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SYNERGISTIC ANTIVIRAL EFFECT OF XANTHATES AND IONIC DETERGENTS

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Abstract—Xanthate compounds have been shown to exhibit antiviral activity against various DNA and RNA viruses under acidic pH conditions. It is now possible to utilize the unique broad range antiviral spectrum of these compounds under physiological pH conditions (pH 7.4) by simultaneous administration of certain ionic detergents. When used in conjunction with tricyclodecan-9-yl-xanthate (D609), sodium deoxycholate, sodium dodecylsulfate and certain fatty acids, which have no antiviral activity of their own, inhibit the replication of various DNA and RNA viruses (such as herpes simplex, vesicular stomatitis and Coxsackie B 4) *in vitro* at pH 7.4. Among saturated fatty acids of various chain lengths there was a marked size restriction in that the efficiency of undecanoic acid (11 C atoms) was three orders of magnitude greater than that of shorter (6 C atoms) or longer (18 C atoms) monocarbonic acids. Dose–response kinetics revealed a synergistic interaction between the xanthate and the monocarbonic acid. A dose that inhibited the replication of herpesvirus by a factor of 1000 still permitted mitotic activity in uninfected growing control cultures.

Xanthates are capable of inhibiting the growth of various taxonomically unrelated DNA- and RNA-containing viruses [1]. The antiviral activity, however, is exerted in acidic pH conditions both *in vitro* [1] and, as was shown recently in the case of herpesvirus, upon topical application *in vivo* [2]. Physiological pH values (e.g. pH 7.3–7.4 in the blood) have precluded systemic antiviral administration of the xanthates. As the substance does not undergo structural alterations *per se* in either acidic or alkaline environments (pH 6.5–7.5), we refer the requirement of an acidic pH to the cell rather than to the xanthate compound. It has also been established that the antiviral xanthates are located mainly, if not exclusively, in the cellular membrane (unpublished). We have attempted to influence the surface charge of the tissue culture cells by the addition of various charged and uncharged detergents which we expected to become incorporated into the cellular membrane. Only the combination of xanthates with certain negatively charged detergents exerted striking antiviral activity at alkaline pH. This finding means that the xanthates can now be used in physiological pH conditions.

MATERIALS AND METHODS

Cells, viruses and media. Rita cells (Italdiagnostics, Rome) and *Cercopithecus aethiops* cells in the second passage (obtained from Behring, Marburg) were grown in a 5% CO_2/95% air atmosphere in Eagle's basal medium with Earle's salts and 2× the standard concentration of amino acids and vitamins, 10% fetal bovine serum (FBS), 1% penicillin and 1% streptomycin. The latter cells were used for the experiments with Coxsackie B4 virus.

The virus strains used were herpes simplex virus (HSV-1) strain Ang [3], vesicular stomatitis virus (VSV) strain Indiana (obtained from the American Type Culture Collection) and Coxsackie B4 (obtained from Prof. H. J. Eggers, Institut für Virologie der Universität Köln).

Two types of media with different pH values were used for antiviral tests and for the assessment of cytotoxicity of the compounds: (a) pH 6.8—bicarbonate-free Eagle's basal medium was supplemented with 0.85 g/l $NaHCO_3$, 2 ml 1 N HCl, and 10% FBS; (b) pH 7.4—Eagle's basal medium containing 2.2 g/l $NaHCO_3$ was supplemented with 5% FBS. It was essential to equilibrate all media overnight in a 5% CO_2/95% air atmosphere to achieve the final pH value.

Infection of cells. Cells in 6 cm plastic petri dishes (1.3×10^6) were infected at a multiplicity of infection (MOI) of 0.01 plaque forming units (pfu) per cell. After 1 hr adsorption at 37° in a 5% CO_2/95% air atmosphere, 8 ml medium with or without inhibitor was added. If not otherwise stated the viral progeny was harvested 24 hr after infection (cultures buffered at pH 7.4). The virus yield was determined by plaque assay on Rita cells in Linbro plates.

Labelling of cells. Tissue culture medium containing 1 μCi/ml ^{3}H-thymidine (Amersham Buchler, 60 Ci/mmol) was added to the cultures for 24 hr. After removal of the medium the cells were washed twice with isotonic salt solution and lysed with 0.5% SDS and 10 mM EDTA. Then 10% trichloroacetic acid was added to give a final concentration of 5%. The precipitate was collected on Whatman glass fiber filters, and the radioactivity was determined in a liquid scintillation counter.

Compounds. The xanthates tricyclodecan-9-yl-xanthate (D609) and cyclododecylxanthate (D435) were kindly provided by Merz & Co. (Frankfurt).

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Table 1. pH-Dependence of the antiviral effect of D609

pH	HSV-1 Ang		VSV	
	Virus yield from control culture	Virus yield from treated* culture (% of uninhibited control†)	Virus yield from control culture	Virus yield from treated* culture (% of uninhibited control†)
6.8	$(1.53 \pm 0.25) \times 10^7$‡	0.1‡ ± 0.009††	$(5.6 \pm 1.3) \times 10^7$§	0.05§ ± 0.032††
7.0	$(7.2 \pm 1.7) \times 10^5$	5.3 ± 0.03¶		
7.1	$(3.8 \pm 0.88) \times 10^6$	6.1 ± 2.6††	$(6.5 \pm 0.5) \times 10^6$	3.5‖
7.3	$(3.7 \pm 0.51) \times 10^6$	48.6 ± 14.6**	$(2.8 \pm 0.54) \times 10^6$	82.1 ± 5‖
7.45	$(2.6 \pm 0.2) \times 10^7$	33.1 ± 11.5**		
7.53	$(6.1 \pm 1.46) \times 10^6$	59 ± 17.2¶	$(7.7 \pm 1.8) \times 10^6$	35.1 ± 2.3**

* Treated with 20 μg/ml D609.
† Results were compared using Student's *t*-test.
‡ Harvested 48 hr after infection.
§ Harvested 96 hr after infection.
‖ Significant at $P > 0.05$.
¶ Significant at $P < 0.05$.
** Significant at $P < 0.01$.
†† Significant at $P < 0.001$.

Hexanoic acid, octanoic acid, pelargonic acid, decanoic acid, undecanoic acid, lauric acid and sodium deoxycholate were purchased from Merck (Darmstadt). Tridecanoic acid, myristic acid, pentadecanoic acid, palmitic acid and sodium dodecylsulfate and Tween 80 were purchased from Serva (Heidelberg), dodecylphosphate and dodecylphosphonic acid from Ventron (Danvers, MA) and dodecyltrimethylammoniumbromide from Sigma (Deisenhofen). Each day, 1% stock solutions (w/v) were prepared from all substances in 80% acetone, 20% H_2O (v/v). All compounds were administered to the tissue culture media as separate solutions.

RESULTS

As can be seen from the data in Table 1, the compound tricyclodecan-9-yl-xanthogenate (code name D609) looses its inhibitory effect on herpesvirus type 1 (HSV-1) and on vesicular stomatitis virus (VSV) with increasing pH of the tissue culture medium. Over the range of pH 6.8 to 7.53 a difference of two to three orders of magnitude was noted in the inhibitory effect of D609.

However, we have found that the failure to inhibit the growth of both viruses in physiological pH conditions (pH 7.4) can be overcome by the simultaneous treatment of infected cells with D609 and certain detergents.

Subconfluent Rita cell cultures were treated for 1 day after infection with HSV-1 with D609 and at the same time with one of the detergents listed in Table 2. (A D609-to-detergent ratio of 1:4 or 1:2 was selected on the basis of preliminary experiments.) Then the yield of viral progeny was determined by plaque assays and compared with the yield from untreated infected cultures. Combined treatment with D609 and any one of the first three detergents listed in Table 2 led to a striking inhibition of HSV-1 growth at pH 7.4. The antiviral effects cannot be attributed to increased cytotoxicity: between 62.5% and 83% of the uninfected control cells subjected to the same treatment as infected cultures were still able to undergo mitotic division (Table 2, also see below).

Dodecylphosphate and dodecylphosphonic acid and the positively charged detergent dodecyltrimethylammoniumbromide displayed either only a moderate or no inhibitory effect. Furthermore, the organic detergent Tween 80 as well as 1-decanol and 1,10-decanedioic acid turned out to be ineffective.

Apart from undecanoic acid, numerous other fatty acids qualified as suitable supplements that led to the desired antiviral effect at pH 7.4; their chain length (number of carbon atoms) turned out to be of paramount importance. In order to screen for the most efficient monocarbonic acid, Rita cells were infected with HSV-1, and after adsorption, D609 (10 μg/ml) together with 40 μg/ml of each monocarbonic acid was added simultaneously to parallel cultures. The chain lengths of the monocarbonic acids tested ranged between 6 and 18 C atoms. We repeatedly found that undecanoic acid (11 C atoms) caused a marked synergistic antiviral effect when used in combination with D609, the titer of HSV-1 progeny being reduced by almost three orders of magnitude (Fig. 1). Undecanoic acid (C-11), being the most efficient "adjuvant", was chosen in all further experiments. Although we choose in the experiments (Fig. 1) equal weights of the monocarbonic acids rather than equimolar amounts we wish to emphasize that, at least in the range between 6 and 12 C-atoms essentially similar results were obtained in the latter case. Only when myristic acid and increasing larger monocarbonic acids were assayed in equimolar amounts there was an increasing cytotoxicity observed. All synergistically active detergents (including monocarbonic acids of various chain lengths) failed to display antiviral activity when administered to cultures of HSV-1 infected cells (assayed up to 40 μg/ml) in the absence of D609 (Fig. 3 and Table 3).

The quantitation of cytotoxic effects on uninfected Rita cells is indicated on the same scale by the triangles in Fig. 1. As stated in a recent publication [1], we consider the ability of treated cells to undergo

Table 2. Antiviral activity of D609 in combination with different detergents at pH 7.4

Designation of compound	Structure	Factor of inhibition	
		Antiviral activity*	Cytotoxicity†
Undecanoic acid	$CH_3(CH_2)_9COOH$	5.8×10^3	1.6
Sodium deoxycholate	$C_{23}H_{39}O_2COONa$	1.0×10^3	1.2
Sodium dodecylsulfate	$CH_3(CH_2)_{11}OSO_3Na$	7.2×10^3	1.6
Dodecylphosphate	$CH_3(CH_2)_{11}OPO_3H_2$	10.4	n.d.
Dodecylphosphonic acid	$CH_3(CH_2)_{11}PO_3H_2$	2.8	1.4
Dodecyltrimethylammoniumbromide‡	$CH_3(CH_2)_{11}N(CH_3)_3Br$	15	1.6
Tween 80‡		1	2.6

* Infected (HSV-1) Rita cell cultures in duplicate were treated separately for 23 hr after the period of adsorption (1 hr) with 10 μg/D609 and 40 μg/ml of each detergent. The viral progeny was titrated in duplicate plaque assays and compared with the yield from untreated cultures. The antiviral activity is expressed as the factor of inhibition of virus production.

† Uninfected Rita cells were treated in duplicate as described above, and the cell number was determined by counting in a Neubauer counting chamber. The ratio of untreated to treated cultures is indicated (mean values from two cultures in each case).

‡ 20 μg/ml of the detergent were combined with 10 μg/ml D609, because higher concentrations of the adjuvants alone were cytotoxic.

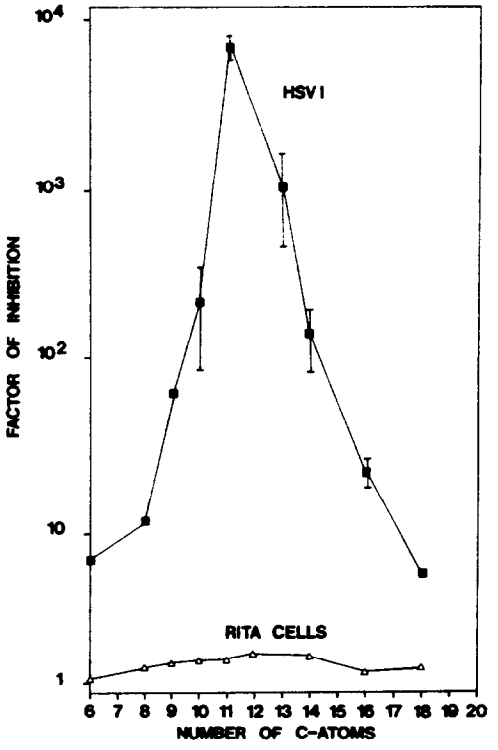

Fig. 1. The influence of the chain length of monocarbonic acids on the antiviral activity and cytotoxicity in combination with D609. Uninfected (△) and HSV-1 infected (■) Rita cells were treated with 10 μg/ml D609 and 40 μg/ml of each monocarbonic acid (at pH 7.4). The virus yield of two cultures was examined individually by plaque assay in duplicate. Error bars indicate standard deviation. Cell densities of duplicate uninfected treated and untreated Rita cell cultures were determined after staining with trypan blue by counting with a hematocytometer.

mitotic division the most stringent criterion for cytotoxicity. Accordingly, uninfected Rita cell cultures were subjected to the same treatment with the D609/monocarbonic acid mixtures as infected cultures, and the living cells were counted after 24 hr. In the case of C-11 a factor of inhibition of 1.57 was determined. This indicates that a treatment which had inhibited the increase in the cell number of uninfected growing cultures by only 36% had led to the inhibition of HSV-1 growth by a factor of 5.8×10^3. Combinations of D609 with other monocarbonic acids, such as pelargonic acid, despite their lower antiviral effect (100-fold lower than D609/C-11), displayed comparable cytotoxic effects on uninfected cultures. The effect of increasing concentrations of C-11 together with a constant amount of D609 (20 μg/ml) on Rita cells was also determined by the ability of the cells to incorporate ^{3}H-thymidine into their DNA. Parallel cultures were incubated for 1 day with the combination D609/C-11 and then labeled for another day in the absence of the inhibitor. As can be seen from Fig. 2, up to 40 μg/ml C-11 altered the level of ^{3}H-thymidine incorporation to only a small extent, indicating that the cells had tolerated the treatment rather well despite the large dose of 20 μg/ml D609.

We would like to stress at this point that Rita cells have consistently turned out to be quite resistant to treatment with the combination of D609 and monocarbonic acids. As will be dealt with in the discussion and in a separate publication (*Cancer Letters*, in press), there are various transformed cell lines that are markedly more vulnerable to such doses as those indicated above. Calibration of the degree of resistance of uninfected cells (which is, in addition, proportional to the cell density in the culture) is mandatory in every single case when other virus–host cell systems are to be employed.

Next, the dose–response relationship between various concentrations of undecanoic acid and a constant D609 moiety and the ensuing antiviral effect (against HSV-1) was established. As shown in Fig. 3, there is clearly a synergistic effect between the two components: the lower the D609 moiety was, the more monocarbonic acid had to be added in order to attain a comparable antiviral effect. For example, the combination of D609 and undecanoic

Table 3. Antiviral effect of the combination D609/undecanoic acid on RNA viruses at pH 7.4

Virus species	D609 (μg/ml)	Undecanoic acid (μg/ml)	Virus yield* (pfu/ml)
VSV†	0	0	$(4.3 \pm 1) \times 10^6$
	5	0	$(5.6 \pm 0.05) \times 10^6$
	0	40	$(4.3 \pm 0.3) \times 10^6$
	5	40	$(1.3 \pm 0.2) \times 10^4$
Coxsackie B4	0	0	$(1.15 \pm 0.2) \times 10^6$
	10	40	$(7.7 \pm 0.25) \times 10^4$

* Values were obtained by assaying duplicate cultures, and the resulting virus yields were determined in duplicate.
† VSV progeny was harvested 10 hr, and Coxsackie B4 at 24 hr after infection.

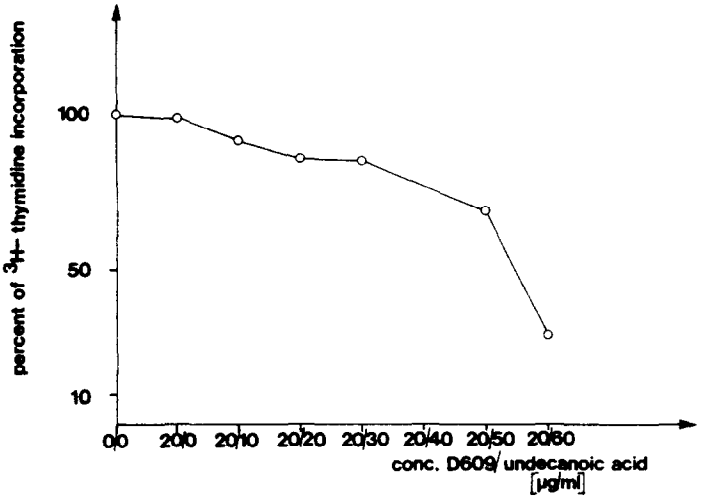

Fig. 2. DNA synthesis in Rita cells after treatment with D609 and increasing amounts of undecanoic acid. Prior to labeling the cells were treated for a period of 24 hr. Each point represents the mean values from four cultures.

acid (15 + 15 μg/ml) inhibited the multiplication of HSV-1 by a factor of 9.6×10^2. In contrast, the factor of inhibition displayed by either component alone was lower by almost three orders of magnitude (D609: 5.0; undecanoic acid: 3.0).

The antiviral effect of the combination of D609 and C-11 is not only confined to HSV, but also concerns various RNA viruses. Both the negative-strand VSV and Coxsackie B4 virus with positive strand polarity are effectively inhibited at pH 7.4 (Table 3). It is also shown that neither C-11 nor D609 alone displays any inhibitory activity.

DISCUSSION

We have shown in this report that certain ionic detergents are capable of extending the broad range antiviral activity of xanthate compounds to "physiological" pH values (pH 7.4). This observation extends our previous report [1], in which the antiviral activity of appropriately substituted xanthates was shown to occur only at acidic pH values (pH 6.8).

The synergistic inhibitory effect detailed above in particular for C-11 also holds true when the antiviral xanthate D435 [1] is used instead of D609. As might be expected, it is not observed in the case of xanthates that have been shown to lack antiviral properties, such as propylxanthate (unpublished). Furthermore, we found that simultaneous administration of the xanthate and the detergent was mandatory for the desired inhibitory effect to be achieved. Subsequent separate administration of either component failed to inhibit virus replication (unpublished). Amongst all tested detergents those bearing negatively charged sulfate and carboxy groups displayed the best synergistic antiviral effect in combination with D609. In contrast the tested cationic detergent and the anionic dodecylphosphate turned out to be only moderately efficient. No effect was obtained by dodecylphosphonic acid.

In an attempt to analyze the mechanism of the synergistic interaction between detergents and the

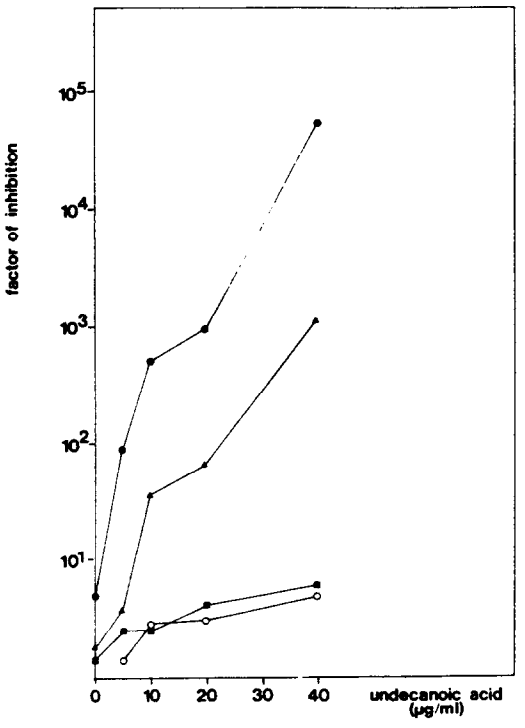

Fig. 3. Dose–response relationship between D609/undecanoic acid combinations. HSV-1 infected Rita cells were treated with different concentrations of D609 (5 (■), 10 (▲), 15 (●) μg/ml) and increasing amounts of undecanoic acid. Empty circles indicate treatment with undecanoic acid alone. The inhibitory effect was determined by plaque assays.

xanthates, it might be assumed that direct inactivation of the viral infectivity might take place. This, however, is not the case, as was evidenced by the incubation of VSV (which has a plasma membrane-derived envelope) for 1 hr at 37° with up to 80 μg/ml of C-11 and subsequent plaque titration. No loss of infectivity (as compared with mock-incubated VSV) was encountered.

With the aid of custom-labeled ^{3}H-D609 (spec. act. 6.3 Ci/mmol), the kinetics of binding of the compound both under acidic (pH 6.8) and alkaline (pH 7.4) conditions to either Rita or mouse embryo fibroblasts was studied, and no difference was detected. Within 6–10 min, about 50% of the label was bound (i.e. it withstood two washings with buffered saline) to the cells in either condition.

We suspect that the detergents integrate into the outer layer of the plasma membrane, thereby exposing their charged sulfate or carboxy groups to the surface. As a corollary to this, the surface charge of the cells would be altered. In addition, further changes might ensue; for example, the fluidity of the plasma membrane might be affected. Possibly, certain hydrophobic protein–lipid interactions might be modulated by the intercalation of the various detergents. We know that ^{3}H-D609, although withstanding repeated washing procedures with saline buffer, can be readily removed from the cell when the membrane is destroyed by organic solvents such as acetone (as shown by autoradiography).

As the xanthates do not only exert broad antiviral activities but also, when combined with the appropriate detergents, are capable of selectively killing transformed cells [4], some central biological mechanism which is probably involved in growth control must be affected. Thus, properties that distinguish transformed cells from their normal ancestor cell lines must be recognized. Whether or not the antiviral and the selective cytotoxic effects are based upon identical mechanisms is not known, although this is likely to be the case. Data hinting at a possible mechanism of activity were obtained while investigating the inhibitory effect of D609 on the multiplication of VSV. As the phosphorylation (however, not the *de novo* synthesis) of the NS protein (which is regulatory active in the transcription of VSV) is specifically inhibited by D609, we suspect that cellular protein kinases are interfered with (manuscript submitted for publication). Such protein kinases play a crucial role not only in cellular growth regulation and transformation [5, 6] but may conceivably also be required in viral growth processes in a fashion that is hitherto not yet recognized.

The antiviral activity of D609/undecanoic acid at pH 7.4 renders this combination now available for systemic treatments. Preliminary experiments revealed that mice tolerated continuous infusion of 120 mg kg^{-1} day^{-1} into the lateral tail vein for 3 days. When appropriately administered (90 min infusion period) we found that the acute toxicity appears to exceed 1 g/kg mouse. Hence, rather substantial doses were tolerated.

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