of the respective reagent. At the indicated time, the amounts of total progeny virus in the infected cultures were determined as described previously (Koyama and Uchida, 1994). Briefly, after two or three cycles of freezing and thawing of the infected cells along with the culture media, the number of infectious virus in the lysate was measured by a plaque assay on Vero cells. For the determination of the amount of the virus released from the infected cells to the medium (a cell-free virus), the culture fluid was harvested before freezing and thawing.

2.5. Determination of cytopathic effects or cell death

Monolayers of HEp-2 cells were mock-infected or infected with HSV-1 at the indicated MOI and the infected cells were incubated in the serum-free MEM containing 0.1% BSA and the indicated concentrations of the reagent. The cytopathic effect (CPE) was determined by a microscopic observation of the cells; approximate amounts of rounded cells on monolayers were estimated under phase-contrast microscope.

To determine the extents of cell death in the cultures, monolayerd cells were trypsinized to obtain single cell suspension. After the addition of MEM containing 10% calf serum to the suspension to stabilize the cells, the numbers of the living and dead cells were determined, respectively, by dye-exclusion method with trypan blue.

3. Results

3.1. Antiviral activity of alkyl gallates

Fig. 1 shows the effects of gallic acid and some of its alkyl esters on the relative virus yield of HSV-1 in HEp-2 cells, when the infected cells were incubated in the medium containing various concentrations of the reagents. While even gallic acid showed some inhibitory effects at higher concentrations, all the alkyl esters of gallic acid so far examined showed higher antiviral activities than gallic acid at any concentrations tested. At the plateau, the virus yields were 10,000-fold lower in the presence of alkyl gallates than in their absence. The antiviral effects of

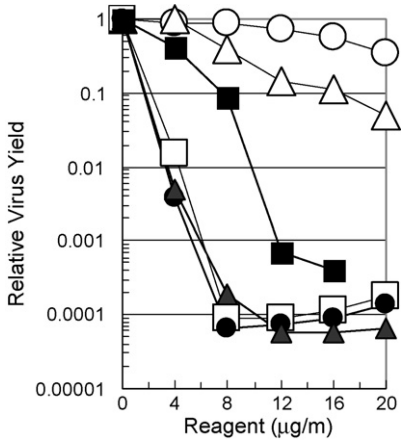

Fig. 1. Effect of gallic acid and its derivatives on the yield of HSV-1 in HEp-2 cells. Confluent monolayers of HEp-2 cells were infected with HSV-1 at an MOI of 14. The infected cells were incubated at 37 °C for about 24 h in MEM containing 0.1% BSA and the indicated concentrations of the respective reagent. The amounts of total progeny viruses were determined as described in Section 2. The virus yield in the absence of the reagent was 6.0×10^8 PFU/ml on an average. (◯) Gallic acid, (△) butyl gallate, (□) octyl gallate, (●) lauryl gallate, (▲) cetyl gallate, (■) stearyl gallate.

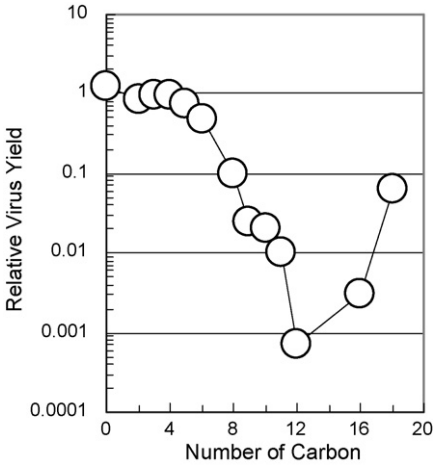

Fig. 2. Effect of the number of carbon in the alkyl group of gallic acid derivatives on the yield of HSV-1 in HEp-2 cells. Confluent monolayers of HEp-2 cells were infected with HSV-1 at an MOI of 14. The infected cells were incubated at 37 °C for about 24 h in MEM containing 0.1% BSA and 15 μM of gallic acid or its alkyl esters (ethyl, propyl, butyl, pentyl, hexyl, octyl, nonyl, decyl, undecyl, lauryl, cetyl and stearyl ester, respectively). The amount of total progeny viruses was determined and was normalized to the virus yield in the absence of any reagent. The virus yield in the absence of the reagent was 5.3×10^8 PFU/ml.

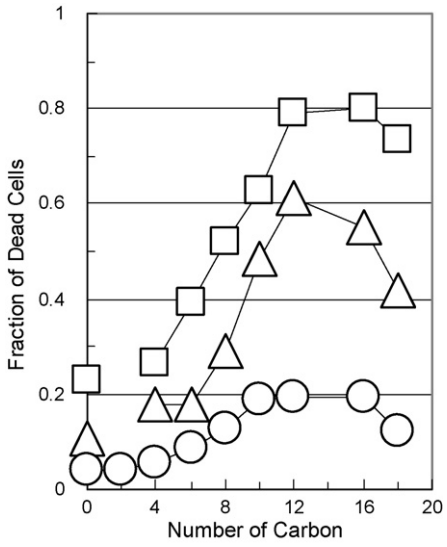

Fig. 3. Effect of the number of carbon in the alkyl group of gallic acid derivatives on the viability of HEp-2 cells. Confluent monolayers of HEp-2 cells were incubated at 37 °C for 24 h (◯), 36 h (△) or 60 h (□) in the MEM containing 0.1% BSA and 15 μM of gallic acid or its alkyl esters as described in the legend to Fig. 2. Then, the treated cells were trypsinized to obtain a single cell suspension and the amounts of the live and the dead cells in each culture were determined, respectively, by a dye-exclusion test with trypan blue.

these compounds increased as the number of carbon in their alkyl moieties increased, reaching maximum antiviral activity at carbon number of 8 (octyl gallate). The carbon number of 8, 12 (lauryl) and 16 (cetyl) of gallate esters showed a nearly identical antiviral activity under these conditions, but stearyl gallate (carbon number of 18) showed a markedly reduced antiviral activity (Fig. 1). The similar results were obtained when Vero cells, instead of HEp-2 cells, were used (data not shown).

The relation between the antiviral activity and the number of carbon in the alkyl group of these compounds was examined by measuring the virus yields in the presence of the reagents at the same molar concentration (15 μM). As shown in Fig. 2, a significant decrease in the progeny virus yields at this concentration was observed for pentyl gallate (five carbons in the alkyl group, i.e., [$n = 5$]). The virus yields steeply decreased as the number of carbon further increased, reaching a maximum at the number of 12 (lauryl gallate). The antiviral activities of the compounds with the alkyl chain longer than 12 (cetyl gallate [$n = 16$] and stearyl gallate [$n = 18$], respectively) were weaker than the activity of lauryl gallate, probably due to the lower solubility and hence lower bioavailability in the aqueous medium as suggested by Savi et al. (2005).

3.2. Cytotoxity of alkyl gallate

In addition to the antiviral activity, these compounds were also observed to induce significant cytopathic effect on HEp-2 cells regardless of whether or not the cells were infected with the virus. Both rounding and shrinkage of the cells as well as detachment from the dish were observed. Apparently, with the increasing antiviral effect, the CPE on the infected cells by the reagents was also enhanced. To examine the relation between the cytocidal activity and the number of carbon in the alkyl group of these reagents, the number of dead cells after the incubation of HEp-2 cells with each reagent for 24, 36 or 60 h was assayed by dye-exclusion test with trypan blue. As shown in Fig. 3, (1) the fractions of dead cells increased with time regardless of the presence or the absence of the reagent, probably because of the lack of serum in the incubation medium, and (2) the number of dead cells as well as the increase in the number with time were more prominent in the presence of the reagent with higher antiviral activity. Although it is not clear whether the cells suffering from CPE still keep the ability to exclude the dye or how long these cells can survive under these conditions, the results in Fig. 3 indicate that the cytotoxity of the reagents was more evident for the reagents with higher antiviral activity. Among these gallate derivatives tested, a particular alkyl-derivative, octyl gallate, turned out to be promising, since it showed a marked antiviral effect (Figs. 1 and 2) with relatively moderate cell death at 24- or 36-h incubation with the reagent (Fig. 3) and moderate CPE on the uninfected HEp-2 cells (data not shown). Thus, the following study focused on this compound.

3.3. Multiplication of HSV-1 in the presence of octyl gallate

Fig. 4 shows a one-step growth curve of HSV-1 in the presence or absence of 15 μM octyl gallate. In the presence of the reagent, the onset of infectious progeny virus production was delayed and the virus yield was suppressed. The most marked effect of the reagent was observed on the production of cell-free virus (Fig. 4, solid triangle); there observed three-fold reduction in the yield of the cell-free virus compared to that of the total virus in the reagent-treated culture.

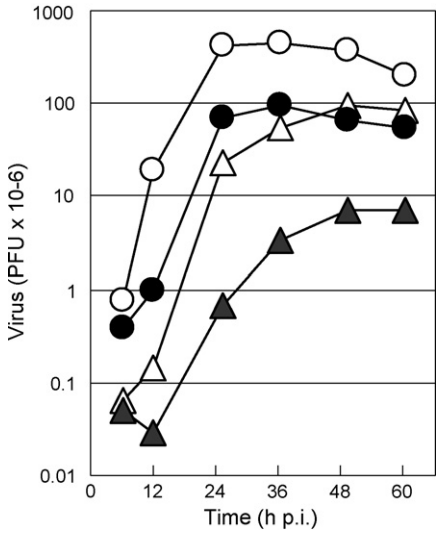

Fig. 4. One-step growth curve of HSV-1 in the presence or absence of octyl gallate. Confluent monolayers of HEp-2 cells were infected with HSV-1 at an MOI of 14. The infected cells were incubated at 37 °C in MEM containing 0.1% BSA (◯, △) or in the medium additionally containing 15 μM octyl gallate (●, ▲). At the indicated time, the amounts of total progeny (◯, ●) and cell-free (△, ▲) viruses were determined separately.

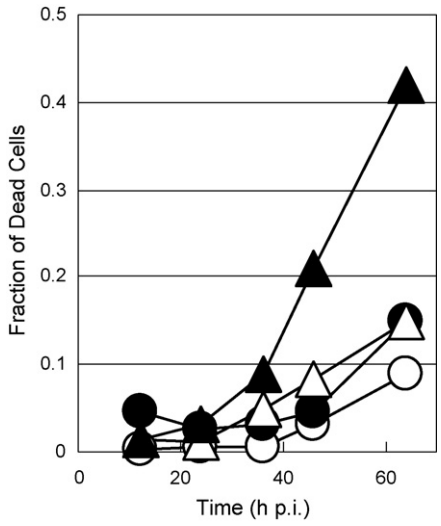

Fig. 6. Effect of octyl gallate on the HSV-induced cell death. Confluent monolayers of HEp-2 cells were mock-infected (◯, △) or infected with HSV-1 at an MOI of 14 (●, ▲). The infected cells were incubated in MEM containing 0.1% BSA (◯, ●) or in the medium additionally containing 15 μM octyl gallate (△, ▲) at 37 °C. At the indicated time, the numbers of dead and living cells were determined by dye-exclusion method with trypan blue.

In addition, probably because of the action of viral antiapoptotic genes (Leopardi and Roizman, 1996; Koyama and Miwa, 1997), HSV-1 usually does not induce recognizable CPE in HEp-2 cells during the productive replication, but induces the CPE in the very late stage of the infection. However, we observed that the virus-induced CPE was significantly accelerated and enhanced in the presence of the reagent. Although morphological studies are often subjective and less accurate, we compared the degrees of the CPE of the mock- or HSV-1-infected cells at 36 and 46 h p.i., respectively, in the presence or absence of octyl gallate (Fig. 5). In the presence of the reagent, the infected cells showed the most marked CPE at both time points while even the mock-infected HEp-2 cells induced significantly enhanced CPE in the presence of the reagent, particularly by a prolonged incubation.

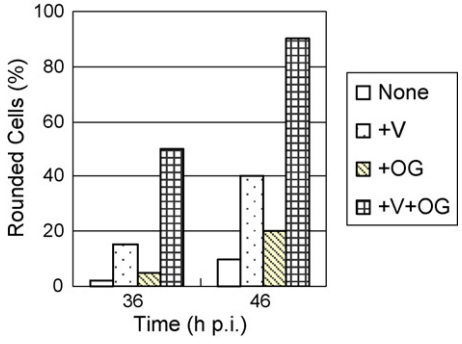

Fig. 5. Effect of octyl gallate on the HSV-1-induced cytopathic effect. Confluent monolayers of HEp-2 cells were mock-infected or infected with HSV-1 at an MOI of 14. The infected cells were incubated in MEM containing 0.1% BSA or in the medium additionally containing 15 μM octyl gallate at 37 °C. At 36 or 46 h p.i., approximate amounts of rounded cells were determined under phase-contrast microscope. None, mock-infected cells incubated in the absence of the reagent; +V, HSV-1-infected cells incubated in the absence of the reagent; +OG, mock-infected cells incubated in the presence of the reagent; +V+OG, HSV-1-infected cells incubated in the presence of the reagent.

Consistent with the results in Fig. 5, induction of infected cell death was notably accelerated in the presence of the reagent (Fig. 6), while the induction of the infected cell death was slow and moderate in the absence of the reagent. Under these conditions, even control mock-infected HEp-2 cells in the absence of the reagent induced some degree of cell death by the prolonged incubation in the serum-free medium and the addition of the reagent to the culture medium enhanced the induction of cell death, although to a limited degree.

3.4. Reagent-sensitive step in the virus multiplication

To examine the reagent-sensitive step in the viral multiplication cycle, we added the reagent to the culture medium of the infected cells at various time post-infection and harvested the progeny virus at the end of virus multiplication (23 h p.i). Results of this "time of addition" study showed that the reagent could completely inhibit the formation of progeny virus by the addition until 6 h p.i., but the addition at 8 h p.i. was markedly less effective (Fig. 7), indicating that there is no critical reagent-sensitive step in the late stage (8 h p.i. or later) of the virus multiplication.

To our surprise, octyl gallate also showed a direct inactivation of HSV-1 at high reagent concentrations. This virucidal activity was concentration-dependent (Fig. 8) and the time course study showed that the inactivation was rapid, as it occurred immediately after mixing the virus preparation with the reagent (data not shown). However, it must be noted that the concentrations required for virucidal activity were much higher (more than 10-fold) than those for the inhibition of the viral multiplication in the infected cells.

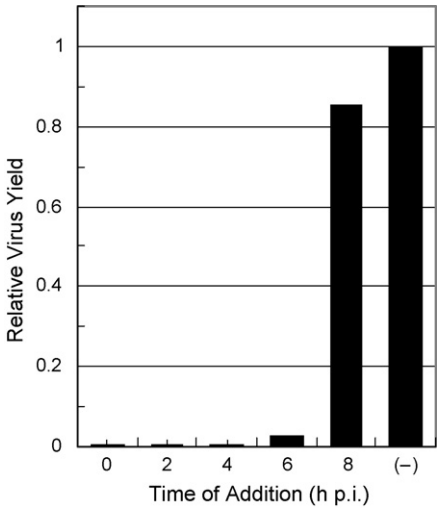

Fig. 7. Effect of time of the addition of octyl gallate on the final virus yield. HEp-2 cells, infected with HSV-1 at an MOI of 14, were incubated in MEM containing 0.1% BSA. At various times after the infection, octyl gallate was added to the culture medium at the final concentration of 4 μg/ml (14.2 μM). At 23 h p.i., the amount of total progeny virus in each culture was assayed. Relative virus yields in the reagent-treated cultures to that in the untreated culture were calculated. The virus yield without the addition of reagent was 6.1×10^8 PFU/ml.

3.5. Effect on RNA viruses

In addition, octyl gallate inhibited the multiplication of some RNA viruses. We examined RNA viruses of two completely different types: vesicular stomatitis virus, an enveloped negative-stranded RNA virus, and poliovirus, a non-enveloped positive-stranded RNA virus. In the presence of 15 μM of octyle gallate, the progeny virus yield of VSV was decreased to approximately one hundredth of the yield in the absence of the reagent, indicating that the reagent-sensitivity of VSV is almost identical to that of HSV-1. On the other hand, the multiplication of poliovirus was also inhibited by octyl gallate, but the sensitivity was much less than that of HSV-1 or VSV (Fig. 9). Furthermore, in contrast to HSV-1, poliovirus was not directly inactivated by the reagent even at the concentration of 200 μg/ml (data not shown).

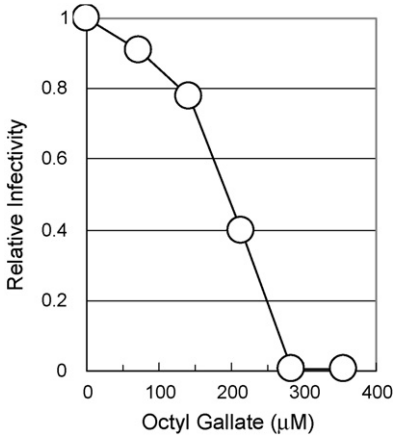

Fig. 8. Direct virucidal effect of octyl gallate on HSV-1. Aliquots of HSV-1 preparation were incubated for 10 min at room temperature with various concentrations of octyl gallate. The number of infectious virus after the incubation was determined by a plaque method.

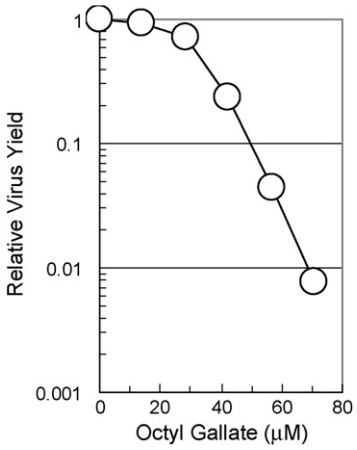

Fig. 9. Effect of octyl gallate on the yield of poliovirus in HEp-2 cells. Confluent monolayers of HEp-2 cells were infected with poliovirus type 1 at an MOI of 10. The infected cells were incubated at 35.5 °C for 22 h in MEM containing 0.1% BSA and the indicated concentrations of the reagent. The amount of total progeny viruses was determined by a plaque method. The virus yield in the absence of the reagent was 1.0×10^8 PFU/ml.

4. Discussion

The present study clearly shows the inhibition of HSV-1 multiplication by octyl gallate. One-step growth curve indicates that the reagent effectively inhibits both the intracellular multiplication and the release of virus in the infected cells (Fig. 4). Although we cannot clarify the mechanism of the inhibition by the reagent, octyl gallate suppresses the HSV-1 infection at two different target steps in virus multiplication cycle. The first one is virion infectivity itself, although this virucidal activity needs the reagent-concentration markedly higher than that required for the suppression of viral growth in the infected cells (Fig. 8). The direct virucidal activity was also found in other gallate derivatives, epigallocatechin-3-gallate and prodelphinidin B-2 3′-*O*-gallate (Cheng et al., 2002, 2003; Song et al., 2005). We have not examined which components of virion would be affected by the reagent, although we found that the reagent also inactivates VSV at the concentrations similar to those used for HSV-1, but does not inactivate poliovirus, a non-enveloped virus, even at higher concentrations than those for VSV or HSV-1. This virucidal activity may result from the antioxidant activity of the reagent, because some antioxidants, such as ascorbic acid and citrulline, also show the virucidal activity (Koyama, unpublished observations).

The second reagent-sensitive step in HSV-1 multiplication is the step(s) prior to the maturation (assembly) of infectious progeny virus. This possibility is supported by the results that, while the addition of the reagent at 6 h p.i. completely suppressed the formation of progeny virus, the addition at 8 h p.i. did not efficiently suppress it (Fig. 7). Previously, we revealed that, in the HSV-1-infected Vero cells, viral DNA rapidly replicates between 3 and 6 h p.i. and the formation of both the nucleocapsids and the progeny infectious viral particles gradually takes place from 5 to

12 h p.i. under these conditions (Koyama and Uchida, 1988). The results in Fig. 8 suggest that, even after the completion of viral DNA replication (at 6 h p.i.), the addition of the reagent completely abolishes the formation of progeny virus. Inhibition of virus multiplication at multiple stages of virus replication cycle, including at the middle or late stage of virus infection, was also observed in studies on the antiviral action of epigallocatechin-3-gallate or prodelphinidin B-2 3′-*O*-gallate (Cheng et al., 2002, 2003; Song et al., 2005; Weber et al., 2003).

On the other hand, when the HSV-1- or mock-infected cells were incubated in the presence of octyl gallate, the onset and extent of the CPE (Fig. 5) and cell death (Fig. 6) were significantly accelerated in the virus-infected cells. Although the HSV-1-induced CPE as well as cell death in HEp-2 cells normally appears at a very late stage of the infection, this acceleration of CPE and cell death, which were observed selectively in the virus-infected cells, can be considered as a result of the preceding disorder of the cellular metabolism in the infected cells by the reagent. The selective action of the reagent on the infected cells likely plays a certain role in the antiviral activity of the reagent, because the acceleration of cell death in the virus-infected cells brings about a significant decrease in the progeny virus production (Koyama et al., 1998, 2000).

In addition to HSV-1, we found that octyl gallate inhibits the multiplication of RNA viruses. Both VSV, an enveloped virus, and poliovirus, a non-enveloped virus, so far tested, showed the decreased virus yield in the presence of the reagent in a concentration-dependent manner, although the sensitivity to the reagent was markedly different between these two RNA viruses. While VSV showed the sensitivity almost similar with that of HSV-1, the multiplication of poliovirus was inhibited by the reagent at much higher concentrations (Fig. 9). Considering the cytotoxity of the reagent at these concentrations, the observed inhibition of poliovirus multiplication by the reagent might be, at least in part, due to non-specific degeneration of the infected cells, while the inhibition of multiplication of HSV-1 and VSV by the reagent cannot be considered to be simply a result of such non-specific degeneration of the infected cells by the reagent.

In addition, we systematically characterized the antiherpetic activity of gallic acid and its *n*-alkyl esters and found that all the alkyl gallates tested have an ability to inhibit the multiplication of HSV-1. The antiviral ability is apparently more evident with the increasing number of carbon in their alkyl moieties, although it reached maximum at the number of 12 (lauryl gallate) and then decreased with the increasing carbon number (Figs. 1 and 2). During the course of our studies, Savi et al. (2005) reported the antiherpetic activity of a series of synthetic alkyl esters of gallic acid. However, they could not find this relationship between the degree of antiherpetic activity and the number of carbon in alkyl moieties of the compounds. Besides, their results are quite different from ours or from Kane et al. (1988). For example, the antiherpetic effective concentration required to inhibit 50% of viral replication is 674 or 2354 μM for octyl gallate (depending on virus strain) in their results, while our data shows it to be approximately 3.5 μM. The discrepancy between these two results comes from the way they determined the antiherpetic activity, i.e., they measured the virus-induced cytocidal effect, not the virus replication, in the infected culture with the use of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). Moreover, they infected the cells with the virus at an MOI of 0.5 (i.e., only half of the cells were infected with the virus). Their method may be convenient and usable for a crude screening of antiviral agent, but may not be reliable for the qualitative and quantitative determination of antiviral activity of the reagents. Here, we determined the antiviral activity of octyl gallate for the first time and analyzed the antiviral activity of alkyl gallates systematically.

5. Conclusions

Gallic acid and its alkyl esters inhibited the growth of HSV-1 in HEp-2 or Vero cells. This inhibition was enhanced by the compounds with higher number of carbon in the alkyl moieties, reaching maximum at carbon number of 12 (lauryl gallate). Although the increase in the carbon number enhanced antiviral effect, cytotoxity of the compounds also increased. Among these compounds, octyl gallate showed a marked antiviral effect with a relatively moderate cytotoxity. Characterization of the antiviral activity of octyl gallate revealed that: (1) this reagent inhibits the multiplication of HSV-1 at early stages (within 6 h p.i.) in the infected cells, (2) it accelerates death of the virus-infected cells selectively, (3) it also suppresses the multiplication of RNA viruses, such as VSV and poliovirus and (4) it can directly inactivate HSV-1 and VSV but not poliovirus at high reagent concentrations.

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